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Par

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Etude des interactions et des propriétés physico-chimiques de mélanges de protéines de pois et de blanc d'oeuf : de l'état colloidal à l'état gélifié.

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Titre : Etude des interactions et des propriétés physico-chimiques de mélanges de protéines de pois et de blanc d'oeuf : de l'état colloidal à l'état gélifié.

Mots clés : protéines végétales et animales, isolat de protéines de pois, protéine de blanc d'œuf, propriétés thermiques, propriétés de gélification, interactions

Résumé: Ces dernières années, en raison de la croissance de la population mondiale, la consommation de protéines animales a augmenté, entraînant une augmentation des émissions de gaz à effet de serre et de l'occupation des sols. Il est donc nécessaire de remplacer partiellement les protéines animales par des protéines végétales pour augmenter la part de protéines végétales dans la consommation quotidienne, et développer des systèmes alimentaires mixtes semble être une solution adéquate. Les protéines de pois en tant que source de protéines végétales ont de bonnes propriétés nutritionnelles mais avec des propriétés fonctionnelles limitées tandis que le blanc d'œuf (EW) a de bonnes propriétés fonctionnelles telles que la gélification et le foisonnement. Ainsi, les interactions physicochimiques des mélanges des deux types de protéines doivent être comprises pour développer des ingrédients protéiques hybrides. Dans le présent travail, des protéines de pois sous forme d'isolat de protéines de pois (PPI) et d'EW ont été préparées à une concentration totale de protéines de 10 % w/w. Les interactions colloïdales en solution, les propriétés thermiques et de gélification des mélanges ont été étudiées en fonction de différents rapports pondéraux (PPI/EW, 0/100, 25/75, 50/50, 72/25. 100/0) et de différents pH (7.5 et 9). Pour comprendre les interactions dans les mélanges PPI-EW, le PPI en mélange avec différentes fractions protéiques EW (ovalbumine (OVA), ovotransferrine (OVT) et lysozyme (LYS)) a été analysé par des méthodes combinées telles que la calorimétrie de titrage isotherme (ITC), la diffusion dynamique de la lumière (DLS), la granulométrie laser, la microscopie De fortes interactions exothermiques entre PPI et LYS conduisent à une agrégation via des interactions électrostatiques. Aucune ou de très faibles interactions n'ont été détectées entre OVT ou OVA et PPI quel que soit le pH. Les propriétés thermiques et de gélification des systèmes mixtes ont été caractérisées par le profil de solubilité avec le pH, la SDS-PAGE, la calorimétrie différentielle à balayage (DSC), la rhéologie dynamique (25-95-25 °C), les analyses de texture, le CLSM et l'ajout d'agents dissociants après gélification. La température de dénaturation thermique (Td) et l'enthalpie sont peu ou pas influencées en fonction du pH. La Td d'OVT, de LYS et de légumineuses a changé en raison des interactions entre les protéines. La formation de gel était régie par les protéines EW et le module élastique (G ') diminuait avec la teneur en EW dans les mélanges. Le point de gélification de l'OVT (~ 59 °C) a augmenté d'environ 3 °C, ce qui donne une indication intéressante pour une application ultérieure du traitement de pasteurisation. Parallèlement, des gels obtenus à partir de protéines mixtes (contenant au moins 50 % de blanc d'œuf) étaient constitués d'un réseau de protéines de blanc d'œuf, incluant des agrégats de protéines de pois. Des interactions fortes incluant des ponts disulfures et des interactions hydrophobes ont été à l'origine de la structure du réseau du blanc d'œuf. Des interactions faibles, y compris plus de liaisons hydrogène, pourraient expliquer les propriétés viscoélastiques inférieures des gels à base de PPI. Dans les gels mixtes, les protéines du blanc d'œuf constituent l'architecture de base du réseau protéique. La gélification de l'EW s'accompagne de la formation

confocale à balayage laser (CLSM) et la microscopie optique.

d'agrégats de protéines qui peuvent être des agrégats de PPI purs ou des agrégats mixtes constitués de globulines de pois et de certaines protéines de blanc d'œuf. D'autres investigations sur la fonctionnalité des protéines (foisonnement) et l'effet des traitements de fonctionnalisation tels que l'étuvage de la poudre sont attendues.

Résumé

Title : Study of the interactions and physicochemical properties of pea and egg white protein mixtures: from the colloidal to the gelled state

Keywords : Pea protein isolate, Egg white protein, Functionality, Thermal properties, Gelation properties, Interaction

Abstract : In recent years, due to the growth of world population, people's consumption of animal protein has increased, leading to the increase of greenhouse gas emissions and land occupation. Therefore, it is necessary to partially replace animal proteins with plant proteins to increase their proportion in daily consumption and developing mixed food systems seems to be one adequate solution. Pea proteins as a source of plant proteins have good nutritional properties but with limited functional properties while egg white (EW) has good functional properties such as gelling and foaming. Thereby, the physicochemical interactions from mixtures of both types of protein needs to be understood to develop hybrid protein products. In the present work, pea proteins as pea protein isolate (PPI) and EW were prepared at total protein concentration of 10% w/w. The colloidal interactions in solution, thermal and gelation properties of the mixtures were studied as a function of different weight ratios (PPI/EW, 0/100, 25/75, 50/50, 72/25. 100/0) and different pH (7.5 and 9). To understand the interactions in PPI-EW mixtures, PPI in admixture with different EW protein fractions (ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (LYS)) was analyzed by combined methods such as isothermal titration calorimetry Thermal and gelation properties of the mixed systems were characterized through solubility profile with pH, SDS-PAGE, Differential Scanning Calorimetry (DSC), dynamic rheology (25-95-25 °C), texture analyses, CLSM, and the addition of dissociating agents after gelation. Thermal denaturation temperature (Td) and enthalpy were slightly or not influenced depending on the pH. Td of OVT, LYS and legumin changed due to the interactions between proteins. Gel formation was governed by EW proteins and elastic modulus (G') decreased with EW content in mixtures. Gelation point of OVT (~59 °C) increased around 3 °C giving an interesting indication for further pasteurization treatment application. Meanwhile, gels obtained from mixed proteins (containing at least 50 % egg white) were constituted of a network of egg white proteins, including aggregates of pea proteins. Strong interactions including disulfide bonds and hydrophobic interactions were at the origin of the structure of the egg white network. Weak interactions including more hydrogen bonds could explain the lower viscoelastic properties of PPI-based gels. In mixed gels, the egg white proteins constitute the basic architecture of the protein network. The gelation of EW is accompanied by the formation of protein aggregates which may be pure PPI aggregates or mixed aggregates

(ITC), dynamic light scattering (DLS), laser granulometry, confocal laser scanning microscopy (CLSM) and optical microscopy. Strong exothermic interactions between PPI and LYS lead to aggregation via electrostatic interactions. No or very weak interactions were detected between OVT or OVA and PPI whatever the pH.

consisting of pea globulins and some egg white proteins. Further investigations on protein functionality (foaming) and effect of functionalization treatments such as dry-heating of powder are expected.

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Accomplishments

This thesis was carried out in cooperation between L'institut Agro Dijon, within the PCAV team (physico chemistry of food and wine) belonging to the UMR PAM (mixed research unit for food and microbiological processes) and L'institut Agro Rennes-Angers within the UMR STLO (Science and Technology of milk and egg) research unit.

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Scientific publications

Submitted papers

Jian Kuang[a,b], Pascaline Hamon[b], Florence Rousseau[b], Eliane Cases[a], Saïd Bouhallab[b], Rémi Saurel[a], Valérie Lechevalier[b*] **Interactions between isolated pea globulins and purified egg white proteins in solution 2022-04-28**
Submitted to Food hydrocolloids (IF 11.5)

Jian Kuang[a,b], Pascaline Hamon[b], Eliane Cases[a], Valérie Lechevalier[b], Rémi Saurel[a*]
Thermal behaviour of pea and egg white protein mixtures 2022-09-19
Submitted to Food Research International (IF 7.4)

Jian Kuang[a,b], Eliane Cases[a], Valérie Lechevalier[b], Rémi Saurel[a*]
Nature of protein-protein interactions during gelation of mixtures between pea protein isolates and egg white proteins
Prepared to be submitted to Food Hydrocolloids (IF 11.5)

Poster communications

Jian KUANG, Valérie LECHEVALIER, Rémi SAUREL. **Effects of heat treatment on rheological properties of pea and egg white protein mixtures**
11th NIZO plant protein functionality conference (Visio conference, 21 - 22 October 2020)

Jian KUANG, Pascaline HAMON, Saïd BOUHALLAB, Rémi SAUREL, Valérie LECHEVALIER. **Colloidal interactions between pea globulin isolate and purified egg white proteins**. 18th Food colloids conference: structure, dynamics and function Lund university (April 11th – 13th 2022)

Oral presentations

Jian KUANG, Valérie LECHEVALIER, Rémi SAUREL. (**March 18th, 2021**). **Rheological properties of thermal gels of pea and egg white protein mixtures**
Webinar **« Rheology and Food structuring & destructuring »,** organized by Marco Ramaioli (INRAE-SayFood) & Guy Della Valle (INRAE-BIA), France

Jian KUANG, Valérie LECHEVALIER, Rémi SAUREL. Interactions between isolated **pea globulins and purified egg white proteins**

Protein interactions and assemblies Intra-INRAE Rennes 20th June 2022

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Main abbreviations

AE: Alkaline extraction

AP: Acid precipitation

CLSM: Confocal laser scanning microscopy

CPC: Critical protein concentration

DF: Diafiltration

DSC: Differential Scanning Calorimetry

DTT: Dithiothreitol

ES: Ethanol soluble fraction

EW: Egg white protein

GP: Globular protein

GDL: glucono-δ-lactone

GuHCl: Guanidine hydrochloride

HP: Heat-acid precipitation

IEP: Isoelectric precipitation

IgE: Immunoglobulin E

IgY: Immunoglobulin

ITC: Isothermal titration calorimetry

LYS: Lysozyme

MC: Micellar casein

MGC: Minimum gelling concentration

MP: Micellar precipitation

MPI: Myofibrillar protein isolate

MTG: Transglutaminase

MW: molecular weight

NEM: N-ethylmaleimide

OVA: Ovalbumin

OVT: Ovotransferrin

PG: Propylene glycol

pI: Isoelectric point

PL: Pea legumin fraction

PPC: Pea protein concentrate

PPI: Pea protein isolate

PPIc: Commercial pea protein isolate

PPIn: Native pea protein isolate

PV: Pea vicilin fraction

RP-HPLC: Reverse phase high-pressure liquid chromatography

SE: Salt extraction

SEC-HPLC: High performance size exclusion chromatography

SPI：Soy protein isolate

SPIc: Commercial soy protein isolate

SPIn: Native soy protein isolate

SS : Salt soluble fraction

Td: Denaturation temperature

Tg: Critical gelation temperature

UF: Ultrafiltration

VPP: Vegetable protein materials

WPI : Whey protein isolate

WPC: whey protein concentrate

WS : Water soluble fraction

β-lg: β-lactoglobulin

General introduction

As a result of predicted socioeconomic development, the world population will increase by nearly one-third until 2050 (from 6.9 billion in 2010 to 8.5-10 billion in 2050) and global income will triple (from US$68 trillion in 2010 to US$180-290 trillion in 2050) (Springmann et al., 2018). This results in a twofold need for animal protein among humans. This situation of growing animal protein consumption is a ticking time bomb in terms of sustainability and food security, as noted by various United Nations assessments (FAO, 2011, United Nations, 2015). However, raw animal materials like milk, eggs, meat, and seafood continue to be the most important sources of protein recently employed by food companies, followed by plant sources like legumes and nuts (Alves & Tavares, 2019). Meanwhile, animal protein production is connected with high greenhouse gas emissions and increased land requirements, whereas plant proteins have a lower economic cost and lower ecological footprint (Davis, Sonesson, Baumgartner & Nemecek, 2009; Alves & Tavares, 2019). Legumes proteins, on the other hand, are produced for animal feed yet having physicochemical features that make them valuable for human consumption (Boye, Zare, & Pletch 2010). Furthermore, excessive intake of animal proteins can have a severe influence on human health, including the development of illnesses such as obesity, cardiovascular disease, neurological disorders, allergies, and so on (Hertzler et al., 2020). As a result, the partial substitution of animal protein with plant protein is gaining popularity in designed goods. They are frequently sold as "healthier" (than meat) and sustainable new foods as "substitutes" for traditional animal-derived food items (Godfray, 2019).

Soy-based protein food businesses now provide a wide range of food items. Over the last decade, worldwide soybean output has expanded dramatically (Qin, Wang, & Luo, 2022). This vegetable raw material's extensive use in many culinary items has eclipsed other kinds of plant proteins, such as pea proteins. However, food manufacturers' concerns are shifting in favor of other diverse plant sources, like pea protein, to counteract soy protein's monopoly. Recently, there has been a lot of attention in pea proteins (Pisum Sativum L), which have a lot of promises in the food supply because of their high yields and low pricing (Munialo et al., 2014a; O'Kane et al., 2004b). Peas are one of the world's most frequently farmed and consumed legumes, namely in Canada, France, China, Russia, and the United States (Boy et al., 2010; Burger & Zhang, 2019). Pea proteins have comparable functional qualities as soy

proteins, such as emulsification, however it is non-allergenic (Aluko, Mofolasayo, & Watts, 2009). This protein source is thought to be a viable alternative to animal and soy proteins (Liang & Tang 2013; Burger & Zhang, 2019). However, there are some limits for pea proteins to be used as an ingredient, primarily due to a lack of understanding of their structure and functional features (Adebiyi & Aluko, 2011; Liang & Tang, 2013).

Egg is well-known for its high nutritional content, great digestibility, and full essential amino acid supply. Moreover, egg proteins are the most sustainable animal proteins. The application of egg is thus wild in food industry: for example, whole egg is used in bakeries; egg yolk in mayonnaise, salad dressing, noodles or ice creams, egg white in bakeries, confectioneries, meringue. Different egg products used as food ingredients can be found on the market such as whole egg, egg yolk and egg white, either in pasteurized liquid, frozen or powder state.

Egg white, especially is widely used for its foaming and gelling properties. Proteins indeed account for more than 90% of the dry substance in egg white, giving it its single functional properties. The water-holding capacity or gel strength of food items can be increased by using egg white as an additive. Because of its low lipid content, egg white is frequently favored over egg yolk for its gelling capabilities (Mine, 2014). Many food items' textural and rheological features, such as meringues and angel food cakes, are dependent on the heat coagulation or gelation capabilities of egg proteins, particularly their irreversible heat coagulation (Ren et al., 2010). A thermally irreversible gel is a viscoelastic solid created by heat that does not reheat to a viscous liquid.

Studies dealing with partial substitution of animal protein by plant protein mainly deals with milk or meat proteins as animal sources. There is thus currently a lack of research on egg white protein as an animal source of protein blended with plant protein. Few studies, however, were dedicated to the study of gelation and thermal aggregation of soy protein mixed with egg white protein (Su et al., 2015; Zhang et al., 2020), or hemp seed protein and egg protein cold gel (Alavi, Emam-Djomeh, & Chen, 2020. Moreover, traditional process for ovoproducts such as egg white which requires a liquid pasteurized form or dried form to produce the products, leading us to think use a process similar to ovoproduct by using a mixed ingredient. Therefore, the knowledge of thermal properties of the mixture systems is required, which will provide more information for further pasteurization. Furthermore, there are some products on the market that using

egg white as glue in meat analogue applications, such as vegetable steaks and burgers, meat balls….; that justifies the gelling properties study. In addition, no studies have been reported on mixtures of pea and egg white proteins. Pea proteins doesn't have nice gelling properties as good as soy proteins, and it seem interesting to use mixture with egg white to improve this property. As a result, the usage of pea proteins combined with egg white proteins as an ingredient requires thorough investigations of the nature of the interactions between these proteins of different nature. Our research is centered on determining the nature of intermolecular interactions involved in the probable connection of pea proteins and egg white, thermal properties of such mixtures, as well as their effects on the meso-structural and textural aspects of mixed gels. Pea globulins was preferred in this thesis as it higher content in pea flours and lack of the studies of albumins regarding gelling and thermal behaviour.

This thesis has the primary objective to study the interactions between egg white and isolated pea globulins under different experimental conditions. Secondly, we will be interested in the thermal properties of mixed systems and the formation of heat-induced gels from egg white and pea globulins. To have a better understanding of the intermolecular interactions of the egg white and pea globulins, we used purified ovalbumin, ovotransferrin, and lysozyme, as well as pea globulins obtained by alkaline-isoelectric extraction. Subsequently, egg white proteins and pea globulins were mixed at different ratios and pHs to study the thermal properties and gelation properties through various scales (molecular, microscopic, and macroscopic), in order to better understand the mechanisms involved during this gelation.

In the following sections, we will firstly introduce the primary data to understand the materials in the bibliographic review (Chapter 1), including a full description of the technique employed in the objective of the study section. The second section of the thesis will describe the materials and methods employed in the execution of this work (Chapter 2 Materials and methods). The experimental section of the manuscript will be separated into four chapters (Chapter 3: Preparation and characterization of protein materials; Chapter 4: Interactions between isolated pea globulins and purified egg white proteins in solution; Chapter 5: Thermal behavior of pea and egg white protein mixtures; Chapter 6: Nature of protein-protein interactions during gelation of mixtures between pea protein isolates and egg white proteins). A final chapter of the manuscript will be

the conclusions on the thermal and gelation properties of the mixed system, as well as the perspective of this study.

Chapter 1 Literature review

1.1 Pea protein

Peas, notably the yellow or green cotyledon variants known as dry, smooth, or field peas, are naturally dried seeds of Pisum sativum L. and are one of the world's oldest domesticated crops, which are farmed for human and animal nutrition all over the world (Smýkal et al., 2012). It belongs to the Leguminosae family (Genus: Pisum, subfamily: Papilionoideae tribe: Fabaceae), and covers more than 34.2 % of the total area under dry pulse (Smýkal et al., 2012; Eurostat, 2020).

The yellow pea seeds contain approximately 60-65 % of carbohydrates of which 35-40% of starch (24-49 % amylose), 23-30 % of proteins, 1-2 % of lipids, and minor elements such as minerals, vitamins, polyphenols, phytic acid and so on, depending on the difference of cultivar, culture conditions and the stage of maturity of the grain at the time of harvest (Lam et al., 2018; Bogahawaththa et al., 2019; Lu et al., 2019; Eurostat, 2020; Saurel, 2020). In addition to starch, its carbohydrate also comprises dietary fiber (10-15 % insoluble part and 2-9 % soluble part) as well as non-starch carbohydrates for instance cellulose, sucrose, and oligosaccharides (Lam et al., 2018; Hoover et al., 2010).

Table 1-1 Classification and chemical properties of pea protein.

Types	Content	Protein	Svedberg Unit	Molecular weight (MW) kDa	No. of subunit	MW of subunit kDa	Isoelectric point
Globulin	55-65%	legumin	11S	360-410	6	60-65	4.8-4.9
		vicilin	7S	150-200	3	48-50	4.6-4.8
		convicilin	7S	210-280	3	70-71	4.6
Albumin	18-25%	Albumin PA1	2S	11	2	5-6	9.8 e 5.3
		Albumin PA2	2S	52	2	25 or 26	5.2
Prolamin	4-5%	prolamin	/	/	/	/	/
Glutelin	3-4%	glutelin	/	/	/	/	/

Cited and summarized from Adebiyi and Aluko (2011); Barać et al. (2010); Beghdadi, (2021); Gressent et al. (2011); O'Kane et al. (2004a, 2004b); Reinkensmeier et al. (2015); Tzitzikas et al. (2006), etc.

According to the classification methods in Table 1-1, pea proteins can be categorized into 4 types: globulins (salt soluble), albumins (water soluble), gliadins (alcohol soluble), and glutenins (insoluble).

1.1.1 Pea globulins

Pea globulins (Glob) are so-called storage proteins that provide nutrients (nitrogen, sulfur, and carbon) for seedlings during germination (Tzitzikas et al., 2006). In most dicotyledonous plants, the nitrogen-containing portion of the protein origin is dominated by globulins (7S / 11S), which account for 65 % to 80 % of total protein nitrogen, while the remainder is attributable to albumin (2S) and other insoluble proteins (Tzitzikas et al., 2006; Kimura et al., 2008). According to Table 1-1, globulins are soluble in a salt solution, which can be consumed during seed germination to supply nutrients to plant development. Globulin dissociates into subunits under high pH and ionic strength. In general, the sedimentation coefficient can be used to classify pea globulins into vicilin / convicilin (7S) and legumin (11S) (Barać et al., 2010). The content of these two primary divisions is influenced mostly by genetic and environmental factors. The legumin/vicilin (Lg/Vn) ratio rises throughout seed development, because of the differing rates of synthesis of the 11S and 7S protein fractions. Several studies have illustrated the variability of these fractions (7S and 11S) by studying different pea genotypes. Tzitzikas et al. (2006) investigated genetic diversity in pea seed globulin protein composition of 59 different distinct varieties, discovering that vicilin proteins were predominated with Lg/ Vn ratios ranging from 0.12 [variety: NGB 102149 Iran] to 0.77 [variety: P arvense (CGN 10193)]. According to Boye, Zare, & Pletch. (2010), Lg/Vn ratios ranged from 0.23 to 0.50 for wrinkled pea cultivars and from 0.31 to 1.67 for smooth pea cultivars. Previous works of literature conducted by O'Kane et al. (2004b) and Mertens et al. (2012) found that the ratio of legumin to vicilin is close to 2:1 and legumin contains more sulfur-containing amino acids than vicilin per unit of protein. Differences in content, composition, and structure between legumin and vicilin show up in both nutritional and functional features, with their association dissociation properties and surface structures being the most critical elements in understanding pea protein functioning (Barać et al. 2010). It should be mentioned that pea protein has a comparable protein structure to soybean proteins in three-dimensional structures and protein sequences, resulting in similar functional properties, except for pea protein having fewer allergenic aspects, a lower amount of sulfur amino acids, and being less digestible (Fischer, Cachon, & Cayot, 2020).

1.1.1.1 11S legumin

Pea legumin has a compact hexametric quaternary structure that is supported by electrostatic and hydrophobic interactions and has a molecular weight of 360 to 410 kDa (Marcone, Kakuda, & Yada, 1998b; Boye, Zare, & Pletch, 2010). They are composed of 60 kDa monomers at pH levels ranging from 7 to 9. Each legume monomer is made up of two acid and basic polypeptides of 38-40 kDa and 19-24 kDa, respectively, that are covalently connected by a disulfide bond (Croy et al., 1980; Tzitzikas et al., 2006) (Figure 1-1). Plietz et al. (1983) proposed that all 11S globulins from legumes had highly similar quaternary structures, using a bipyramidal trigonal structural model. Meanwhile, previous literature conducted by Gueguen & Barbot (1988), Karaca, Low, & Nickerson (2011), and Marcone et al. (1998b), proved that basic polypeptides with a hydrophobic nature were found in the protein's core, whereas acidic polypeptides are found on the protein's surface. Therefore, this arrangement would clarify the orientation of the α polypeptides, which are more hydrophilic on the exterior, and β polypeptides, which are more hydrophobic on the inside of the oligomeric protein (Figure 1-1). The monomers' secondary structure is composed of 45% β-sheets and 15% α-helices (Matta et al., 1981).

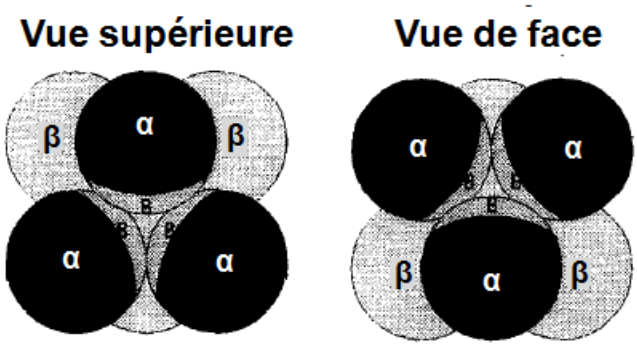


Figure 1-1 Model of the bipyramidal trigonal quaternary structure possible for dicotyledon 11S globulins. Each sphere includes an acidic and basic polypeptide that makes up the legume components (Marcone, Kakuda, & Yada, 1998a).

Acidic polypeptides have an isoelectric point (pI) between 4.5 and 5.8, whereas basic polypeptides have a pI between 6.2 and 8.8 (Matta et al., 1981; Heng et al., 2004). Thus, the typical pI value of legumes is between 4.8 and 4.9 (Chihi, 2016). Ionic strength and pH have a deep influence on legumin structure, according to the report of Subirade, Gueguen, & Schwenke (1992), Gueguen, Chevalier, Barbot, & Schaeffer

(1988) and Gueguen (1989), legume dissociated into a mixture of trimers, dimers, and monomers even though it was in the form of non-aggregated at pH 7 with an ionic strength higher than 0.1 M, and extreme acidic or basic pHs depending on ionic strength. Thus, this kind of change in the state increased the surface hydrophobicity of these proteins.

1.1.1.2 7S vicilin

Vicilin is a glycosylated trimeric protein with a molecular weight of 150-200 kDa, nevertheless, although having lower molar weights, the group of 7S vicilins looks more diverse, and its structure is not well known (O'Kane et al., 2004b; Saurel, 2020). Vicilins are abundant in glutamic acid, aspartic acid, and lysine with a low methionine concentration but are devoid of cysteine, resulting in the non-formation of intramolecular or intermolecular disulfide bonds (Gatehouse, Croy, Morton, Tyler, & Boulter, 1981; Shewry, Napier, & Tatham, 1995). Therefore, Sikorski (2001) reported that vicilin is bound together by hydrophobic contacts rather than covalent disulfide bonds in contrast to legumin.

The vicilins are primarily composed of three subunits of ~50 kDa, each monomer has a more hydrophilic surface than legumin and can be cleaved into a variety of low molecular weight fragments (α,β, and γ), as illustrated in Figure 1-2 (Gatehouse, Lycett, Croy, & Boulter, 1982; O'Kane et al. 2004a, 2004b; Tzitzikas et al., 2006). In detail, the 50 kDa precursor has two potential cleavage sites, denoted by the letters α: β and β: γ (Matta et al., 1981; Tzitzikas et al., 2006). Cleavages at both locations result in fragments of 20 kDa (α), 13 kDa (β), and 12-16 kDa (γ). The cleavage between α and β results in pieces of 20 kDa (α) and 25-30 kDa (β+γ), whereas cleavage between β and γ results in fragments of 30-36 kDa (α+β) and 12-16 kDa (γ) (Gatehouse et al., 1982). In addition, the γ-subunit is occasionally N-glycosylated around the C terminus (Spencer et al., 1983). Serine, glutamic acid, and aspartic acid are the most common N-terminal amino groups (Sikorski, 2001). While amino acid fingerprinting revealed 70-80 % commonality between legumin and vicilin from the Vicieae (or Fabaceae) tribe, N-terminal analysis revealed more variability (Jackson, Boulter, & Thurman, 1969). Secondary structures of the legumin and vicilin proteins are dominated by β-sheets (Sikorski, 2001). Despite its structural diversity, vicilin's pI ranged between 5.4 and 5.5 (Derbyshire, Wright, & Boulter, 1976).

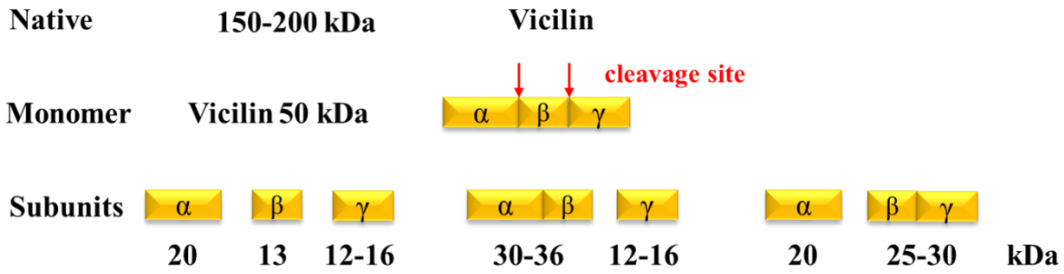


Figure 1-2: Assembly of 7S vicilin fragments, and potential cleavage sites according to Tzitzikas et al., 2006.

1.1.1.3 7S convicilin

Convicilin is a third-storage protein that may be found in peas and other pulses. The convicilins are made up of subunits of around 71 kDa that are most likely connected in trimers with a molar mass of 210-280 kDa (Barać et al., 2010; Boye, Zare, & Pletch, 2010; Tzitzikas et al., 2006). According to the report of Tzitzikas et al. (2006), vicilin had an amino acid content that is generally 80 % similar to that of vicilins, as well as a particularly prolonged and charged N-terminal chain of 122 to 166 amino acid residues, except for one cysteine and one methionine residues per convicilin subunits.

1.1.2 2S albumins

Albumin (2S), accounting for 18-25% of total protein, is water soluble metabolic and enzymatic protein including plenty of important amino acids such as tryptophan, lysine, threonine, and methionine (Lam et al., 2018). According to Klupšaitė & Juodeikienė (2015), albumin comprises components, for instance, enzymes, protease inhibitors, amylase inhibitors, and lectins. Nevertheless, albumins are particularly susceptible to oxidation due to their high cysteine content. The 2S albumin fraction is exceedingly variable in composition and has a low molecular mass, ranging from 5 to 55 kDa. Within this protein group, two primary fractions have been found: a bigger albumin protein called PA2 (5-10 % of total protein) composed of two polypeptides (molecular mass of ~ 26 kDa) and a smaller one called PA1 (4-7 % of total protein) with a molecular mass of 4-6 kDa (Boye, Zare & Pletch, 2010). Notable in the soluble fraction is the presence of lipoxygenase, an enzyme responsible for the oxidation of fatty acids with a molecular mass of 93 kDa; this protein is frequently associated with lipids due to its affinity for fat (Saurel, 2020).

PA1 albumins (10-11 kDa) are composed of two different polypeptides of 6 kDa

(PA1a, pI 5.3-5.4) and 4 kDa (PA1b, Pi = 9.8) connected in dimers through non-covalent interactions (Bérot, Le Goff, Foucault, & Quillien, 2007). Both PA1a and PA1b contain extremely high cysteine levels (7.5 and 16.2 %, respectively), suggesting that PA1b might be utilized as an insecticide in biocontrol (Gressent, Da Silva, Eyraud, Karaki, & Royer, 2011; Eyraud, et al., 2013). PA2 albumins have a molecular mass of (a) 25-26 kDa or (b) 24-25 kDa, linked by non-covalent bonds, with an isoelectric point of 5.2 （O'Kane et al., 2004b). Each subunit includes three cysteine residues in the chain with an intra-disulfide bond and a free sulfhydryl group.

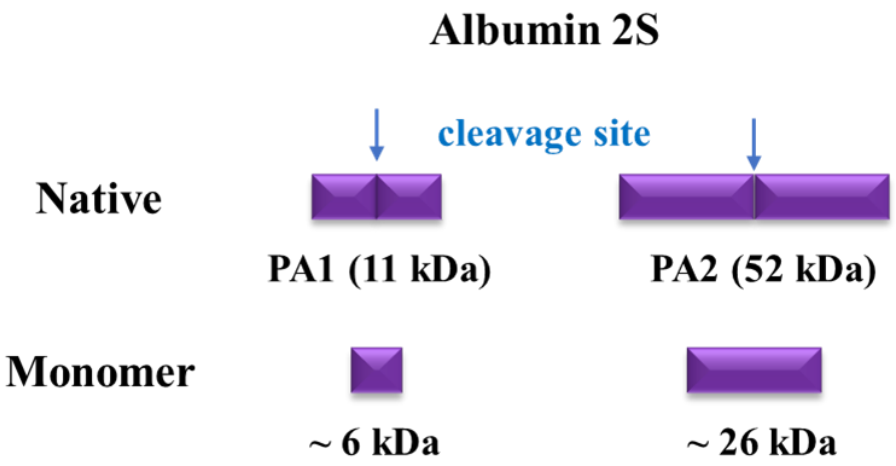


Figure 1-3 Assembly of constituent 2S Albumin according to Tzitzikas et al., 2006.

1.1.2 Amino acids of pea proteins

Table 1-2 displays the mean amino acid content of separated pea legumin, vicilin, and convicilin fractions, as well as soybean 11S (glycinin) and 7S (conglycinin) fractions. The protein composition of legumes is high in essential amino acids (lysine, isoleucine, glutamic and aspartic acids, and arginine) (Savage & Deo 1989).

The amino acid contents of pea globulins appear to be very comparable to those of soy globulins. According to O'Kane et al. (2004b), the concentration of sulfur amino acids in soy globulins is low and considerably lower in pea globulins. It has been found that the amino acid sequences of legume globulins (7S and 11S) are identical; however, the content of these amino acids differs from species to species (Kimura et al. 2008) and relies on cultivar and environmental conditions, as well as the process of protein extraction (Stone et al. 2015b).

Table 1-2: Mean amino acid composition of the protein fractions of peas and soy 100 g

of protein (according to [1]Derbshyre, Wright, & Boulter, 1976; [2] Emmert & Baker, 1995; [3]Gueguen, 1991; [4]Savage & Deo, 1989; [5]Jackson, Boulter, & Thurman, 1969; [6]O'Kane et al., 2004b; [7]Riblett et al., 2001; [8]Helmick et al., 2021).

Amino acids	Pea protein			Soy protein	
	Legumin 11S[1,4,5]	Vicilin 7S[1,4,6]	Convicilin[8]	Glycine 11S[6,7]	Conglycinin 7S[2,7]
Essential					
Threonine	3.4±0.1	3.4±0.6	2.87	1.3±0.0	1.3±0.1
Valine	4.7±0.3	5.1±0.8	5.73	4.3±0.9	3.4±0.2
Methionine	0.6±0.1	0.5±0.2	0.36	1.1±0.1	0.9±0.2
Isoleucine	4.8±2.2	5.1±0.1	5.72	4.3±0.9	3.8±0.1
leucine	7.8±0.3	9.1±0.1	11.98	11.7±5.5	5.3±0.6
Tyrosine	2.5±0.7	2.7±0.6	2.71	3.1±0.4	3.1±0.4
Phenylalanine	4.1±.7	6.1±0.1	4.64	7.7±3.2	6.2±0.7
Lysine	4.8±0.3	7.3±1.1	7.66	6.6±2.9	7.7±1.0
Histidine	2.6±0.2	2.4±0.5	1.82	1.7±0.4	1.9±0.4
Arginine	1.25±0.2	6.9±0.8	6.85	6.1±1.4	9.1±0.5
Tryptophan	0.8±0.4	0.1±0.0	0.0	0.7±0	0.1±0.1
Not essential					
Cysteine	0.7±0.1	0.1±0.0	0.26	1.8±0.5	1.1±0.1
Proline	5.0±0.6	4.0±0.9	4.79	7.6±1.4	7.3±0.2
Aspartic acid	12.1±0.6	12.0±0.0	4.58	9.0±4.4	8.8±1.5
Serine	5.9±1.0	6.2±0.7	7.17	5.8±1.7	7.9±0.1
Glutamic acid	19.6±2.1	18.5±1.3	10.12	16.2±0.5	22.1±2.3
Glycine	6.2±2.0	3.7±1.1	4.64	5.3±1.4	5.8±0.4
Alanine	5.5±1.2	3.7±1.2	4.48	5.7±0.3	5.5±0.1

1.1.3 Extraction processes of pea protein

According to Grandviewresearch (2019), the pea global market is expected to grow at a compound annual growth rate (CAGR) of 7.6% between 2020 and 2027. In order to facilitate pea proteins application in products, the structural and functional properties of peas after different extraction methods must first be explored (Lam et al., 2018). Protein isolates of varying quality can be obtained using various procedures, on which their future physicochemical characteristics will depend, for example, pH, ionic strength, number of washes, temperature, extraction equipment, duration of solubilization, filtering or purifying process (Feyzi, Milani, & Golimovahhed, 2018; Shanthakumar et al., 2022). Figure 1-4 summarizes the main steps in the extraction of pea globulins. The seeds are first treated, and then the proteins are extracted by either a dry or wet process from concentrations acquired by the dry method. According to the previous paper by Stone et al. (2015b), based on the extraction methods, pea proteins are available in a variety of forms, including flours (20-50 % of protein), concentrates

(50-65 % of protein), and isolates (70-92 % of protein). In addition, non-protein ingredients such as water, starch, lipids, and mineral salts can be reduced or removed by extraction step to obtain what are known as raw vegetable protein materials (VPP).

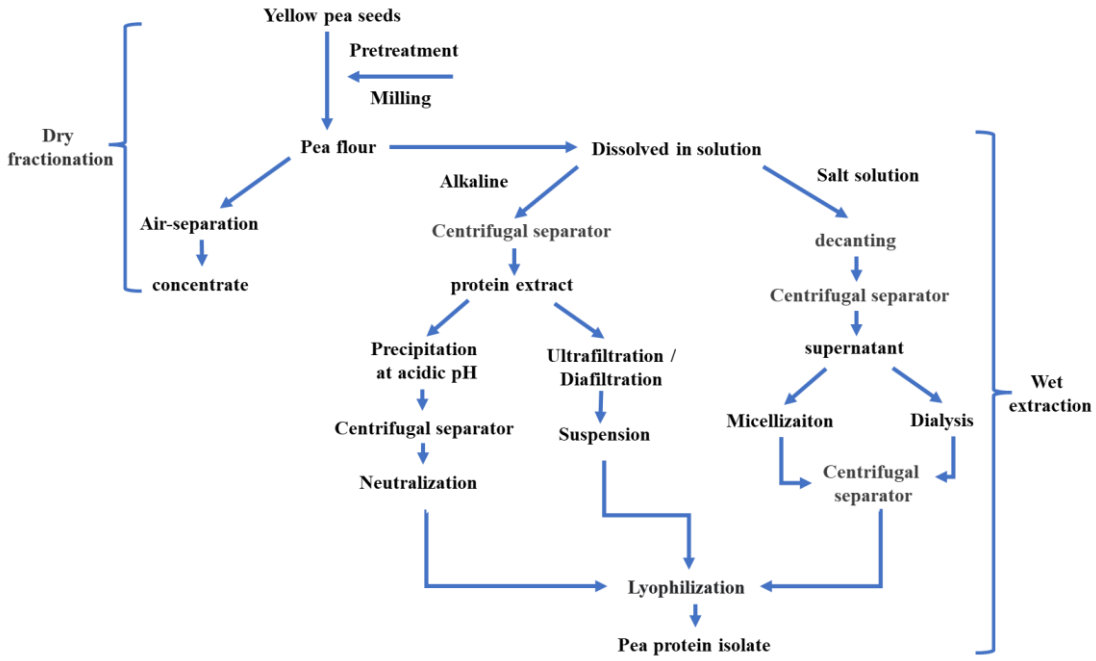


Figure 1-4: Main steps in the extraction of pea protein materials, according to Boukid, Rosell, & Castellari, 2021; Lam et al., 2018; Saurel, 2020.

1.1.3.1 Dry Fractionation: air classification and size reduction

As shown in Figure 1-4, after pretreatment, pea seeds will follow the dry fractionation which contains two keyways: milling to reduce the particle size and air classification to separate the particle based on their sizes. Thus, pea seeds will be milled through different methods (roller, pin milling, hammer, and stone) to form smaller structures to facilitate the separation of starch granules from the protein matrix, where roller miller is the most utilized technique (Boukid, Rosell, & Castellari, 2021). However, different milling parameters can result in two possible consequences: the obtained flour can be too small to separate protein and starch, or the granules can be coarse to not be separated as it is attached to protein and starch. Hence, optimal milling parameters should be chosen to produce consistent size while avoiding disruption of starch granule structure, which might impair starch gelatinization capabilities. In an air separation process, pea flour can be separated into two parts by turboseparation: small particles (protein-rich particles with a size of 1 - 3 µm) and coarse particles (starch particles with a size of 2 - 40 µm), based on size, shape, and density (Boukid, Rosell,

& Castellari, 2021). At the end of the dry extraction process, around 50-55 % of pea protein concentrate (fine fraction) based on dry matter, and around 67 % of pea starch (coarse fraction) based on the dry matter can be obtained (Pelgrom, Boom, & Schutyser, 2015; Saurel, 2020). Nonetheless, the purity of protein concentrate (50-55 %, dry basis) obtained from dry extraction is lower than protein isolates (over 80 %, dry basis) obtained by wet extraction (Pelgrom, Boom, & Schutyser, 2015; Rempel, Geng, & Zhang, 2019). The fundamental benefit of dry fractionation over wet extraction is that it preserves the intrinsic quality, nutritional and functional properties of the protein while consuming less energy and water, making it more cost-effective and hence more sustainable (Rempel, Geng, & Zhang, 2019; Saurel, 2020; Boukid, Rosell, & Castellari, 2021).

1.1.3.2 Wet extraction

The most commonly used typical method for producing pea protein isolates (highly concentrated protein fractions) is alkaline extraction/isoelectric precipitation (AE/IEP), which takes advantage of the high solubility of legumin proteins under alkaline conditions and the minimal solubility at isoelectric point (pI) between 4 and 5 using the same solubility for legumin and vicilin (Boye, Zare, & Pletch, 2010; Qiaoyun et al., 2017; Shanthakumar et al., 2022). Some factors during the extraction process, such as the solvent employed, pH, extraction duration, particle size, and flour/water ratio, impact the isolate yield, purity, and functionality of proteins, even though wet extraction may provide high isolation yields of 80-94 % (Hoang, 2012; Feyzi, Milani, & Golimovahhed, 2018). The water/flour ratio (v/w or w/v) and extraction pH were determined as the most critical factors by Hoang (2012). Boukid, Rosell, & Castellari, (2021) obtained the highest protein yield around 80 % at pH 9.96 and water/flour ratio at 15 v/w. However, at high extraction pH (> 10), pea protein extraction has been linked to increased starch swelling, leading to starch contamination in pea protein isolate products as reported by Lam et al. (2018) and Hoang (2012). Furthermore, while the procedure is under high alkaline pH, high temperature, or longer holding durations, the isolate is more prone to protein denaturation, decreasing its functionality and solubility, despite these conditions could lead to an increase in isolate production (Lam et al., 2018). The size of the flour particles and the type of solubilizing agent used also have an impact on the isolated yield. The ideal AE flour particle size is 100-150 μm, while

alkali-like NaOH and KOH are commonly used to enhance protein recovery and yield (Owusu-Ansah, & McCurdy, 1991; Del Mar Contreras et al., 2019).

As shown in Figure 1-4, defatted pea flour is dispersed in water, the pH is adjusted to an alkaline range using NaOH or KOH and stood for 30-180 min to maximize the protein solubility (Boye, Zare, & Pletch, 2010; Shanthakumar et al., 2022). Afterwards, the supernatant is collected by a centrifugal separator to remove the insoluble residues and adjusted to pH close to the pea protein pI (4.5 – 5.2) with hydrochloric or sulfuric acid solutions to precipitate globulins. Finally, the precipitate is collected, washed, resuspended in neutralized water at pH 7, and dried to obtain pea protein isolate (PPI). Another technique can be used to isolate proteins called ultrafiltration (UF) with diafiltration (Figure 1-4). Previous protein extraction and removal procedures for starch are identical to those used in IEP, then, expected protein isolates can be extracted through UF membranes with specific molecular weight cutoffs (Vogelsang-O'Dwyer, Zannini, & Arendt, 2021). This approach allows to produce more native protein extracts with superior functional characteristics and greater yields than isoelectric precipitation (Mondor, Tuyishime, & Drolet, 2012). Furthermore, this extraction approach has been demonstrated to be beneficial in eliminating antinutrient chemicals from isolate proteins (Boye, Zare, & Pletch, 2010). However, one disadvantage of this technique was that it could not modify the amino acid composition of PPI (Hadidi, Boostani, & Jafari, 2021).

Salt extraction (SE) extracts proteins from seed materials in salt solutions at neutral Ph, using the advantage of salting-in and salting-out phenomena of proteins (Lam et al., 2018; Shanthakumar et al., 2022). It is generally followed by a desalting progress to reduce the ionic strength of protein (Lam et al., 2018). Pea flour is mixed in a salt solution with a predetermined ionic strength for 10-60 minutes at a ratio of 1:10 (w/v). By settling, pouring, screening, and centrifuging, insoluble matter is removed. Since proteins precipitate at a variety of ionic strengths, the supernatant is desalted and dried before, subsequently, the concentration and salt combination are determined depending on the salting-in features of the protein to be extracted as well as the salting-out qualities of any undesirable proteins (Stone et al., 2015b). In general, protein salting-in occurs at low ionic strength, between 0.1 and 1 M. SE has the advantage of not requiring excessive alkaline or acidic pH or high temperatures. The extraction occurs at a natural pH level of 5.5-6.5. SE is more suitable for extracting vicilin and convicilin, because

legumin is less soluble in diluted salt solution than vicilin, while alkaline extraction tends to extract a slightly higher legumin content (Hadidi, Boostani, & Jafari, 2021; Shanthakumar et al., 2022).

The micellization technique causes protein precipitation by adding cold water in a 1:3 to 1:10 (v/v) ratio of high salt protein extract to water (Lam et al., 2018). Dilution of the protein solution causes solubilized proteins to regulate the low ionic strength via a sequence of dissociation events, resulting in the formation of reduced molecular aggregates. When the aggregates reach a critical protein concentration (CPC), they unite to form micelles, which are comparably low molecular weight entities that precipitate from liquids. The diluted solution is permitted to stand for some time to enhance micelle production (Shanthakumar et al., 2022). The precipitated protein can then be retrieved by centrifugation, washing, resuscitation, and spray drying. This method has the benefit of less denatured protein during extraction due to less dramatic pH swings, however, the poor protein recovery owing to a lack of protein solubilization is a drawback of the micellization approach (Stone et al., 2015b; Muranyi et la., 2016).

1.2 Egg white proteins

Eggs are abundant in nutrients that are needed daily for body tissues growth and maintenance and are also one of the few foods that are consumed worldwide, regardless of religion or ethnic group (Abeyrathne, Lee, & Ahn, 2013; Stadelman, Newkirk, & Newby, 2017; Zhu et al., 2018). The nutritional content in eggs comprises not only proteins and lipids, but also vitamins such as thiamin, riboflavin, vitamins A, B, D, E, and minerals such as Ca, P, K, Na, Mg, Fe, and Zn (Zhu et al., 2018). Eggs also offer various functional features that are significant in a variety of culinary items, such as foaming and emulsifying, as well as a distinct color and flavor.

Eggs are made up of three primary parts: eggshell (9 – 11 %, containing shell and shell membrane), egg white (60 – 63 %), and yolk (28 – 29 %), the introduction of each structure is demonstrated in Figure 1-5 (Li-Chan, & Kim, 2008; Abeyrathne, Lee, & Ahn, 2013; Mine, 2014). In brief, the eggshell is the egg's outermost layer, and it is made up of a frothy cuticle layer, a calcium carbonate layer, and two flexible membranes (inner and outer membrane) (Guha, Majumder, & Mine, 2019). This whole system keeps the egg white or albumen and egg yolk inside the egg, while also

preventing dangerous microorganisms from invading (Burley & Vadehra, 1989). The organic matter of eggshell and eggshell membranes is a complex mixture of proteins and polysaccharides, with proteins accounting for over 70% of the total organic matter (Tullet, 1987). Many bacteriolytic enzymes, including N acetylglucosaminidase and lysozyme, have been discovered in the eggshell membrane, as well as other components that may play a role in inhibiting Gram-negative and Gram-positive bacterial invasion (Guha, Majumder, & Mine, 2019).

Egg yolk is made up of plasma and granules that are suspended between the thin and thick albumen and supported by the chalazae. It is a rich source of vitamins and minerals. Despite containing a high level of cholesterol (11 mg/g of edible part) and lipids, serum yolk serves as a reservoir for huge amounts of hen's immunoglobulin (IgY), which might be deployed as an alternate material of antibodies for infectious disease prevention and therapy (Mine & Kovacs-Nolan, 2002; Puertas & Vázquez, 2019).

Egg white has been one of the well-known and wide-used protein sources all over the world since the dawn of recorded history. For human beings, egg white is an important reference protein source because it is rich in essential amino acids, has excellent functional properties, and remarkable nutritional values (Mine, 1995). Proteins are distributed in all parts of the eggs, but the majority of them are in egg white and egg yolks, containing 50 % and 40 % of egg proteins, respectively. Hence, egg white proteins are introduced in detail in the following sections.

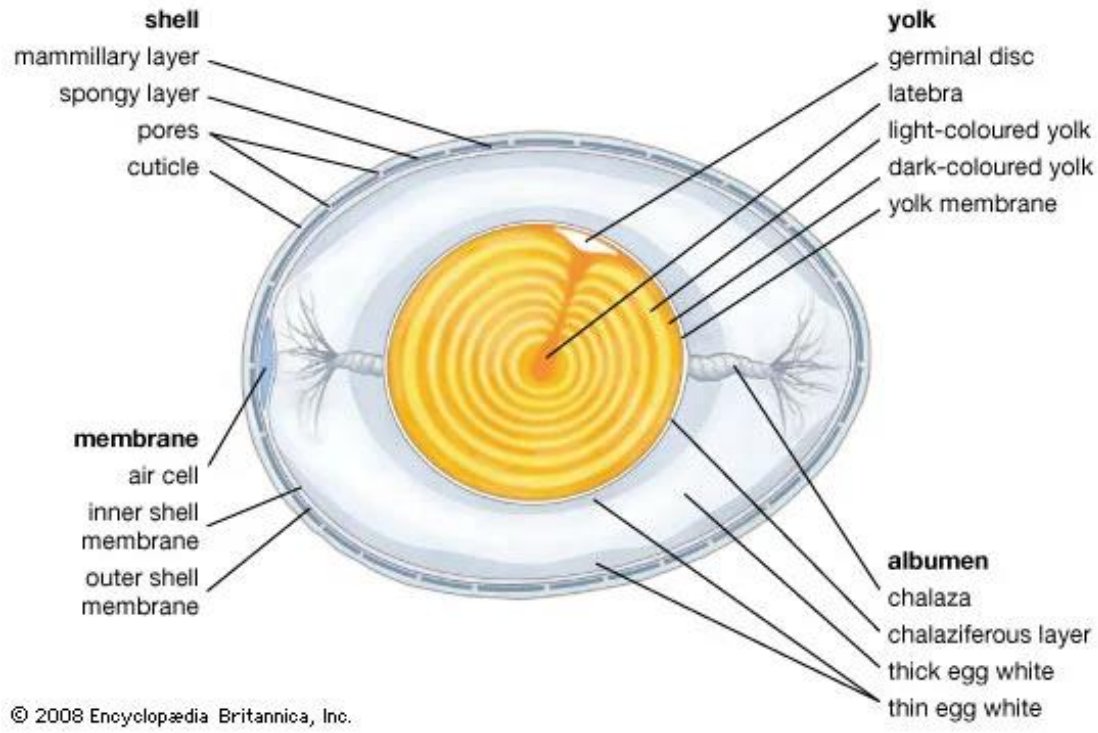


Figure 1-5 The structure of different components in an egg (Froning & Singh, 2021).

1.2.1 Composition of egg white (albumen)

Egg white usually contains about 11 % proteins which consist of more than 300 different kinds of proteins (Guérin-Dubiard et al., 2006; Mann, 2007). Many of them are still uncharacterized because of their low concentration. In general, egg white is composed of four individual components: chalaziferous layer, thin egg white, thick egg white, and chalazae (Figure 1-5). The thin layer comprises about 23.3 % of the egg white, which is further divided into two layers, namely the inner and outer thin layers. The thick or viscous layer accounting for the majority of egg white, around 57.3 %, separates the outer and inner thin layers (Brake et al., 1997; Li, 2006; Guha, Majumder, & Mine, 2019).

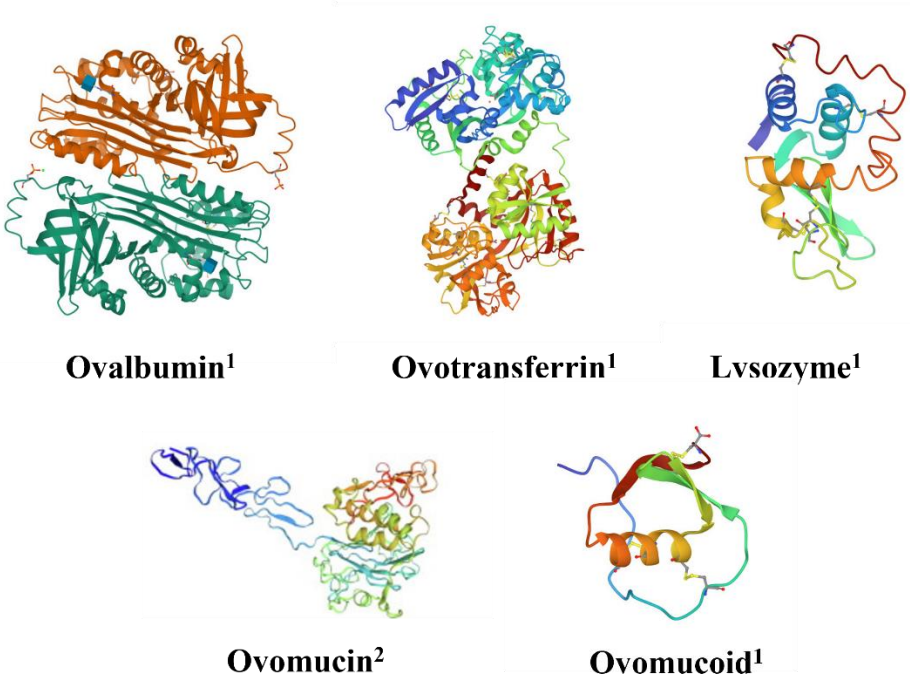


Figure 1-6 3D-structure of five major egg white proteins: ovalbumin, ovotransferrin, lysozyme, ovomucin, and ovomucoid. 1: download from Protein Data Bank, 2: Jalili-Firoozinezhad et al., 2020

Water is the major component of egg white, accounting for around 84 % - 89 % of total egg white weight. According to Li-Chan (1989), carbohydrates constitute 0.9 %, lipids occupy 0.03 %, protein content is around 10 to 11 %, and the rest is vitamins and minerals when talking about the solid part inside egg white. Egg proteins are widely known for their high nutritional quality and availability of all critical amino acids required for human nutrition and growth (Alleoni, 2006). However, among egg white proteins, ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme have been extensively studied due to their abundant presence in egg albumen. Physicochemical characteristics and structure properties of egg white protein are provided in Table 1-3.

Egg white proteins are globular (Figure 1-6) The structure and chemical composition of these proteins are presented in detail in the following sections.

Table 1-3 Physio-chemical and structural properties of proteins from egg white[1]

Protein	Content % of egg white	pI	MW / kDa	T_d / °C	characteristics	structures
Ovalbumin	54	4.5	45	84	Heat-stable phosphoglyco-protein, emulsifying and foaming agent, could change to S-ovalbumin	Serpin-like structure with a three-turn α-helical reactive center loop
Ovotransferrin	12	6.1	77.9	61	binds metal ions	An α-helix interconnect with two (N- and C-) lobes
Ovomucoid	11	4.1	28	70	Trypsin inhibitor, thermal stable	Three domains
Ovomucin	3.5	4.5-5.0	α: 150-220 β: 5500–8300	-	Structural protein maintaining viscosity and structure of egg white	Two (α-and β-) subunits linked by disulfide bonds
Lysozyme	3.4	10.7	14.4	75	Highly soluble and stable	Monomer, two (N- and C-) domains segregated by a helix-loop-helix motif
Ovoglobulin G2	4	5.5	36	92.5	Good foam agent	
Ovoglobulin G3	4	5.8	45	-	Good foam agent	
Ovomacroglobulin	0.5	4.5	760-900	-	Strongly antigenic protein, inhibits serine and cysteine proteinases	Four subunits linked by disulfide bonds
Ovoglycoprotein	1	3.9	24.4	-	An acidic glycoprotein	13.6% hexose, 13.8% hexosamine, and 3% sialic acid
Flavoprotein	0.8	4	32-	-	binds riboflavin	Two domains: N-terminal with the

			36			riboflavin-binding site and C-terminal with a negatively charged amino acid portion
Ovoinhibitor	1.5	5.1	49	-	Serine protease inhibitor, trypsin inhibitor	Seven domains
Cystatin	0.05	~5.1	12.7-13	-	Ficin and papain inhibitor	No carbohydrates
Avidin	0.05	10	68	85	Biotin-bingding	Four subunits

T_d: denaturation temperature; pI: isoelectric point; MW: molecular weight

1: Burley, 1989; Belitz, Grosch & Schieberle, 2009; Abeyrathne, Lee & Ahn, 2014; Sunwoo & Gujral, 2015; Rao, Klaassen Kamdar & Labuza 2016; Guha, Majumder & Mine, 2019; Wu, 2019; Jalili-Firoozinezhad, et al., 2020.

Table 1-4 amino acid composition of major egg white proteins*, **

	Ovalbumin *(1,3,4,7)	Ovotransferrin *(2,4,7)	Ovomucoid *(2,4)	Lysozyme *(2,4)	Ovomucin **(5,6)
Alanine	6.4	5.4	2.3	5.6	5.6
Valine	7.1	7.6	6.0	4.8	6.6
Leucine	9.5	8.6	5.1	6.9	6.4
Isoleucine	6.3	4.5	1.4	5.2	5.1
Proline	3.6	4.7	2.7	1.4	4.3
Phenylalanine	6.9	5.5	2.9	3.1	4.7
Tryptophan	1.2	3.4	0.3	10.6	-
Methionine	4.9	2.0	1.0	2.1	1.8
Tyrosine	3.5	4.6	3.2	3.6	3.5
Glycine	3.1	5.2	3.8	5.7	7.6
Serine	7.8	6.1	4.2	6.7	9.1
Threonine	4.2	5.1	5.5	5.5	7.7
Cystine/2*	1.1	3.4	6.7	6.8	6.4
Arginine (+)	5.7	7.3	3.7	12.7	3.2
Histidine (+)	2.3	2.4	2.2	1.0	2.1
Lysine (+)	6.3	9.1	6.0	5.7	6.3
Aspartic acid (-)	9.3	12.1	13.0	18.2	10.1
Glutamic acid (-)	15.4	11.7	6.5	4.3	9.7

*: mean amino acid value calculated from literature measured by g of amino acid per 100 g of protein,

**: mean amino acid value calculated from literature measured by the number of residues / 100 amino acid residues, -: not determined

1: Ovalbumin Tristram, 1949; **2**: Lewis et al., 1950; **3**: Smith & Back, 1970; **4**: Messier, 1991; **5**: Adachi et al., 1973; **6**: ITOH et al., 1987; **7**: Liu, Liu & Zhang, 2012.

1.2.1.1 Ovalbumin

Ovalbumin (OVA) occupies around 54 % of the total egg white proteins (Abeyrathne, Huang, & Ahn, 2018). Ovalbumin is a monomer, phosphorylated glycoprotein made up of complete three subunits having different phosphate groups along with a carbohydrate group attached to its N-terminal (Li-Chan, Powrie, & Nakai, 2017). Three ovalbumin fractions (A1, A2, and A3) were discovered by electrophoretic techniques according to Vadehra, Nath, & Forsythe (1973); Osuga & Feeney (1977), and Zabik (1992). Differences between these fractions are the phosphorus content of

their molecules, A1, A2, and A3 having 2, 1, or 0 phosphate groups, respectively (Vadehra, Nath, & Forsythe, 1973; Zabik, 1992). Ovalbumin has a molecular weight of 45 kDa and 386 amino acids, as well as two genetic polymorphisms at 290 (Glu/Gln) and 312 (Asn/Asp) (McReynolds et al., 1978). The isoelectric point (pI) of ovalbumin is 4.5 (Nisbet et al., 1981). Ovalbumin is distinct from other egg albumen proteins in that it includes 3.5 % carbohydrates and six cysteine residues, two of which are connected by a disulfide bond, while the remaining four residues have free sulfhydryl groups. According to Fothergill & Fothergill (1970), one of the four sulfhydryl groups is only reactive when the protein is denatured, the other three staying in their original states. Also, Vadehra, Nath, & Forsythe (1973) said that these kinds of the group could be denatured by heat, surface absorption, in films, agitation, or the action of numerous denaturant agents.

In addition, the N-terminal and C-terminal amino acid end with acetylated glycine and proline, respectively (Abeyrathne, Lee, & Ahn, 2013). Half of the molecule's residues are hydrophobic, while 30% are acidic and charged amino acid residues, leading to the isoelectric point of 4.5 (Huopalahti et al., 2007). Ovalbumin has a heat-stable form, S-ovalbumin, which formation depends on temperature, duration time, and pH (Vadehra, Nath, & Forsythe, 1973; Alleoni, 2006). Huang et al. (2012), demonstrated that the conversion of ovalbumin to S-ovalbumin during storage had been related to an increase in pH and storage time. Donovan & Mapes (1976) performed a differential scanning calorimeter (DSC) to identify the Td of S-ovalbumin, which showed that it was 92.5 ℃, which is higher than the Td of ovalbumin (84 ℃). S-ovalbumin has a slightly lighter molecular weight than ovalbumin and its relative proportion in egg white may rise with the duration of storage, from 5 % in fresh eggs to 81 % after 6 months of storage, despite the eggs being stored at a low temperature, around 4 ℃ (Huang et al., 2012). Furthermore, OVA's structure and features might change during storage. For example, the proportions of α-helix and β-sheet are reduced, while the proportions of β-turns and random coils are increased (Sheng et al., 2018).

Ovalbumin is also the main allergen in egg white, causing immunoglobulin E

(IgE)-mediated allergic responses (Caubet & Wang, 2011). The ovalbumin epitopes, which bind IgE are mostly composed of polar, charged, and hydrophobic amino acids and these sequences are generally composed of β-sheet and β-turn structures. The only allergenic epitope which comprises an alpha helix is Asp95-Ala102 (Kim, 2002).

1.2.1.2 Ovotransferrin

Ovotransferrin, also known as conalbumin, was shown to have the ability to bind iron (Williams, 1968). It is a monomeric glycoprotein containing 686 amino acids with a molecular weight of 77.9 kDa (Wu & Acero-Lopez, 2012). It accounts for around 12 % of the total egg white proteins (Désert et al., 2001; Bou Abdallah & El Hage Chahine, 1998). Ovotransferrin has approximately 15 disulfide bonds (6 in N-lobe and 9 in C-lobe) and no free sulfhydryl groups (Zabik, 1992). One molecule of ovotransferrin can bind 2 iron molecules and transports iron throughout the body. It was reported that Fe^{+3} can be successfully linked to ovotransferrin at a pH higher than 7 but easily released at a pH lower than 4.5 (Ko & Ahn, 2008; Guérin-Dubiard et al., 2006). Ovotransferrin is divided into two globule lobes (N- and C- lobes) linked by an α-helix, with each lobe consisting of two different α-domains and β-domains. These two domains are connected by antiparallel β strands that open and close through a hinge (Huopalahti et al., 2007). The N and C lobes are linked through noncovalent, mainly hydrophobic interactions (Charter & Lagarde, 2014). According to Lambert et al. (2005), each lobe may reversibly bind one Fe^{3+} ion and one bicarbonate anion but with different iron-binding capabilities, in which the iron-binding constant for the C-terminal lobe is 1.5×10^{18} and 1.5×10^{14} for the N-terminal lobe.

The structure stability is governed by disulfide bonds (Rabbani et al., 2011), and the iron binding capability confers antimicrobial properties to ovotransferrin (Alleoni, 2006). Ovotransferrin is the most heat-sensitive protein, denatured and aggregated between 53 and 65 ℃, and altering egg white viscosity and early gelation (Chaiyasit et al., 2019; Iwashita, Handa, & Shiraki, 2019; Jalili-Firoozinezhad et al., 2020). Ovalbumin, on the other hand, can suppress the thermal aggregation of ovotransferrin

at temperatures lower than its T_d (Iwashita, Handa & Shiraki, 2019). Moreover, Iwashita, Handa, & Shiraki, (2019) proved that non-covalent interactions enabled native lysozyme to precipitate soluble ovotransferrin aggregates and suppressed electrostatic repulsion between soluble ovotransferrin aggregates. Ovotransferrin operates similarly to lactoferrin in milk, and both of them have iron scavenging and iron delivery functions (Abdallah & Chahine, 1999).

1.2.1.3 Ovomucoid

Ovomucoid is a glycoprotein, widely recognized as a trypsin inhibitor (Abeyrathne, Lee, & Ahn, 2013). It constitutes 11 % of the total egg white proteins and is thermally stable (Li-Chan, 1989). The protein consists of 186 amino acids with a molecular mass of 28 kDa and a pI of 4.1 (Kovacs-Nolan et al., 2000; Benedé et al., 2013). Ovomucoid structure contains 9 disulfide bonds and three distinct domains (I, II (α-type), and III (β-type)) which are crosslinked only by the intra-domain disulfide bonds and lead to structure stability (Figure 1-6) (Stevens, 1991). Domain II has the active site for the trypsin inhibitory activity inside. A previous study by De Oliveria et al., (2009), found that ovomucoid was classified as a single-headed inhibitor of trypsin, which means that each ovomucoid molecule binds trypsin at a ratio of 1:1, and its 3D structure was held together by three disulfide bonds. Domain III differs from domains I and II in that the disulfide bonds between its first and second cysteine residues are shorter (Zhu et al., 2018).

Chicken ovomucoid is one of the major egg white allergens, which plays an important role in the pathogenesis of IgE-mediated allergic responses (Mine & Zhang, 2001, 2002a; Mine & Rupa, 2004). According to Kovacs-Nolan et al. (2000), peptides produced from ovomucoid utilizing pepsin have IgE binding activity and preserve trypsin inhibitory properties. Besler, Steinhart, & Paschke (1997) discovered that the ovomucoid epitopes which were responsible for the IgE binding were exclusively found on the protein backbone and not the carbohydrate groups. It was discovered through epitope mutational research that charged amino acids (lysine, glutamic acid, and

aspartic acid), polar amino acids (cysteine, tyrosine, threonine, and serine), and hydrophobic amino acids (glycine, leucine, and phenylalanine) are essential for antibody binding (Mine & Zhang, 2002b). Numerous experiments have been done to change the content and the structure of the allergic ovomucoid epitopes (Mine & Rupa, 2003), such as heating with wheat flour (Kovacs-Nolan et al., 2000; Kato et al., 2001), gamma irradiation along with heating (Lee et al., 2002), deglycosylation by endo-beta-N-acetylglucosaminidases (Yamamoto et al., 1998), and genetic modifications (Rupa & Mine, 2006). Under thermal or enzymatic hydrolysis, the allergenic action of ovomucoid remains constant (Julià et al., 2007). This kind of feature, however, may be exploited to identify eggs in food items even after heat processing.

1.2.1.4 Ovomucin

Ovomucin is another important protein in egg white as it accounts for about 3.5 % of the total egg white proteins (Abeyrathne, Lee, & Ahn, 2013). It is a sulfated glycoprotein that is responsible for the gel-like structure of egg white. The protein is divided into two parts: the soluble part (the primary component of the inner and outer egg white), and the insoluble part (only in the thick egg white portion) which is accounting for the insoluble gel-like fraction of thick albumen (Huopalahti et al., 2007; Omana & Wu, 2009). According to Tominatsu & Donovan (1972), the molecular weight of insoluble ovomucin in 6.5 M guanidine hydrochloride was calculated to be 23×10^3 kDa. The molecular weight of soluble ovomucin has been reported to be in the range of 5.6 to 8.3×10^3 kDa (Miller, Kato, & Nakai, 1981; Hayakawa & Sato 1976). These discrepancies in molecular weights are most likely attributable to variances in measurement methods and circumstances, as well as ovomucin heterogeneity.

Both soluble and insoluble fractions are made up of two subunits, α-ovomucin and β-ovomucin, with different carbohydrate levels. β-ovomucin is richer in carbohydrates (60 %) than α-ovomucin (16 %) (Omana & Wu, 2009; Shan et al., 2020). Previous research has revealed that ovomucin has at least three types of carbohydrate chains, which are constituted of galactose, galactosamine, sialic acid, and sulfate with a

molecular ratio of 1:1:1:1. On average, 33 % of ovomucin is carbohydrates (Mine, 2008). α-ovomucin is homogeneous with an MW of 150-220 kDa, whereas β-ovomucin is heterogeneous with an MW of 5500-8300 kDa (Itoh et al., 1987; Omana & Wu, 2009; Shan et al., 2020; Jalili-Firoozinezhad et al., 2020). The soluble fraction contains 40 α-subunits and 3 β-subunits, while the insoluble fraction consists of 84 α- and 20 β-subunits (Robinson & Monsey, 1971; Omana & Wu, 2009). There are two distinct subunits of the α-subunit, α1, and α2 (Abeyrathne, Huang, & Ahn, 2018). The α-subunit is mostly made of acidic amino acids such as glutamic acid and aspartic acids (Omana & Wu, 2009), whereas the β-subunit is primarily composed of serine and threonine (Robinson & Monsey, 1971). A study by Toussant & Latshaw (1999) discovered that the fraction of β-subunit or its glycosylation was positively correlated with the quantity of ovomucin present in the thick albumen. Many of the functional and biological features of egg white are attributed to ovomucin. It has an important function in the thinning of egg white during long-term preservation. Ovomucin has high thermal stability and a proclivity to interact with other proteins (Lesnierowski & Stangierski, 2018). Egg white thinning can be caused by either the disruption of the ovomucin-lysozyme complex or the decrease of disulfide bonds, which leads to the degradation of ovomucin (Abeyrathne, Lee, & Ahn, 2014). It is also well-known for its excellent emulsifying and foaming properties (Mann, 2007). Furthermore, ovomucin's physiologically active glycoproteins make it an appealing candidate for food and nutraceutical research.

1.2.1.5 Lysozyme

Lysozyme is a highly basic protein and is enriched in egg white compared to other forms of lysozyme found in nature. Egg white lysozyme (3.5 % of total egg white proteins) is made up of a single polypeptide chain of 129 amino acids and has a molecular weight of 14.4 kDa with a pI of 10.7 (Guha, Majumder, & Mine, 2019). Lysozyme differs from other proteins found in egg white and other foods due to its high solubility and stability. Although the lysozyme is a monomer in eggs, it is also frequently found as a dimer with thermal stability. Lysozyme contains two domains in

its three dimensions: an N-domain containing antiparallel β-sheets and a C-domain composed of 4 α-helices (Figure 1-6) (Lesnierowski & Kijowski, 2007; Tokunaga et al., 2013). Recent studies found that a helix-loop-helix motif located on the top side of the enzyme's active site separated the two domains (Ibrahim, Thomas & Pellegrini, 2001; Mine, Ma, & Lauriau, 2004). All of its polar groups (hydrophilic) are on the surface, while the majority of its hydrophobic groups are buried in the enzyme particle's core. Egg lysozyme is cross-linked through four disulfide bonds to stabilize its tertiary structure as well as cohesion (Kato, 2006).

Lysozyme preferentially binds to negatively charged proteins in the egg albumen for instance ovalbumin, ovomucin, and ovotransferrin (Wan, Lu, & Cui, 2006; Abeyrathne, Lee, & Ahn, 2014; Iwashita, Handa, & Shiraki, 2019). The chalaza and the chalaziferous layer are mainly made up of the lysozyme–ovomucin complex. However, many studies are still being conducted to further investigate its structure and function. Several studies have also been carried out to explore the structural changes of lysozyme caused by various conditions, such as aqueous-organic solvent combinations (Griebenow & Klibanov, 1996), pH variations (Babu & Bhakuni, 1997), co-crystallization in presence of different alcohols (Deshpande, Nimsadkar & Mande, 2005), sorbitol addition (Petersen et al., 2004), in presence of thiol reagents (Raman, Ramakrishna & Rao, 1996), and supercritical CO_2 treatment followed by heat treatment (Liu, Hsieh & Liu, 2004). Huopalahti et al. (2007), investigated that action of lysozyme remained unaffected by high temperatures (100 ℃) or acidic solutions (pH 3.0–4.0). It may, however, be readily and quickly deactivated by interacting with thiol compounds. Furthermore, lysozyme aggregates at temperatures below 80 °C, and neutral pH affects its functional properties (Iwashita, Handa, & Shiraki, 2017).

1.2.2 Methods to purify egg white

Several methods have been used to separate and purify proteins from egg white. Initially, salts such as ammonium sulfate, sodium chloride, or potassium chloride were used to separate ovalbumin (Warner & Weber, 1951), ovotransferrin (Fraenkel-Conrat

& Feeney, 1950), ovomucin (Brooks & Hale, 1959), and phosvitin (Mecham & Olcott, 1949). However, the purity of these isolated proteins was very low. Chromatography methods are now commonly used to separate egg proteins. Ovalbumin (Sakakibara & Yanagisawa, 2007), lysozyme (Li-Chan et al., 1986), ovotransferrin (Croguennec et al., 2001), and phosvitin (Lei & Wu, 2012) were separated by ion exchange chromatography. Ovotransferrin (Al-Mashikhi & Nakai, 1987), hen's immunoglobulin (IgY) (Jiang et al., 2016), and lysozyme (Junowicz & Charm, 1975) can be separated by affinity chromatography. These chromatographic methods can improve the yield of high-purity proteins. Nonetheless, they are not suitable for large-scale production because of the high cost, the slowness of the process, and the low capacity. Ultrafiltration is a suitable method for the separation and purification of egg proteins. Many egg proteins such as ovalbumin (Datta et al., 2009), lysozyme (Wan, Lu & Cui, 2006), and IgY (Hernandez-Campos et al., 2010) can be separated by ultrafiltration. However, these ultrafiltration methods are highly complex and are strongly affected by operating and physicochemical conditions; therefore, even though they yield purity values greater than 90%, they cannot be scaled up.

The first purification of lysozyme was done using a high concentration of ammonium sulfate; however, it led to the modification of the characteristics and morphology of the protein due to the high salt concentration used during extraction (Liu et al., 2004; Abeyrathne, Lee, & Ahn, 2013). The purification technique which is commonly used nowadays for lysozyme is cation exchange chromatography which makes use of the high pI value of the protein (Abeyrathne, Lee, & Ahn, 2013).

1.3 Type of interactions

It is vital and fascinating to investigate the partial substitution of animal proteins in processed goods with plant proteins to minimize the intake of animal proteins while keeping good nutritional qualities. To make this sort of partial replacement possible, animal and plant proteins should be able to interact to generate homogenous molecules

with functional qualities. The energy (intensity), type, and specificity of interactions found in protein assemblies might vary depending on the structure of the protein assembly and the physicochemical environment. There are 2 main molecular forces involved in protein-protein interactions, covalent disulfide bonds and non-covalent forces which encompass electrostatic interactions, hydrogen bonds, hydrophobic interactions, and Van der Waals forces (Alrosan et al., 2022). Furthermore, these types of protein interactions are likely to play a role in the formation and stability of protein assemblies, as well as modifying protein functional properties, for instance, solubility (Wang, Xu et al., 2019), interfacial behavior (Liang et al. 2016; Wang et al., 2020), and gelation (Sun, Wang, & Guo 2018). In the following section, we will introduce details information on disulfide bonds, hydrophobic interactions, electrostatic interactions, hydrogen bonds, and Van der Waals forces.

1.3.1 Disulfide bonds

Covalent bonds are strong chemical bonds and are formed by the sharing of electrons between atoms to generate electro pairs. These electron pairs are known as shared pairs or bonding pairs, and covalent bonding is the stable equilibrium of attractive and repulsive forces between atoms when they share electrons. Covalent interactions can occur inside the same protein as well as between proteins (Whitten et al., 1992).

In the case of the globular protein, this sort of connection exists between two portions of the polypeptide in the form of disulfide bonds formed between the thiol groups of the cysteine residues to produce inter or intramolecular bonds (Kinsella, 1982). Covalent bonds can be formed between the carboxylic groups and amine groups of amino acids in proteins to produce peptide-type linkages. This kind of connection typically necessitates the use of a catalyst, which can be physical (temperature, pressure), biological (particular enzymes such as transglutaminase), or even chemical (glutaraldehyde). They can also be formed between cysteine residues via their thiol groups, resulting in intra- and/or intermolecular disulfide bonds (Visschers & de Jongh,

2005). High temperatures and the presence of catalysts can conduce the creation of covalent bonds (Creighton, 1984). During prolonged heating, intramolecular disulfide bonds gradually convert to intermolecular bonds (Chaplin & Lyster 1986).

Covalent bonds have an important function in protein structure and stability. Intramolecular disulfide bonds, for example, provide protein stability by establishing the backbone's stability and structure while leaving flexibility to the side chains. (Scharnagl, Reif, & Friedrich, 2005). The protein aggregates of egg white are stabilized due to the formation of disulfide bonds by exposed SH groups caused by high pH, heat, and a combination of heat- and high-pressure treatment (Van der Plancken, Van Loey, & Hendrickx, 2005). Zhao et al. (2016) proposed that ionic and disulfide connections might potentially help to stabilize alkali-induced gels. Moreover, according to a previous paper conducted by Chen et al. (2015), the stability of alkali-induced egg white gel was shown to be closely linked to protein disulfide links. Therefore, these linkages have a favorable impact on the mechanical characteristics of heat gels and aggregates of egg white proteins (Wang et al., 2020; Alavi et al., 2020). Several investigations on pea proteins have demonstrated the importance of these interactions in stabilizing and sustaining the structure of heat aggregation (Mession et al. 2013, Wu et al., 2020).

1.3.2 Hydrophobic interactions

Hydrophobic interactions allow proteins to bind and assemble to create complexes. They are endothermic attractive and are formed in aqueous fluids between apolar molecules or molecules with apolar domains. These hydrophobic interactions are entropic in nature and very temperature dependent; they prefer high temperatures (Ray, 1971; Chandler, 2005).

The phrase "hydrophobic interaction" is commonly used in biochemistry to characterize the attractive interaction between hydrophobic elements of a system (i.e., proteins); these interactions are the consequence of the London dispersion (Holtrop et al., 2020). Although the exclusion of water from hydrophobic pockets is induced by both entropic contributions and water molecules' inclination to associate with one another via favorable hydrogen bond formation, the attraction between the hydrophobic

sections is produced by the London dispersion forces. These attractive forces play an important role in protein stability and folding (Privalov & Gill, 1988). With regard to globular protein, apolar amino acids are found inside it attributing to form hydrophobic interactions between these amino acids. Because of the way proteins are organized and folded, the majority of these amino acids are found buried in the core of proteins, where solvent accessibility is limited (except for membrane proteins whose apolar amino acids are exposed to the protein surface). As a result, the creation of hydrophobic linkages between proteins typically necessitates conformational and structural changes in the latter to expose the apolar amino acids on their surface. In the case of globular proteins, exposing solvent hydrophobic regions might cause aggregation via hydrophobic interactions.

Hydrophobic interactions play an important role in the effects of processing on the aggregation of egg white proteins, such as heat treatment (Matsudomi, Takasaki, & Kobayashi, 1991; Iwashita et al., 2017), alkali treatment (Chen et al., 2015; Zhao et al., 2016), pulsed electric field (Wu et al., 2015 & 2016), high pressure (Van der Plancken et al., 2005; Singh, Sharma, & Ramaswamy, 2015), high intensity ultrasound (Xiong et al., 2016). Several previous studies have demonstrated that when it comes to the thermal denaturation of soybean and pea proteins, high levels of exposure of hydrophobic groups favor protein aggregation and gelation of these systems at high protein concentrations, regardless of ionic strength or pH (Shand et al. 2007; Sun & Arntfield, 2010; Sun & Arntfield, 2012a; Tang et al., 2008).

1.3.3 Electrostatic interactions

Electrostatic interactions are considered weak bonds. These interactions occur between particles with a constant electric charge, such as dipoles or ions. These interactions can be either attractive or repulsive depending on the charges carried by the molecules or chemical groups; interactions are repulsive when the charges have the same sign, whereas interactions are attractive when the charges have opposite signs. The implementation and strength of electrostatic contacts are determined by the charges

of the proteins, particularly their intensity and distribution at the protein level, as well as the physicochemical parameters of the medium in which the protein complexes develop (pH, ionic strength, nature of the ions and temperature).

According to a previous study by Ben-Harb et al. (2018), protein aggregation dynamics and the electrostatic interactions between protein aggregates have a major impact on the rheological properties of pea and PPI-milk protein gels, prepared at a total protein concentration of 14.8 % at a ratio of 1:1 between pea and milk protein with 1 % NaCl. Salt concentration has a considerable impact on electrostatic interaction during gel formation as the higher the salt concentration, the more pronounced the electrostatic interaction (Tolano-Villaverde et al., 2016). Archer & Wang (1990) illustrated that at higher temperatures, electrostatic interactions may be expected to have a more favorable contribution to folding stability. Electrostatic repulsions between aggregates are often relatively weak at pH close to the isoelectric point (pI) of proteins and/or at high ionic strength, where aggregation/gelation of serum proteins happens via some other non-covalent interactions (Alting et al., 2002).

1.3.4 Hydrogen bonds

The hydrogen bonds are a dipole-dipole attraction in nature. A hydrogen bond is created through the interaction of a hydrogen atom (electron acceptor) with an electronegative atom (N, O) and another electronegative atom (electron donor) (Britannica, 2019). This bond is weaker than an ionic or covalent bond, but stronger than van der Waals forces. The energy of these bonds is determined by the electronegativity of the atoms as well as their orientation in the bond: the energy is greatest when the electronegative atom and the hydrogen atom engaged in the bond are aligned (MacLeod & Rosei, 2011). Kinsella & Whitehead (1989) demonstrated that hydrogen bond was not responsible for globular protein aggregation, instead of playing an important role in stabilizing the formed structure. This kind of stabilization has been observed for pea protein thermal gels (Sun & Arntfield, 2010), elastic pea protein hydrogels (Zhu et al., 2021), as well as heated egg protein gels (Chang & Chen, 2000).

1.3.5 Van der Waals attraction

Van der Waals interactions are non-specific bonds that exist between permanent dipoles, induced dipoles, and permanent and induced dipoles. It contains attraction and repulsion depending on the distance between the interaction partners. Essentially, when two atoms go closer, their attraction grows stronger until they are separated by the van-der-Waals contact distance. When two molecules are too close to each other, the potential energy due to repulsion increases dramatically, causing the assembly to become unstable, and repulsion to occur even when these molecules are neutral (Righetti & Boschetti, 2013). Van der Waals interactions contribute to the stability of the tridimensional protein structure. It is also involved in the self-assembly together with hydrogen bonds or covalent interactions between polymer chains during the formation of gels (Jaberi et al., 2020). At lower temperatures, van der Waals as a short-range interaction led to the gel reinforcement and continues to enhance the gel network with hydrogen bonding and covalent bonds (Ikeda & Nishinari, 2001; Shevkani et al., 2015).

1.4 Techno-functional properties

Proteins have physico-chemical functional qualities that contribute to the desired aspects of food as well as its physical behavior during preparation, transformation, and storage (Alleoni, 2006). Proteins' functional qualities are essentially determined by their physical, chemical, and structural features, which include size, shape, amino acid content and sequence, net charge, and charge distribution (Mine, 2014). These factors will determine the exposure of hydrophobic groups on the molecular surface and their interactions with oil (emulsion), air (foam), or other protein molecules, such as in gels or coagula (Li-Chan & Nakai, 1989; Alleoni, 2006); the architecture of their secondary (i.e., -helix, -sheet, and random structures), tertiary and quaternary structures; the presence of inter- or intrasubunit bonds (i.e., disulfide crosslinks), and ultimately reflect the nature of their functional properties (Damodaran, 1997).

Pea proteins are highlighted for their technological-functional features such as hydration properties (solubility, water, and oil holding capacity), surface properties (emulsifying and foaming capabilities), and protein structure and rheological properties (gelation and viscosity), in addition to their nutritional significance (Klupšaitė & Juodeikienė, 2015; Lam et al., 2018). These various features explain how these proteins work in a variety of food-related applications. The techno-functional qualities and applications in food of pea protein can be significantly influenced by protein concentration, concentration in distinct protein families, extraction procedure used, and processing conditions (pH, temperature, pressure, ionic strength, etc.) (Rui et al., 2011; Tang & Sun, 2011; Lu et al., 2019).

In terms of egg white, there are three well-known functions, such as gelation, foaming, and emulsion ability. Therefore, it is necessary to understand the different functional qualities of eggs leading to a significant implication for egg consumption and future use in the food industry. For egg white, it exists excellent research papers or reviews in the literature describing foaming (Li-Chan, & Nakai, 1989; Murray & Ettelaie, 2004; Lomakina & Mikova, 2006; Murray, 2007; Lechevalier et al., 2005; Lomakina, & Mikova, 2006; Raikos, Campbell, & Euston, 2007a; Lechevalier et al., 2017; Chen et al., 2019; Duan et al., 2018; Li et al., 2018a; Sheng et al., 2020; He et al., 2021) and emulsion properties (Drakos & Kiosseoglou, 2006; Niu et al., 2016; Xiong, Zhang, & Ma, 2016; Chang et al., 2017; Zhao, Cao et al., 2020) of egg white.

However, in this section, we will introduce the techno-functional properties of pea protein isolate and egg white, especially in terms of solubility (PPI) and gelation (PPI and EW).

1.4.1 Solubility of pea protein

Solubility is one of the most widely evaluated functional qualities of food proteins as it affects their functional properties such as foaming, emulsification, and gelation (Bogahawaththa et al., 2019). Protein solubility can be described as Protein-Solvent ↔ Protein-Protein + Solvent-Solvent, which is the equilibrium between protein–protein

(hydrophobic) and protein–solvent (hydrophilic) interactions (Hall, 1996; Heredia-Leza, Martínez, & Chuck-Hernandez, 2022). Water or buffer is the most widely used solvent in solubility. Several parameters can impact solubility including pH, ionic strength, temperature, solvent type, and protein concentration (Klupšaitė & Juodeikienė, 2015; McCarthy et al., 2016). Protein surface properties, especially the amount and distribution of hydrophilic and hydrophobic amino acid residues on the surface, can influence how a protein behaves in solution (Lam et al., 2018). Hydrophilic amino acid residues are more orientated toward the solvent interface in water. In contrast, most of the hydrophobic residues are buried in the inner of the protein to reduce free energy. Hydrophobic patches are formed on the protein surface by the remaining hydrophobic residues, which hinder solubility (Stone et al., 2015b).

The solubility of PPI is strongly pH-dependent with a minimal solubility between pH 4 and 6 regardless of the extraction method or pea cultivar, and the solubility profile performs a classic “U” shape (Boye, Aksay et al., 2010; Taherian et al., 2011; Withana-Gamage et al., 2011; Shand et al., 2007; Zhao, Shen et al., 2020). Solubility increases at pH levels above and below the pI due to electrostatic repulsion caused by positive and negative net charges on the protein surface (Lam et al 2018; Shanthakumar et al., 2022). A protein has the lowest solubility at its isoelectric pH because it carries a zero net charge, thereby decreasing electrostatic repulsive forces (Vihinen, 2020). Under these circumstances, hydrophobic interactions between nearby proteins can cause aggregation, and if the aggregates are large enough in size and number, precipitation is developed. Extraction and dehydration alter protein solubility by changing the hydrophobicity of the protein surface, exposing hydrophobic regions, and strengthening hydrophobic interactions between proteins (McCarthy et al., 2016). Boye, Aksay et al. (2010) demonstrated that a substantial difference in the solubility of pea protein isolates extracted by AE/IEP and UF, with AE/IEP resulting in solubility of 90 % at pH 1, but 29 % at pH 3, whereas UF isolates displayed solubilities of 60 % and 56 %, respectively. In addition, a study conducted by Stone et al. (2015b) highlighted that those isolates created by SE had the maximum solubility (86–91 %), followed by AE/IEP (63–64 %),

and by micellization (43–49%). The authors hypothesized that the lower solubility was caused by protein-protein hydrophobic interactions in the AE/IEP and micellization samples. Commercial pea protein may have decreased solubility in wet extraction due to heat-induced denaturation (and possible aggregation) during spray-drying (Chao & Aluko, 2018). Salts in solution can operate to screen the electric double-layer (i.e., diffuse, and stern layers) around the protein, thereby lowering the zeta potential and the quantity of electrostatic repulsive forces that occur. As a result, proteins assemble via hydrophobic interactions, hydrogen bonding, and Van der Waals attractive forces as if they had decreased or low net charges (Lam et al., 2018). In the case of salting-in, thiocyanate, perchlorate, barium, and calcium salts promote protein–water interactions and ordering of hydration layers surrounding the protein to improve solubility (Hall, 1996; Walstra, 2003; Damodaran, Parkin, & Fennema, 2007). In the case of salting-out, sulfate, hydrogen phosphate, ammonium, and potassium salts increase ion–water interactions, which operate to destabilize the hydration layers around the proteins, allowing the exposure of hydrophobic moieties to be exposed (Hall, 1996; Walstra, 2003; Damodaran, Parkin, & Fennema, 2007). Table 1-5 describes the main published studies on the solubility of pea proteins based on the extraction method used and the experimental physicochemical conditions applied to measure the solubility.

Several researcheres have used solubility comparisons to compare pea protein isolates to other plant protein isolates. In general, soy protein isolates were more soluble than pea protein isolates (Shand et al., 2007; Kimura et al., 2008; Zhao, Shen et al., 2020). The study by Ladjal-Ettoumi et al. (2016) highlighted that pea protein isolates had similar solubility to lentil protein or chickpea protein at pHs ranging from 2 to 8. As a result, it is extremely difficult to position the solubility of pea proteins concerning the large range of legume species that exist, depending on a variety of criteria (cultivar, technique of extraction, experimental settings, etc.). This techno-functional feature can be optimized by using different extraction methods that are more respectful of protein structure such as gentle fractionation (Figure 1-4) (Kornet et al., 2021b) or using high hydrostatic pressure, associated with solubility enhancement techniques, for instance,

ultrasound, microwaves, germination, enzymatic hydrolysis, and pH-shifting (Boukid et al., 2021; Jiang et al., 2017; Zhu et al., 2021).

Table 1-5: Summary of the main works on the solubility of pea protein isolate (from Lam et al., 2018; Ge et al., 2020).

Comparison	Materials used	Extracted conditions	Conditions for solubility assessment	Major results	References
Comparison between different pulses	CDC Golden peas, CDC Grandora Green chickpeas, Common Blaze Red and Green lentils, Mylese desi chickpea and Xena kabuli chickpea	Ultrafiltration (UF) and alkaline extraction (AE) /isoelectric precipitation (IEP)	10 mg /ml in water, pH 1-10	PPC-UF (60%) < PPC-AE/IP (90%) at pH 1.0; PPC-UF (56%) > PPC-AE/IP (29%); At pH 3.0 solubility of two chickpea was similar to each other, green lentil was similar to red but different at pH 4	Boye, Aksay et al., 2010
	Chickpea (CDC Frontier, Kabuli), faba bean (CDC SSNS), lentil (CDC Grandora), and pea (CDC Leroy) Defatted soy flour	IEP and salt extraction (SE)	1.0%, w/v 10 mm sodium phosphate buffer (pH 7.00)	IEP: Soy (97 %) > chickpeas (91 %) > lentil (97 %) > field beans (90 %) > pea (61 %); SE: soy (97 %) > lentil (90 %) > field beans (53 %) > pea (38 %) > chickpea (29 %); Extraction process significantly affected PPI	Karaca, Low, & Nickerson, 2011
	PPIc, PPI, SPIc, Soy protein isolate (SPI)	AE/IEP	0.5 mg/ml, pH 3-10	At pH 4.5, minimum solubility of PPIc, PPI, SPI and spic; >4.5 or <4.5; Solubility of PPI > PPIc, and SPI> spic; Solubity of SPI > PPI at all pH	Shand et al. (2007)
	Pea (Pisum sativum), chickpea (Cicer	AE/IEP	pH 2-8	At pH 4.5, minimum solubility of pea, chickpea and lentil; the	Ladjal-Ettoumi et

	arietinum) and lentil (Lens culinaris) cultivated in Algeria			solubility profiles for protein were pH-dependent	al., 2016
Combination of different pea cultivars and extraction methods	Commercial pea protein isolate (PPIc)	Pea protein isolate (PPI), different extracted solutions: water soluble fraction (WS), salt soluble fraction (SS), alkaline soluble fraction (AS), ethanol soluble fraction (ES)	1% (w/v) 0.01 M phosphate buffer pH 3-8	WS with the highest solubility at pH range ES insoluble in aqueous solutions all fractions had better solubility than PPI for pHs ≥ 7	Adebiyi & Aluko, 2011
	Pea (CDC Striker, CDC Meadow, and CDC Dakota) and PPIc	AE/IEP; SE; micellar precipitation (MP)	pH 7	Solubility: SE (85.7% to 91.1%) >IEP (62.7% to 64.4%) > MP (42.8% to 49.0%) > PPIc (5.0%) Extraction process significantly affected PPI solubility	Stone et al., 2015b
	Commercial soy protein isolate (spic) and PPIc	UF, Heat-acid precipitation (HP), acid precipitation (AP)	1g in 75 ml biditilled water pH 2-10	Solubility (at all pH) UF>AP>HP>PPIc; Minimum solubility between pH 3 and pH 5; Solubility for pH ≥ 7: UF > SPI > PA > PTA > PPI c	Fuhrmeister & Meuser, 2003
	Pea seeds under different lactic acid fermentation and time (analyzed total 12	AE, IEP	1.25% (w/v) protein concentration in 0.1 M NaCl	pH 4.5: low solubility (<11%) for extracts of all cultivars; pH 7: solubility (60-62%) for extracts of all cultivars	Arteaga et al., 2021

	numbers) and PPI		solution pH: 4.5 and 7		
	7 different pea cultivars CDC Striker (green), CDC Golden (yellow), Cooper (green), CDC Dundurn (dun), MRF042 (marrowfat), CDC Meadow (yellow), and Kaspa (dun)	IEP	pH 7	PPI (seven cultivars) solubility was significantly different, with 54% to 76% (pH 7.0)	Stone et al., 2015a
PPI, PV and PL	Pea flour	PPI; Fraction (PV) rich in vicilin (7S) purified from PPI then isoelectric precipitation; Fraction (PL) rich in legumin (11S) purified from PPI.	pH 2 - 10	PPI had the lowest solubility at all pH values; Solubility (at pH < 5): PL > PV > PPI; Solubility (at pH > 7): PL ≈ PV > PPI Minimum solubility of the 3 fractions between pH 5 and 6	Liang & Tang, 2013

1.4.2 Gel properties

1.4.2.1 Gelling properties of Pea protein

Gelation is an essential functional property of globular proteins because it is used to alter the texture of food and plays an important role in the sensory of many food products as well (Ge et al., 2020; Shanthakumar et al., 2022). Protein gelation is the formation of a three-dimensional network of molecular structures by protein molecules immersed in an aqueous solvent (Tomé et al., 2015; Shanthakumar et al., 2022). The mechanism of globular protein gelation is a complicated process that involves partial denaturation of protein molecules, progressive association or aggregation, and network creation (Mession et al., 2015; Ge et al., 2020).

In general, gelation can be generated by a physical process (heat-induced, high pressure) or a (bio)chemical process (chemical acidification or microorganisms, enzymatic crosslinking, salt addition) (Totosaus et al., 2002; Nicolai, 2019). For heat-induced gel, when a protein is heated above its denaturation temperature, at a higher concentration than its minimum gelling concentration (MGC), this causes partial unfolding of the protein and exposes the interaction sites, leading to intermolecular interactions, and eventually resulting in clustering of protein aggregates to form a spatial gel network. In terms of cold-induced gel, it performs a specific pre-heating treatment for a low-concentrated protein suspension at pH far from protein pI to prepare soluble protein aggregates in the absence of salts, and then cooled, the cold-set gelation is carried out by adding salts, acidifying agents, or enzymes, allowing it to assemble into network structure (Ge et al., 2020; Shanthakumar et al., 2022). Regarding cold gelation of pea protein, non-covalent and new disulfide linkages were involved in gelation (Mession et al., 2015). Several parameters have a great impact on gelation, such as extrinsic factors related to gelling process, containing pH, temperature, ionic strength, and intrinsic factors related to protein including protein conformation, concentration, the cultivar, and extracted procedures (Shand et al., 2007; Mession et al.,

2015; Ge et al., 2020; Shanthakumar et al., 2022).

Table 1-6: Main research work on the heat-induced gelation of pea proteins.

Method of gelation	Type of pea protein	Physico-chemical parameters of the initial dispersion	Parameters of the applied gelation process	Major conclusion	References

Heat-induced gelation	Three soluble pea extracts prepared in the laboratory A) Extract rich in globulins B) Purified legumin extract C) Purified vicilin extract	25- 30 µL of protein in 30 mm tris-HCl buffer, pH 7.2. Mixed globulin: 30 mm tris- HCl buffer, at different pH: 5.95- 8.6. Mixed globulin: 15%, 30 mm tris- HCl buffer at pH 7.1, with different NaCl concentrations: 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 m	Heated at 87 °C at different holding times: 0-45 min. Heated at 87 °C 20 min	A) Vicilin formed gel while legumin didn't gel under the same conditions B)Gel hardness of globulin decreased then the concentration of NaCl was $\geqslant$ 0.05 m C) Increase in the strength of the gel with the heating time (maximum strength after 20 min of heating) whatever the protein concentration. D) Formation of a gel for mixed globulin at pH 6.4 (maximum strength at pH 7.1)	Bora, Brekke, & Powers, 1994

	2 vicilin fractions: vicilin 1 and vicilin 2, from 2 pea cultivars (Silara and supra)	A) Minimum gelling concentration (MGC): protein concentration (6-16 %, w/v), pH 7.6, in 75mm potassium phosphate buffer b) Various parameters of samples in 75 mm potassium phosphate buffer: protein concentration 10% w/v, at pH 6.1; protein concentration: 2, 4, 6, 8, 10 %, w/v, at pH 3.8; protein concentration 10% w/v, NaCl (0-1 m), at pH 7.6; mixed of legumin/vicilin: 1/0.22, 1/0.57, 1/1.2, final concentration is 11% w/v, at pH 7.6	A) Heated at 100 °C for 30 min, cooled to room temperature, and stored at 4°C overnight b) Heated at 100 °C for 20 min, cooled to room temperature, and stored at 4°C overnight	A) Vicilin 1: MGC 10% w/v, turbid gels; vicilin 2: MGC 14% w/v, transparent gels b) Turbid gels were stronger than transparent ones c) Vicilin 1 (supra) gel was stronger than gel made of vicilin 1 (Silara) d) Concerning NaCl, a gel formed byvicilin2: at 0-0.1 m transparent, 0.2 m turbid, 0.5 m phase separation, and 1 m NaCl smooth brown gel; gels by vicilin 1: turbid and opaque at from 0 to 1 m NaCl e) No gelation after the addition of vicilin2 to the legume dispersion for the ratios 1/0.57 and 1/1.2; gelling not impacted by the addition of vicilin (1) to the legume dispersion	O'Kane et al., (2004a)

	Purified legumin fraction from peas by non-denaturation fractionation progress; glycinin purified from soybean by acid precipitation and	A) Minimum gelling concentration (MGC): protein concentration (8-16 %, w/v), pH 7.6, in 75mm potassium phosphate buffer b) legumin (8.4%), glycinin (6.6%), at pH 7.6, in 75 mm potassium phosphate buffer	A) Heated around 100 °C for 30 min, cooled to room temperature (1 h), and stored at 4 °C overnight b) Heated from 45 °C to 98 °C, holding at 98 °C for 30 min, cooling to 25 °C, and holding at 25 °C for 30 min, with different heating and cooling rate (0.5, 1 °C/min)	A) MGC: 8.4% for pea legumin, 6.6% for soy glycinin b) For both protein gels, a lower heating rate (0.5 1 °C/min) did not affect gel formation compared to a higher rate at 1 °C/min, while a lower cooling rate led to the formation of a stronger gel c) After reheating/recooling, pea legumin gels became stronger due to the rearrangement of gel networks, while glycinin gel was not	O'Kane et al., (2004b)

	Pea protein isolate (PPI) was prepared by alkaline and isoelectric precipitation from 5 pea cultivars (Solara, Supra, Classic, Finale, and Espac)	18% w/v protein concentration, in 75 mm potassium phosphate buffer at pH 7.6 20mm of n-ethylmaleimide (NEM), block thiols-blocking agent	Heated from 45 to 98 °C, kept at 98 °C (30 min), cooled to 25 °C, and kept at 25 °C (30 min) with different heating and cooling rate: standard: heating and cooling at 1 °C/min a) 0.5 °C/min heating, 1 °C/min cooling b) 1°C/min heating, 0.2 °C/min cooling	A) No impact of lower heating rate on gelation of all the cultivars b) With the highest legumin content, Solara formed a firmer gel at a lower cooling rate c) Supra and Classic only formed stronger gels with the presence of NEM at a lower cooling rate, indicating gel strength depended on the disulfide bond d) Strong and self-supporting gels cannot be formed in Finale and Espace due to rich vicilin level	O'Kane et al., (2005)

	Pea protein isolate, PPI (Pisane HD, Cosucra, Belgium), lupin protein isolate, LPI (lupie, Fraunhofer Inst., Germany), soy protein isolate, SPI (S974, ADM, Netherlands)	PPI (12.5, 16 %, w/w), SPI (10%, w/w), LPI (16% w/w) dispersed in demineralized water at pH 5.5	Heated to 90 °C for 15 min	A) Gelling ability: SPI > PPI > LPI, although SPI in lower protein concentration b) At 16% protein concentration, both PPI and LPI showed weak gel, with no significant difference on G' and G" c) In texture, SPI (1.23 N, 10% w/w) and PPI (1.34 N, 16% w/w) had firmer gels than LPI (0.43 N, 16 % w/w)	Batista et al., 2005
	Native pea protein isolate (PPIn), and soy protein isolate (SPIn) extracted by alkaline (pH 8) and acidic precipitation (pH 4.5) Commercial pea protein isolate (PPIc), soy protein isolate (SPIc)	A) Comparison of PPIc, and PPIn gels: 19.6 % w/w in 1% NaCl deionized water, pH 6.5 B) Optimization of the gelation of PPIc: 19.6 % w/w, PH 7.1 in demineralized water with different NaCl concentrations from 0-2%	A) Heating at 82 or 92 °C for 45 min, cooling and incubation at 4°C for 14 h B) Heating at 79, 82, 87, 92, or 95°C for 25 min, cooling and incubation at 4°C for 14 h	a) Gels stiffness: PPIc > PPIn; gels elasticity: PPIn > PPIc at studied two temperatures b) Firmer PPI c gels for higher heating temperatures c) Under the same conditions, SPIc gels are stiffer and more elastic than PPI c gels	Shand et al., 2007
	Soy protein isolate (SPI) obtained by alkaline	A) Study the influence of MTG: 1% w/w of	A) Heated at 92°C for 45 min (storage	a) Increase in stiffness and elasticity of PPIc gels with:	Shand et al., 2008

	extraction/ isoelectric precipitation; commercial pea protein isolate (PPIc), soy protein isolate (SPIc)	NaCl, 19.6 % w/w protein content, pH 6.5; various parameters: 0-0.7% w/w of MTG, incubation temperature (22-78 °C) and incubation time (10-80 min) B) Comparison with PPIc and PPIn gels: with 0.6 % w/w of MTG, deionized water, pH 6.5, 10% w/w protein content	at 4°C for 14 h) B) MTG heat-treatment at 50 °C (0, 30 min), 35 to 95 °C at a rate of 2 °C	- Increase in MTG concentration - The increase in the incubation temperature - Increased incubation time Elasticity and stiffness of gels: PPIc with 0.7% w/w showed a close value to SPIc without MTG b) gelling point for PPIn decreased in the presence of MTG 0.6 % w/w - No significant differences for PPIc gels with or without MTG	
	Commercial pea protein isolate (PPIc), soy protein isolate (SPIc); pea protein isolate (PPIs) prepared by salt extraction and dialysis	A) Minimum gelling concentration: 0.3M NaCl, 4–18% (w/v) PPIs; 0.3M NaCl, 8–20% W/V PPIc B) Gelling point: PPIs 5.5-18% w/v pH 5.65-5.7 0.3 M NaCl C) Comparison of PPIs, PPIc, SPIc, 14.5% w/v, 0.3M NaCl, pH 5.7	A) 95 °C in a water bath for 10 min B) Influence of heating and cooling rate: 25–95–25 °C at a controlled rate (0.5, 1, 2, 4 °C/min C) Heating and cooling: 25–95–25 °C at 2 °C/min	a) MGC: 5.5% PPIs, 14.5% PPIc b) Gelling point was independent of protein concentration; the gelling point was dependent on the heating rate at a lower heating rate (0.5-1.0 °C/min); the gelling point was independent of the heating rate at a high	Sun & Arntfield, 2010

				heating rate (2-4 °C/min c) Gel strength: PPIs > SPIc > PPIc	
	Commercial pea protein isolate (PPIc), soy protein isolate (SPIc); pea protein isolate (PPIs) prepared by salt extraction and dialysis	A) Influence of heating rate: 14.5% (w/v), 0.3 M NaCl B) Influence of cooling rate: 14.5% (w/v), 0.3 M NaCl C) Comparison of PPIc, PPIs, and SPIc: 10.5% (w/v), 0.3 M NaCl	A), B) Influence of heating and cooling rate: 25–95–25 °C at a controlled rate (0.5, 1, 2, 4 °C/min C) Heating and cooling: 25–95–25 °C at 2 °C/min.	a) At the same cooling rate (2 °C/min), the gelling temperature increased with increasing heating rate, but no statistical changes on G' b) At the same heating rate (2 °C/min), G' decreased with increasing heating rate, but no changes in gelling temperature c) Gel strength: SPIc > PPIs > PPIc	Sun & Arntfield, 2011a

	Pea protein isolate (PPIs) obtained by salt extraction and dialysis Commercial soy protein isolate (SPIc)	A) Minimum gel concentration (MGC): 2–5% (w/v) of PPIs, in 0.3 M NaCl buffer with 10 U MTG B) MTG treatment: 0.3 M NaCl pH 5.65, 10.5% w/v, 0-30 U/g C) comparison between PPIs, SPIc: 10.5%w/v, 0.3M NaCl, with or without MTG 10 U/g	A) i: Heated at 40 °C for 30 min; ii: from 40-95 °C, and kept at 95 °C 10 min, storing at 4 °C (for MGC) b) Heated and cooled: from 40-95-25 °C, (0.25, 0.5, 1, 2, 4 °C/min) c) Heated and cooled: from 40-95-25 °C, 2 °C/min	a) MGC around 3% w/v, with 10 U MTG b) Increased MTG concentration means increased G* led to stronger gel without changing relative elasticity; at the same concentration of MTG, lower rate of heating and cooling produced stronger PPI gels c) Rigidity of gels: with MTG: PPIs > SPIc, without MTG: SPIc > PPIs	Sun & Arntfield, 2011b
	pea protein isolates prepared by salt-extraction followed by dialysis	pea protein dispersion (10.5%, w/v) dissolved in solutions with two combined parameters: pH: 3-11 NaCl concentration: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1.0, 2.0 M	Heated: 25–95 °C at a rate of 2 °C/min, cooled: 95–25 °C at a rate of 2 °C/min	a) Pea protein gelation influenced by both NaCl and pH b) Higher NaCl concentration inhibited pea denaturation c) Significant stiffer gels formed at higher pH and lower salt concentration, while the significant stiffest gel achieved at 0.3 M NaCl at pH values below 6	Sun & Arntfield, 2011c

				d) Pea protein gelation was entirely inhibited at a NaCl concentration of 2.0 M.	
	Commercial pea protein isolate (PPIc); Four pea protein isolates were obtained by saline extraction or water extraction followed by ultrafiltration (UF) and diafiltration (DF) on membranes, different combinations of pHs of 6 and 7.5 were used at each extraction step: - PPI-a (0.06 M KCl, pH 7.5/6 for UF/DF, 25°C) - PPI-b (0.06 M KCl, pH 7.5/7.5 for UF/DF, 25°C) - PPI-c (H_2O, pH 7.5/6 for UF/DF, 25°C) - PPI-d (H_2O, pH 7.5/7.5 for UF/DF, 25°C)	A) Text 20 wt.% was first dispersed in phosphate buffer adjusted at pH 6.5 B)	A) Heated at 100°C for 60 min (storage at 4°C for 24 h) B) Heated from 20 to 90°C, (10°C/min)	a) Firmness of the gel: PPI-a > PPI-c > PPI-d > PPI-b > PPIc-no gel b) Rigidity of the gel: PPI-c > PPI-a > PPI-d > PPI-b c) gelling temperature: PPI-a > PPI-a > PPI-c > PPI-d > PPI-b > PPIc-no gel	Taherian et al., 2011

	Pea protein isolate prepared by salt extraction	A) Study of the effect of salts Protein dispersions = 14.5% (w/v) in either: distilled water, 0.3 M NaCl (pH 5.7), 0.3 M Na_2SO_4 (pH 5.9), 0.3 M CH_3COONa (pH 6.3) and 0.3 M NaSCN (pH 6) B) Study of the non-covalent bonds contributing to gelation by adding to the protein dispersions at 14.6% and 0.3 M NaCl: guanidine hydrochloride GuHCl (0, 0.3, 1.0, 3.0 M; interrupts hydrogen bonds and hydrophobic interactions; Urea (0, 2, 5, 8 M; interrupts hydrogen bonds and hydrophobic interactions) PG (0%, 5, 10, 15, 20%; interrupts hydrophobic	Heated from 25 to 95°C (0.5, 1, 2, 4 °C/min), cooled from 95 -25 °C (0.5, 1, 2, 4 °C/min)	a) Increased gel stiffness in the presence of various salts in water: Na_2SO_4 < CH_3COONa < NaCl < NaSCN b) Involvement of hydrophobic, electrostatic, and hydrogen bonding interactions in the formation and stiffness of pea protein gels c) Non-involvement of covalent bonds in the formation of pea gels d) Small contributions of disulfide bridges in improving the characteristics of gels	Sun & Arntfield, 2012a

		interactions and promotes hydrogen bonds and electrostatic interactions) C) Study of the covalent bonds (disulfide bridges) contributing to gelation at 14.6% and 0.3 M NaCl via: DTT (0, 0.05, 0.1, 0.15 M); NEM (0, 10, 15, 20, 25, 50, 100 mM); β-mercaptoethanol (0, 0.1, 0.2, 0.3 M)			

	Pea protein isolated prepared in 0.2 m NaCl followed by IEP from commercial green peas	150 mg/ml, ph3, 5, 7, 9 at different ionic strengths: 0.01 m $cacl_2$, 0.1 m $cacl_2$, and 0.3 m NaCl	Heated at 95 °C (30 min), cooled to 20 °C	a) Fine-stranded networks were formed at pH levels away from IEP and low ionic strength. b) Coarse networks were produced at a pH close to IEP and at high ionic strength. c) The capacity of the networks to elastically store energy was connected to the microstructure d) Higher ability of gels to elastically store energy when they have a less coarse structure	Munialo, et al., 2014b

	Pea protein isolate (PPI) extracted by IEP	Protein concentration: 100-150 mg/mL, at pH 3; Protein concentration: 10%, pH from 3-4.2	Heated at 95 °C, 30 min, cooling to 20 °C,	a) pH changes led to changes in pea protein structural network b) At pH 3.7, structural transitioning occurs in pea protein gels. c) Increase in firmness as pH increases d) Increase in the firmness of the gels and no significant alteration of the microstructure of the gels when protein concentration increases	Munialo et al., 2015
	A protein fraction from pea flour (Alimex) A protein fraction from the coarse fraction of pea flour A protein fraction from the fine fraction of pea flour	30% w/w Transglutaminase (MTG) present with a ratio (MTG/protein: 1/17.3)	heated firstly at 60 °C for 1 h, then heated from 60-90 °C for 30 min (0.1-6 °C/min), cooled to 25 °C (at 0.3 °C/min)	a) Starch-rich pea mixes might be used to make firm heat-induced gels b) The rates of heating and cooling were effectively employed to modulate gel stiffness. c) A solid protein gel was produced by enzymatic crosslinking of natural pea protein d) MTG makes it possible	Pelgrom, Boom, & Schutyser, 2015a

				to cross-link the protein from the fine fraction of flour (rich in protein) forming a firmer gel	
	Pea protein concentrate (PPC) Hydrolysis on PPC with different times: 25min (PPCH25), 120min (PPCH120)	Dissolved in water 12% w/w pH: 2, 6.5, 8	20 °C to 90 °C (5 °C/min), hold at 90°C (30 min), cooled to 20 °C (5 °C/min)	a) Hydrolysis and pH significantly impacted gelling qualities b) Very low gelling capacity at pH 2. c) Stiffer and firmer gels at pH 6.5 and 8	Felix et al., 2017
	Pea protein isolate (PPI) prepared by AE PPI with oil (PO) PPI with oat fiber: (PF) PPI with both: (POF)	PPI: 10.83% (w/w), pH 6.5, in 0.025 M HCl PO: 11.27 % (w/w) in 0.025 M HCl PF: 13.96 % (w/w) in 0.025 M HCl POF: 14.64 % (w/w) in 0.025 M HCl	Heated to 60 °C for 60 min, cooling at room temperature for 15 min	a) Structure development is accelerated by an increase in relative protein content b) A weak pea-protein gel is the dominant structure in multi-component systems c) There are just a few direct impacts of oil and fiber addition on gel characteristics	Klost & Drusch, 2019

	Commercial pea protein concentrate (PPC) prepared by air classification	PPC concentration: 8, 12, 16, 20, and 24 g protein / 100 g water) ~ pH 6.0	High-pressure treatment (HPP): 250, 350, 450, and 550 MPa (15 min hold time) Heat treatment (HTT): t 95 °C/ 15 min, cooled for 15 min	a) Gel formed at 16 g/g (250 MPa, HPP), 12g/g (HTT) b) Gel strength increased with increased pressure and protein content c) HTT-treated gels had higher gel strength than HPP-treated gels under the same protein content d) Higher pressure led to more protein denaturation, aggregation, and developed network because of changes in protein tertiary and quaternary conformation	Sim, Karwe, & Moraru, 2019

	Two commercial pea protein isolates (PPIc): PPIc-A (PEVESA): extracted by AE/IEP/drum drying PPIc-B (COSUCRA): extracted by solubilization in water and concentration of soluble protein after denaturized insoluble residue (fibers and starch), followed by spray drying	Protein concentration: 20% and 23%, dispersed with 2% NaCl (w/w of PPIc), 4.5% sodium caseinate in water microbial transglutaminase WM (MTG) was added (0 and 5 U/g) pH: 6.40 and 6.98 for PPIc-A and PPIc-B,	Heated at 80 °C/5 min, cooled down to 40 °C before adding enzyme	a) PPIc-B created a gel network (more flexible, solid-like, and cohesive) b) PPIc-A gels had a coarse network and a greater strength. c) MTG addition to a 20% PPI solution increased the rheological gel characteristics. d) PPIc-B had the same protein composition as PPIc-A but with more fat, carbs, and starch concentration.	Moreno et al., 2020
	Pea protein concentrate and isolate (PPC and PPI) prepared in the laboratory	15%wt protein concentration pH 7	Heated at 95 °C for 15min, cooled to room temperature	a) Gelling capacity impacted by the sugar content during lyophilization and ash content b) Firmer gel and more homogeneous structure for PPC compared to PPI (heterogeneous gel structure) c) PPC had more gelling	Kornet et al., 2021b

				capacity than PPI	
	Pea (PPC), Faba bean (FPC), and lentil protein (LPC) concentrates extracted by AE/IEP/spray-drying	Protein concentration:7.5% to 15% (w/w) at pH 7.0 different solutions (distilled water, 0.5 M NaCl, 0.25 M $CaCl_2$)	Minimum gel concentration (MGC): heated at 95 °C for 60 min, cooled immediately, and stored at 4 °C Rheology: heated up to 95 °C and cooled to 25 °C at a ramp rate of 2 °C/min and frequency of 1 Hz	a) Higher MGC of PPC b) Under the same protein concentration, G' was the highest ($CaCl_2$) c) G′ increased with increased protein concentrations d) More ordered gel structure and firmer and denser gels for LPC and FPC compared to PPC	Guldiken, Stobbs, & Nickerson, 2021
	PPI prepared by different extraction methods -Alkaline extraction/isoelectric precipitation (AE/IEP)	0.1 mol/L phosphate buffer (pH 7) with different PPI concentrations (12-	heated at 90 °C for 1 h, cooled by an ice bath, and stored at 4 °C overnight	a) Extraction methods significantly affect gelling properties b) MP or UF-extracted PPI	Yang et al., 2021

	-Alkaline extraction/ultrafiltration (AE/UF) -Salt extraction/dialysis (SED) -Salt extraction/ultrafiltration (SE/UF) -Micellar precipitation (MP)	20%, w/v)	heating the protein suspension from 25 to 90 °C (2.5 °C/min), held at 90 °C (1h), cooled to 0 °C (5 °C/min)	formed a better gel c) AE/UF, SE/UF, and MP gels show strong strength as heat-induced soy protein gel d) SED formed weak gel via prevented protein unfolding	
	Pea protein isolate (PPI) extracted from pea protein concentration (PPC) by AE/IEP/dialysis	suspension in phosphate buffer saline (1x PBS, pH 7.4) with 0.02% (w/v) sodium azide several parameters involved: -protein concentration: 17%, 21% -alkaline (at pH 12) treating time: 1, 24, 48h -heating time: 1, 2h finial suspension after treatment was adjusted to pH 7	heated at 92 °C for 1 or 2 h to form gels, cooled to room temperature, and kept at 4 °C overnight	a) Increased alkalization time formed stronger, elastic, and cross-linked gels b) Strong gels formed with pH-shift treatment c) Involvement of hydrophobic interactions and hydrogen bonds in gel formation after alkalinization d) longer alkaline treating time, more hydrogen bonds formed to enhance the gels e) heating time did not affect the mechanical properties of gels f) Partial protein unfolding	Zhu et al. 2021

				at pH 12 before heating + more holding alkaline time during heating formed a polymer-like network and a strong gel	
	Pea protein concentrate (PPC) prepared by AE/IEP/dialysis	Protein concentration: 12 % wt Atmospheric cold plasma (ACP) and after being adjusted to pH 6.9	Gels: heated at 70, 80, and 90 °C for 30 min, ice-water bath for 10 min Rheology: 25 to 90 °C, held at 90 °C for 30 min, cooled down to 4 °C, and held at 4 °C for 30 min	a) ACP-PPC formed gel compared to PPI (no gel formed) b) ACP-treated PPC: performed lower denaturation temperature (due to protein unfolding), homogeneous 3D network, good mechanical strength (due to hydrophobic interaction and disulfide bonds), viscoelasticity and high-water holding capacity	Zhang et al., 2021

	Pea flours (18.27% protein based on dry basis), PPI was prepared by salt extraction and micellar extraction,	15% protein solution, dissolved in 0 M NaCl; 0.3 M NaCl; 0.6 M NaCl; 0.9 M NaCl, and 1.5 M NaCl at 4 different pH ranges (pH 3, 4.5, 7, 9)	25℃ around 10 min with amplitude strain of 0.02, heated from 25 °C to 95 °C (2 K/min), kept at 95 °C (10 min), cooling from 95-25 °C (2 K/min), hold at 25 °C (10 min)	a) Both pH and ionic strength affected the protein gelation - the impact of pH > ionic strength b) Ionic strength (minor effect at pH 7 and 9; the major effect at pH 3 and 4.5) C) Brittle gel at pH 4.5 with high G' and low tan δ	Tanger et al., 2022
	PPI extracted by isoelectric precipitation a) Ohmic heating (OH): OH 20 kHz 5 V/cm, OH 20 kHz 10 V/cm, OH 20 kHz 20 V/cm, OH 50 Hz 5 V/cm, OH 50 Hz 10 V/cm, and OH 50 Hz 20 V/cm b) CH heating: heating including come-up 5min, holding 10 min,	12% (w/v) in phosphate buffer (0.01 M, pH 7) with $CaCl_2$ (final concentration 0.2 mol/L)	Heat at 95 °C for 1h by Ch and OH treatments, cooling down to 4 °C test: frequency scanning (0.1-10 Hz) at a strain of 0.02 at 25 °C	a) G'> G", indicating elastic behavior in all treatments b) OH-treated formed weaker gel c) OH-treated reduced G' and G" d) Viscoelasticity increased with increasing electric field strength (5 V/cm-20 V/cm)	Chen et al., 2022

Comparison of previous gelation progress (heat-induced, acidic, enzymatic)	Commercial pea protein isolate (PPIc, NUTRALYS S85F, Roquette) and skim milk powder (SMP, Lactalis)	Dissolved in 1 % NaCl at pH 7.17 7.4 or 14.8 % (p/p) et ratio 1/1	Heat treatment: heated at 80 °C (1 h), cooled to 20 °C; heated at 90 °C (1 h), cooled to 20 °C Acid treatment: glucono-δ-lactone (GDL) (1, 2%, w/v) incubated at 30 °C for 20 h Enzymatic treatment: chymosin (0.1; 0.5; 1 and 2%), transglutaminase (0.05; 0.1 and 0.3%) and CaSO4 (0.3; and 1%), alone or in combination	a) PPI: G'-acidic > G'-enzymatic > G'-heat b) Thermal gelation: PPI-milk formed firmer gel than only milk-gels but equal to only PPI-gel c) Under transglutaminase, the elasticity of mixture gels < PPI-gel or milk-gel	Ben-Harb et al., 2018

Table 1-6 summarizes the published work concerning the heat-induced gelation of purified pea proteins or pea protein isolates.

Heat-induced gels are the most common kind of pea protein gelation, with just a few cold-set gels and enzymatic gels described (Table 1-6). According to Table 1-6, numerous conclusions may be derived from the study on the various approaches to the gelation of pea proteins, which can allow for the optimization of the different gelation parameters to form well-defined gel structures with desirable rheological and textural features. The commercial pea protein isolate (PPIc) displayed poor gelling capabilities after applying multiple gelling procedures, for instance, an increase in the minimum gelation concentration (MGC) and a decrease in the modulus of elasticity (G'), as compared to the pea protein isolate (PPI) prepared by laboratory methods. This was related to pea protein denaturation during large-scale manufacture (Shand et al., 2007; Adebiyi & Aluko, 2011; Mession et al., 2015; Ben-Harb et al., 2018). However, Sun & Arntfield (2010) obtained the opposite result, that adding NaCl led to an MGC of PPIc around 14.5 % which was much lower than other reports. NaCl might have promoted gel formation by increasing intermolecular hydrophobic interactions and decreasing electrostatic repulsion and changing the water structure surrounding PPIc proteins, therefore increasing protein hydration and reducing the MGC. Zhao, Shen, et al. (2020) found similar results, with the MGC of PPIc being 14 % (w/v). On the other hand, several studies focused on the comparison between PPI and soy protein isolate (SPI), that, SPI, generally, showed greater gelling ability than PPI, with harder, stiffer gels and more organized structures (Batista et al., 2005; Munialo et al., 2014a; Shand et al., 2007). This was related to the makeup of fractions from these proteins. For instance, the composition of the fractions of pea protein can vary from cultivar to cultivar, resulting in significant differences in gelation capabilities (O'Kane et al., 2005). This observation was also corroborated by Shevkani et al. (2015), who discovered that various pea cultivars exhibited variable gels and rheological properties because of differences in their physicochemical and structural composition even though utilizing

the same extraction procedures. Pea protein is a mixed protein that may be fractionated into vicilin (7S) and legumin (11S) fractions, as well as water-soluble, salt-soluble, ethanol-soluble, and alkaline-soluble fractions, using various classification techniques (Ge et al., 2020). Thus, O'kane et al. (2004a et 2005) showed that the isolate from five pea protein isolates recovered at the laboratory scale and from various cultivars, with the greatest legumin content formed stiffer gels. However, Bora et al. (1994) found that no gel was formed for legumin under the same condition as pea globulin. Thus, it could be the difference between the cultivars (Barać et al., 2015). In parallel, it was highlighted that the formation of disulfide bonds was a factor that inhibited the gel network from strengthening. These contradictory findings are in complete agreement with other studies that have concluded that vicilin is the main fraction that allows the gel network of pea proteins to be reinforced due to the predominance of hydrophobic, electrostatic, and hydrogen bonding interactions (Mession et al., 2015; O'Kane et al 2004a; Sun & Arntfield, 2012a). As a result, pea protein isolates with increased vicilin content may be more suited to gel formation (Barać et al., 2015; O'Kane et al., 2005). It was also discovered that the inclusion of starch (high water retention capacity), sugars, and ash in the composition contributed to the gels' hardness (Kornet et al., 2021b; Pelgrom et al., 2015b).

Table 1-6 shows that several extraction and fractionation processes have been thoroughly explored and compared, with salt extraction (SE), alkaline extraction, and isoelectric precipitation (AE/IEP) being the most used. Furthermore, Kornet et al. (2021b) demonstrated that it was necessary to use restricted extractions and fractionations from pea concentrates to produce a greater "degree of the nativity" of pea proteins and hence improved gel firmness and homogeneity. Thus, with the goal of improving the extrinsic elements that can significantly impact the gelling properties of pea proteins, various research has concentrated on identifying the perfect conditions for pea protein gelation, involving heat-treating temperatures, times and the rate of heating and cooling, ionic strength, pH, and the addition of cross-linking enzymes, to produce

final features of improved pea protein gels (Yang et al., 2021; Zhu et al., 2021; Zhang et al., 2021; Tanger et al., 2022). In general, pea protein formed a weak, heat-induced gel (Barać et al., 2015). Pea protein gelation is temperature dependent and is largely regulated by the degree of protein denaturation. A stronger gel is created when the degree of denaturation is lower. Protein concentration is also essential in gelation characteristics (Sim et al., 2019; Guldiken et al., 2021). Gels are often stronger at higher concentrations. Sun & Arntfield (2010) found that the gelling point was concentration-independent. Heating and cooling speeds have a small impact on the gelation characteristics of pea protein. The gelling point was impacted by the heating rate, with greater heating rates resulting in delayed gelling (higher gelling temperatures). Higher heating and cooling rates resulted in a loss of gel elasticity. According to the previous studies conducted by Felix et al. (2017) and Munialo et al. (2014b), thermal gelation capability has been demonstrated to be superior at neutral and alkaline pHs and moderate ionic strengths. Furthermore, all the heat-treating temperatures used ranged between 85 and 100 °C, which surpassed the thermal denaturation temperature of the pea proteins. However, when the heating time was increased, so did the gel strength (Bora et al., 1994). On the other hand, the heating rate did not affect the strength of the gels (O'Kane et al., 2005), although a slower cooling rate permitted in many studies to increase the stiffness of the gels due to the lengthening of the gelation duration, making it feasible to facilitate and promote the intermolecular contacts and structural rearrangement that occurs during cooling (O'Kane et al., 2004b; Sun & Arntfield, 2011a). Furthermore, cross-linking enzymes such as MTG, have been found to be particularly successful in strengthening the network and enhancing the strength and elasticity of gels (Djoullah et al., 2018; Shand et al., 2008; Sun & Arntfield, 2011b). Finally, Ben-harb et al. (2018) examined the rheological parameters of the three gelling procedures listed above on a commercial pea protein isolate to find the differences. The results showed that the acidified gels were substantially stiffer due to electrostatic interactions between the pea protein aggregates, which had a considerable influence on

the gel's characteristics.

1.4.2.2 Gelling properties of egg white

Egg white is frequently used as an additive to improve the water-holding capacity or gel strength of food items. Because of its low lipid content, egg white is frequently favored over egg yolk for its gelling capabilities (Mine, 2014). Many food items' textural and rheological features, such as meringues and angel food cakes, are dependent on the heat coagulation or gelation capabilities of egg proteins, particularly their irreversible heat coagulation (Ren et al., 2010). A thermally irreversible gel is a viscoelastic solid created by heat that does not reheat to a viscous liquid (Mine, 2014).

1.4.2.2.1 Mechanism of gelation

A gel is a transitional state between liquid and solid. Cross-linking between polymeric molecules forms an intermolecular network inside a liquid medium (Alleoni, 2006). Protein gel production may be separated into two steps, which is depicted schematically in Figure 1-7. The first step involves conformational changes or partial denaturation of the protein molecule (typically by heat), leading to the unfolding of proteins and an elastic solid occurs. The second step shows a slow association or molecular aggregation of denatured proteins resulting in an exponential rise in viscosity and the creation of a continuous network. Depending on the physicochemical conditions, this stage of the process creation is slower than the first one and concludes when an orderly network is developed. When the second stage is quicker, a disorderly protein cluster with limited water holding capacity might develop, resulting in a greater degree of syneresis. (Alleoni, 2006).

In this gelation process, non-covalent interactions (electrostatic, hydrophobic, and hydrogen bonds) and intermolecular disulfide cross-linking induced protein aggregation are prevalent (Razi et al., 2022). A significant increase in effective

hydrophobicity during heating indicates protein unfolding, and when too many hydrophobic sites are exposed, interactions between the exposed hydrophobic sites are unavoidable, resulting in protein molecule aggregation (Totosaus et al., 2002). Meanwhile, soluble aggregates of high molecular weight form with intermolecular β-sheet interactions. During cooling, unfolded proteins can refold into a refolded configuration, the gel strength increases due to the creation of hydrogen bonds. What should be paid attention to is that if low molecular weight and low protein concentration circumstances generate an aggregation, the protein solution becomes opaque, whereas high molecular weight and high protein concentration conditions produce a coagulum (due to protein chain entanglement) (thermo-irreversible gel) (Totosaus et al., 2002). By joining the polymers in the case of EWP gels, a 3-dimensional (3D) network arises. At greater repulsive values, the 3D network cannot develop, but at higher attractive strengths, water is forced off the matrix (Alleoni, 2006; Razi et al., 2022).

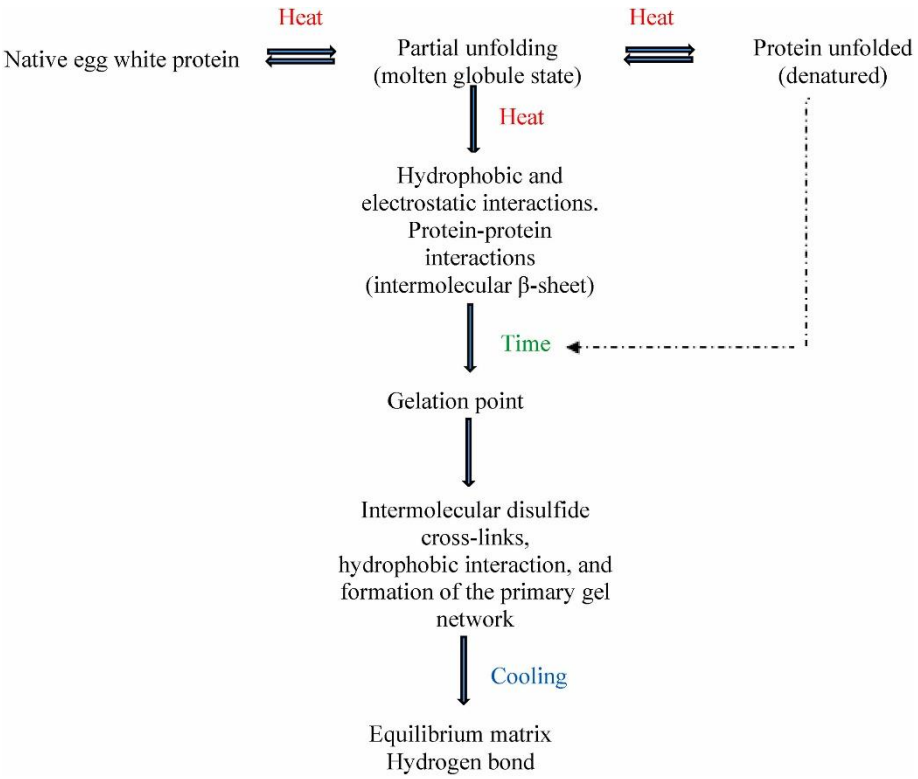


Figure 1-7 Schematic representation of heat-induced gelation of egg-white proteins. (Adapted from Mine, 1995; Campbell et al., 2003; Razi et al., 2022)

1.4.2.2.2 Factors affecting the heat-induced gelation of egg white

Because of the interactions between protein chains, egg white creates a three-dimensional gel network. The physicochemical parameters of the medium, such as ionic strength, salt type, pH, protein content, and interactions with other components, all affect the gel's quality (Croguennec, Nau, & Brule, 2002; Raikos, Campbell, & Euston, 2007b).

1.4.2.2.2.1 Effect of protein concentration

The minimum gel concentration is the starting point for protein cross-linking to create a gel; once this concentration is reached, the protein may form a gel with a three-dimensional network structure (Lv et al., 2022). The concentration mostly influences the hydrophobic contacts and ionic connections formed between protein molecules. Increasing protein concentration can enhance protein gel strength and gel hardness (Hongsprabhas & Barbut, 1997; Quan & Benjakul, 2019). The protein gel structure created at high concentrations is more compact because a large number of surface groups of protein molecules can contribute to the development of gel networks. Iwashita et al. (2015) discovered that heat treatment readily gelled at a high content of albumen. These authors found that strings of beads, characterized as organized soluble linear aggregates of denatured ovalbumin molecule, are more likely to form via constrained attractive hydrophobic contact but substantial electrostatic repulsion, making it easier to form 3D gel networks by the interconnections of these soluble linear aggregates. In contrast, according to the work of Ren et al. (2010), low protein concentrations preferred to form a viscous transparent solution rather than a gel network. Campbell et al. (2003) obtained the creation of a 3D gel network with sufficiently high protein content, high ionic strength, and pH levels near its isoelectric point. Furthermore, the protein content is known to influence the rheological properties and gelling temperature of egg white gels (Quan & Benjakul, 2019). The temperature of gelation

and storage modulus (G') were both found to be concentration dependent, with G' increasing as egg white concentrations rose. This rise is commonly attributed to the consolidation of attractive forces such as van der Waals and hydrogen bonding between protein particles inside the gel network. Furthermore, when the protein content increased, so did the gelation temperature (Eleya & Gunasekaran, 2002).

1.4.2.2.2.2 Effect of pH

The pH has a major influence on the gelling properties of egg white proteins, by influencing the protein net charge and the reactivity of the SH group at higher pH (Quan & Benjakul, 2019; Lv et al., 2022; Razi et al., 2022). The charge distribution of amino acid side chains is affected by the pH of the protein environment. When the pH approaches the protein's isoelectric point, the electrostatic repulsion between protein molecules reduces, and the protein quickly aggregates and solidifies (Campbell et al. 2003; Quan & Benjakul, 2019). The pH of egg white rises during egg storage typically from 7.6 (time of lay) to around 9.5 (Sharp & Powell, 1931). Hickson et al. (1982) and Hammershøj et al. (2002) found that gel elasticity, viscosity index, and penetrating force increased with increasing pH of egg white during storage. Moreover, Raikos et al. (2007b) illustrated that the gelation temperature of egg proteins increased with increasing pH and/or adding NaCl. Croguennec et al. (2002) investigated the gelation of egg white in the presence of salts at different pH. These authors found that protein denaturation at pH 5.0 (the pI of most egg white proteins) produced a coarse aggregation and coagulum with little water holding capacity and viscoelastic characteristics. Lower protein net charge was found at this pH, as well as the least hydrophilicity near the pH close to its isoelectric point. More viscoelastic gels were formed at pH 7 and 9, whereas thermo-coagulation was slowed at both pHs, because of an increase in protein net charge. Because of the unfolding of proteins before aggregation, the gel formed at these pHs exhibited a high water-holding capacity as well. Nyemb et al. (2016) have studied the heat-induced gel of EW influenced by four

different combinations of pH and ionic strength (IS) (pH 2/IS 0.05 M, pH 5/IS 1 M; pH 7/IS 0.05 M; pH 9/IS 0.05 M). It was found that the Granular-spongy EW gel was formed at pH 5/IS 1 M, the intermediate gel was formed at pH 7/IS 0.05 M, the smooth-rigid gel was formed at pH 9/IS 0.05 M, the fracturable gel was formed at pH 2/IS 0.05 M. Minimal elasticity was also observed at pH 5/IS 1 M, as well as a lower cohesiveness than that observed at pH 9 resulted from the heterogeneity of the microstructure, and from the nature of protein interactions which are mainly weak (hydrophobic) in these conditions. The EW gels formed at pH 7 (intermediate gel) have a variety of aggregate structures: ovotransferrin spherical aggregates scattered in an ovalbumin linear-branched aggregate protein network, due to i) random and spherical ovotransferrin aggregates are predominated as pH 7 closed to pI of ovotransferrin (~ 6.5), and ii) development of linear-branched OVA aggregates as pH 7 far way from ovalbumin's pI (approximately 4.5). Gelation is delayed at pH 7 as compared to pH 5 because electrostatic repulsions are higher at pH 7 as compared to pH 5. Gels formed at pH 9 had greater elasticity and cohesiveness values as compared to pH 7 and pH 5 gels and has a smooth-rigid network due to increased net charge and electrostatic repulsions (pH 9 far from pI of ovalbumin and ovotransferrin). Gels formed at pH 2 had a filamentous gel made of linear aggregates due to high net charge and electrostatic repulsions leading to proteins tending to denature rather than aggregate.

Khemakhem et al. (2019) investigated the influence of pH (4.5 and 6.5) on the gelling characteristics of egg white protein and highlighted that at pH 4.5, gels had the lowest hardness and elasticity (near the pI) but cohesiveness and adhesiveness were greater. Protein-protein interactions can increase at pH around pI, but protein-solvent interactions increase at higher pH (above pI). The gel structure was poor at pH 4.5, and the network had big holes (Khemakhem et al., 2019). Protein aggregation is a significant process for gel formation at pH 4.5, on the opposite, at pH 7.0, aggregation is restricted by repulsive electrostatic forces, and produced gels are constituted of organized linear polymers. Chen et al. (2015) studied the changes in gel properties of

egg white treated at strong alkaline and found that gels formed under strong alkaline relied mostly on ionic and disulfide connections, while hydrophobic interactions and hydrogen bonds were rarely involved in gel conformation preservation. According to Li, Zhang, Fan, et al. (2018b), the opacity and particle turbidity of egg white protein gel reduced with increasing NaOH concentration. According to Babaei, Khodaiyan, & Mohammadian (2019), the stiffness of egg white protein gels was higher at pH 4 than at pH 7, which was due to stronger electrostatic repulsion between the proteins at pH 7 compared to pH 4. Raikos et al. (2007b) also highlighted that egg samples at pH 5 and 8 had higher gel strength than egg samples at pH 2. Another analysis by Phillips & Williams (2011) found that coagula or soft/turbid gels of egg albumen were generated at high ionic strength and around the isoelectric point, because of the existence of these coagula inside the gel network, opaque gels formed.

1.4.2.2.2.3 Effect of salts and types of salts

Salts can favor protein aggregation in solutions and allow the formation of a gel network by egg protein. Sodium chloride can influence gel characteristics by strengthening or decreasing connections between protein molecules. It was found that moderate levels of sodium chloride can improve the hydrophobic interactions between protein molecules, but too much salt can disintegrate ionic and hydrogen connections between protein molecules, making the gel weaker (Raikos et al., 2007b). Ma, Zhao, & Chi. (2012) also found that increasing the concentration of NaCl increased the water release of hen albumen gel. In the presence of 120 mM NaCl, the most water was released. The greater NaCl content was linked to the excessive heat-induced aggregation of albumen proteins, most likely via ionic or hydrophobic contact. The effects of inorganic salts on the thermal aggregation of albumen, including NaCl, Na_2SO4, and NaSCN had also been studied by Iwashita et al. (2015). Na_2SO4 and NaSCN are both kosmotropic and chaotropic agents. The kosmotropes enhanced the surface tension of the solution more than the chaotropes did. This might be because

chaotropes destabilize protein tertiary structure, resulting in a lower denaturation temperature, whereas kosmotropes stabilize protein structure. The salting out of protein molecules predominated at salt concentrations greater than 0.5 M. Salt anions attach to the positively charged domains of lysozyme at low concentrations, increasing solubility independent of the type of ions (Iwashita et al. 2017). Denaturation of heat-treated proteins containing NaCl reduced the ordered secondary structure (α-helix and β-sheet) of egg white proteins gel by disrupting hydrogen bonds (Li et al., 2018b, Li et al., 2018c, Li et al., 2018d). The mechanical characteristics of the produced gel are affected by salt ions, which might improve its stability. Salt ions in macromolecules tend to lower protein electrostatic energy and increase protein solubility in aqueous conditions (Li et al., 2018b, Li et al., 2018c, Li et al., 2018d). A reversal effect can be noticed at high salt concentrations. This might be due to greater salt concentrations breaking hydrogen bonds and destabilizing protein molecules, weakening gel structure (Li et al., 2018b, Li et al., 2018c, Li et al., 2018d).

Several studies mentioned that heat-induced and alkaline-induced gelation of egg albumen is also affected by salt type (Croguennec et al., 2002; Zhao et al., 2014; Deng et al., 2020). Croguennec et al. (2002) highlighted that Ca^{2+} and Mg^{2+} altered the viscoelastic characteristics of egg albumen gel, resulting in a less homogenous gel, with particles aggregated in random aggregates and no string of beads formations. The inclusion of cations enhanced the heat stability of albumen proteins, but $CuSO_4$ significantly softened albumen coagulum gel. Gel stiffness was unaffected by $FeCl_3$ or A_1C1_3. These modifications were caused by the negative charge of albumen proteins being shielded (Croguennec et al., 2002). The alkali provides an environment for egg white protein denaturation and aggregation, and high concentrations of metal ions can promote cross-linking and more effective aggregation of aggregates. Monovalent and multivalent ions both protect negatively charged protein molecules from electrostatic interactions, and differing valence levels of metal ions have varied effects on protein gels (Deng et al., 2020; Lv et al., 2021). For example, Deng et al. (2020) investigated

the effect of different ions on alkaline-induced egg white gels. It was found that multivalent ions (Ca^{2+}, Zn^{2+}, and Fe^{3+}) had more positive charges and a higher shielding capacity against electrostatic interactions between protein molecules, which might result in more cross-links when gels were formed at the same concentration. Higher concentrations of the salts, on the other hand, were incompatible with the formation of protein gels. The authors also showed that the performance of the gel generated by monovalent ions (K^{+}) was superior to that of the gel formed by multivalent ions, which was distinguished by increased soluble protein content and water-holding capacity, as well as a denser gel microstructure.

1.5 Mixtures of plant proteins and animal proteins

Raw animal materials such as milk, eggs, meat, and seafood continue to be the most important protein sources lately employed by food companies and humans in the world, followed by plant sources such as legumes and nuts (Alves & Tavares, 2019). However, unlike plant protein production, animal protein production is coupled with high greenhouse gas emissions and a larger requirement for land space (Henchion et al., 2017; Alves & Tavares, 2019; Schmitt et al., 2019; Wu et al., 2021). It has been determined that, in order to achieve long-term development, animal proteins should be more commonly at least partly, substituted by plant proteins in food composition keeping good nutritional qualities. For this sort of partial replacement to be possible, animal and plant proteins must be able to interact with one another to generate homogenous products with intriguing functional features.

Few investigations have been conducted to date to characterize the processes of aggregation and association of plant and egg white protein mixtures. Currently, Alves & Tavares (2019) reviewed the main proteins used in the mixed system: plant protein, soybean, pea beans, and wheat are mainly studied, while in terms of animal proteins, milk containing caseins and whey proteins, egg white protein, and gelatin from cattle or fish are studied. Whey protein also can be divided into two main parts: β-

lactoglobulin (β-Lg) and α-lactalbumin (α-La) occupying around 50 % and 20 % of total whey protein content, respectively (Chatterton et al., 2006). Soy proteins and dairy proteins are mostly employed in protein combination studies in food items due to excellent nutritional content, capacity to gel formation, increased consumers demand for soy protein (Renkema et al 2001), high purity, availability, and high solubility of dairy proteins (Croguennec, Tavares, & Bouhallab, 2017). Particularly, formulations including soy and milk proteins may generate goods with outstanding nutritional value (Ruiz-Henestrosa et al., 2014). However, pea protein has lately gained popularity because of its excellent functional, and nutritional qualities, with a non-allergenic advantage, compared to soy protein (Aluko, Mofolasayo, & Watts, 2009; Burger & Zhang, 2019).

The primary studies on the techno-functional properties of mixed systems produced from plant proteins (mainly pea protein) and animal proteins (mainly egg white) are summarized in Table I-7. In the following sections, co-aggregation and gel formation will be used to highlight the impact on the mixture systems (pea-based, egg white-based).

Table 1-7 Research on the techno-functional characteristics of mixed systems between plant protein and animal protein (According to Alves & Tavares, 2019; Schmitt et al., 2019; Hinderink et al., 2021a; Wu et al., 2021).

Properties	Plant proteins	Animal proteins	Reference
Co-aggregation/ co-precipitation	Pea protein	Whey protein	Kristensen et al., 2021a & b Kristensen et al., 2020
		β-lactoglobulin	Chihi et al., 2016 Kristensen et al., 2022
		Casein micelle	Mession Roustel, & Saurel, 2017a
		Egg white protein	Kuang et al., Unpublished
	Soy protein		Zhang et al., 2020
Gel and film formation	Soy protein	Egg white proteins	Su et al., 2015
	Hempseed protein		Alavi, Emam-Djomeh, & Chen, 2020

	Pea protein	Caseins	Message, Roustel, & Saurel, 2017b Silva et al., 2018 Silva et al., 2019b Silva et al., 2019a
		β-lactoglobulin	Chihi, Sok, & Saurel, 2018
		Bovine lactoferrin	Adal et al., 2017
		Cape Hake sawdust proteins	Tomé et al., 2015
		Myofibrillar proteins	Sun & Arntfield, 2012b Zhu et al., 2022
		Whey protein	Wong, Vasanthan, & Ozimek, 2013 Kornet et al., 2021b
		Gelatin	Hedayatnia et al., 2019
		Milk protein	Yousseef et al., 2016 Ben-Harb et al., 2018
Emulsifying	Pea protein	Caseins	Liang et al., 2016 Hinderink et al., 2019 Yerramilli, Longmore, & Ghosh, 2017
		Cape hake sawdust proteins	Tomé et al., 2015
		Sodium caseinate	Yerramilli, Longmore, & Ghosh, 2017
		Whey protein	Hinderink et al., 2020 Hinderink et al., 2021b
Other results			

Co-precipitation	Egg white lysozyme	β-lactoglobulin	Diarrassouba et al., 2015
Gel	Egg white	Gelatin	Badii & Howell, 2006 Babaei, Mohammadian, & Madadlou, 2019 Bashash, Varidi, & Varshosaz, 2022
		Whey protein	Pu et al., 2022
Foam			Kuropatwa, Tolkach, & Kulozik, 2009
		Gluten	Wouters et al., 2018
		Soy protein	Wang et al., 2012
Phase-separation	Egg white lysozyme	Soy protein	Zheng et al., 2020
	Pea protein isolate	Bovine lactoferrin	Adal et al., 2017

1.5.1 Animal proteins mixed with pea proteins

According to Alves & Tavares (2019), mixture systems containing soy-animal proteins play an important role. Recently, several researchers have focused on the mixtures prepared by animal proteins and pea proteins, due to their excellent techno-functional properties. Many factors influenced the techno-functional properties of the pea protein isolate, as described in Section 1.4. We focused on the heat-induced gelation prepared by pea proteins and animal proteins which will be discussed in the following sections.

Kristensen et al. (2020, 2021a & b, 2022) have studied the mixtures between pea protein isolate (PPI) and whey protein isolate (WPI). Kristensen et al. (2020, 2021a) have defined the processes behind protein-protein interactions in a combination of two commercial isolates of pea protein (PPI) and whey protein (WPI). The previous paper (2020), wanted to optimize the yield and compare the solubility of co-precipitates and protein blends. Samples were prepared at 10 g/L mixed with different volume ratios (WPI: PPI at 20:80, 50:50, 80:20, v/v) then adjusted to pH 4.6, and heated at 60, 80,

98 ℃ for 30 min, respectively. Then the co-precipitates were obtained by centrifuging. The maximum precipitation yield was obtained at a higher WPI ratio in the mixture, high temperature, and alkaline protein solvation. Solubilities were measured at pH 3, 7, and 11.5. Co-precipitates were far less soluble than protein blends. High WPI content in the mixtures, low precipitation temperature, and high pH showed the greatest solubility, indicating various protein-protein interactions in the mixtures. The goal of Kristensen et al. (2021a) was to see how the creation of a co-precipitate of these two proteins from distinct sources affected the functional aspects of the mixtures. The mixtures were made in various ratios in water at a total concentration of 2 % (w/v). The co-precipitates were then obtained by lowering the pH to 4.6 and then centrifuging to eliminate the supernatant, and freeze-dried. The powders generated from the assembly of these two proteins were then distributed at various pH levels (3, 7, and 11.5). Because of the massive aggregates created by interactions between the two proteins, co-precipitation did not further improve the rise in solubility. Furthermore, this study demonstrated that a simple mixing of these proteins under neutral or alkaline pH conditions can increase the solubility, emulsifying, and foaming capabilities of the proteins in the mixture compared to the proteins alone. Moreover, Kristensen et al. (2020) investigated the interactions inside WPI-PPI co-precipitate, by adding N-Ethylmaleimide (NEM) to break disulfide bond formation, 0.5 M NaCl to investigate electrostatic interactions and hydrophobic interactions and 0.2 % SDS to identify hydrophobic interactions. The results showed that the impact of NEM and NaCl was strong and additive when combined, but SDS had minimal to little effect, indicating a multi-reaction of disulfide bonds and electrostatic forces was responsible for the mechanism underlying protein-protein interactions between whey and pea protein isolates. The work of Kristensen et al. (2022) wanted to identify if the β-lactoglobulin (β-lg) and pea legumin played a substantial part in these interactions. The interactions of β-lg and legumin in both co-precipitates and blends were shown to be a synergy of electrostatic interactions and disulfide bonds. Thus, β-Lg and legumin are the major

proteins responsible for previously discovered interactions in whey and pea protein isolates.

Chihi et al. (2016) investigated the formation of heat-induced mixed aggregates (heat treatment at 85 °C for 60 minutes) of serum proteins (β-lactoglobulin obtained by purification of a commercial isolate of whey proteins) and purified pea proteins (pea globulins (Glob) obtained by isoelectric precipitation) mixed at various ratios (30/70, 50/50, and 70/30). The experiment, which was conducted at pH 7.2 in the presence of 5 mM NaCl, indicated the creation of mixed thermal aggregates including both the formation of new covalent connections (disulfide bonds) and non-covalent contacts between the subunits denatured β-lactoglobulin and Glob. The soluble aggregates in the mixes had a larger molecular weight and a smaller diameter than the Globs' thermal aggregates alone. Furthermore, increasing the quantity of β-lactoglobulin in the mixture resulted in a decrease in the size of the soluble aggregates formed following heat treatment. Finally, it was postulated that thermal aggregation of these proteins in a mixture is driven first by β-lactoglobulin denaturation, followed by the creation of complexes stabilized by disulfide bonds between non-refolded β-lactoglobulin and legume subunits (11S) produced after heat treatment. These complexes can then create non-covalent connections with other pea globulins (legumin and vicilin) to form soluble aggregates with sizes ranging from 90 to 110 nm.

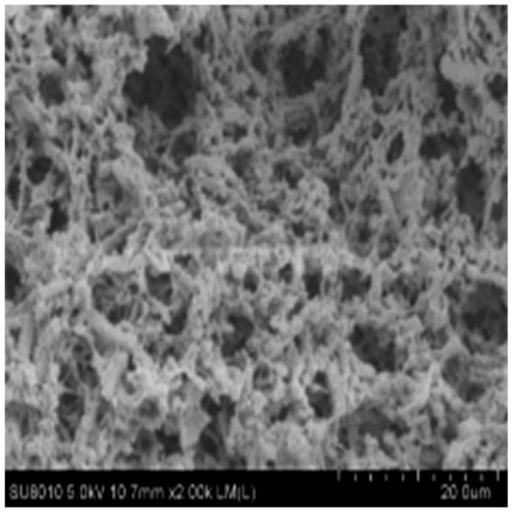
Mession et al. (2017a) investigated the aggregation of proteins after heat treatment of a mixed system constituted of casein micelles and pea globulins separated into vicilin and legumin. To be more specific, casein/vicilin micelles or casein/legumin micelles were generated at pH 7.1 in the absence of salts, with total concentrations of 1.8 and 3.6 % (w/w) and a 50/50 ratio for each dispersion before being subjected to an 85 °C heat treatment for 60 minutes. The denaturation temperature of the combined legume and vicilin rose by roughly 4 °C when compared to the denaturation temperature of these fractions alone, according to the authors. This improvement in thermal stability was linked to the influence of the casein micelle's steric hindrance, which inhibits the

legumin's molecular mobility and flexibility, hence the unfolding of this protein. The solubility of these protein fractions in the mixes was significantly enhanced after heating. Furthermore, it was shown that the acidic and basic legumin subunits formed soluble and insoluble aggregates with the casein micelles in the mixes due to disulfide bonds. Denatured vicilin, on the other hand, generated soluble mixed aggregates mostly through non-covalent interactions. Finally, caseins were not engaged in pea protein aggregation, even though heat-induced pea protein interactions were changed in the presence of micelles.

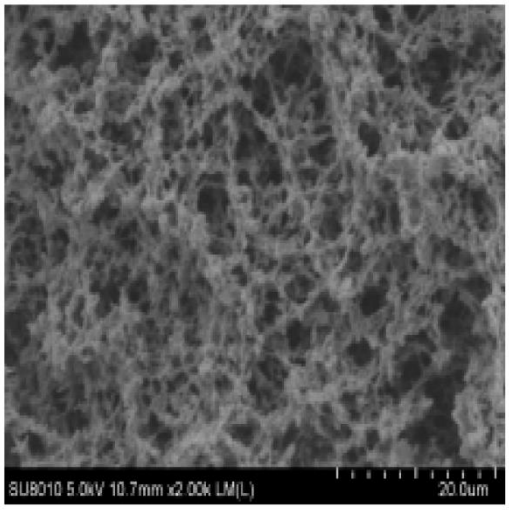
Several literature focused on the gelation properties of the mixed system conducted by pea protein isolate and animal proteins (mainly dairy proteins) (Table 1-7). Tomé et al. (2015) investigated heat-induced gel prepared by the mixture of PPI and Cape hake protein powder (CHPP, prepared by alkaline extraction) at a total protein content of 20 % with various weight ratios (PPI/CHPP: 100/0, 20/80, 50/50, 80/20, 0/100%, w/w). The results showed that heat-induced gels were also produced for all the samples after 30 minutes of treatment at 90°C. A drop in G′ and G″ values as PPI concentration increased in the mixes, suggested that pea proteins inhibited the gelation of fish proteins. During heating, the gel network was quickly generated by the CHPP and then reinforced by pea protein. Thus, increasing the CHPP fraction resulted in stronger and more structured gels.

Mixture gels prepared by myofibrillar proteins (MPI) from different species (chicken, red sea bream, duck) and PPI were studied by Sun & Arntfield (2012b), Lin et al. (2019), and Zhu et al. (2022), respectively. Sun & Arntfield (2012b) studied the heat-induced gel prepared by Chicken-MPI (C-MPI) and PPI, with a total of 4% protein content and different proportion and NaCl concentration. The results showed that the addition of PPI to C-MPI reduced gel strength; with 0.6 M NaCl, the PPI denaturation temperature was higher than the gel formation temperature, restricting interactions between the two proteins. The authors also studied the influence of transglutaminase (MTG) added to the mixture. It was found that the gel strength of a C-MPI/PPI at a ratio

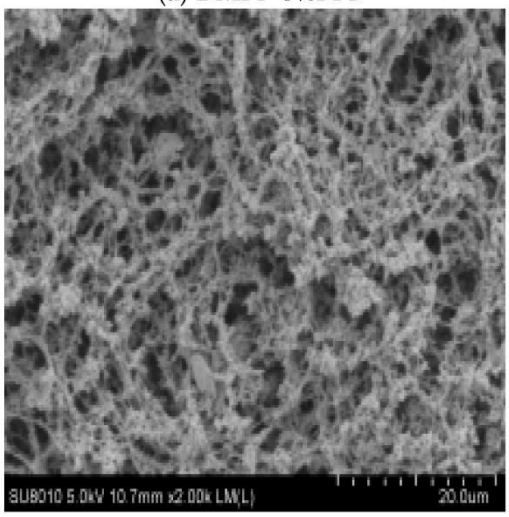
of 3/1 was considerably enhanced in this investigation with the addition of suitable doses of MTG, indicating that partial crosslinking between PPI and C-MPI via glutamyl-lysine bonds with the inclusion of MTG. As a result, MTG improves the gelation characteristics of heat-induced MPI/PPI gels, which was also confirmed by soy-MPI with MTG (Jiang & Xiong, 2013; Han et al., 2015). Zhu et al. (2022) investigated the heat-induced gel obtained by PPI and duck-MPI (D-MPI) with a total protein content of 40 mg/mL and various amounts of PPI. The results showed that with increased PPI addition, D-MPI's gel-forming capabilities, including water retention and textural qualities, increased. Pea proteins help in the conversion of free water to immobilized water. When heated, the α-helix content dropped while the β-sheet content increased, providing the driving force for the gel formation, and generating a more compact and homogenous gel structure (Figure 1-7), which may also have a conductive impact on water entrapment in the gel network. Therefore, Pea proteins may enhance the characteristics and microstructure of duck myofibrillar protein gels, thereby accelerating the transition from a weak to a non-aggregated, rigid form.



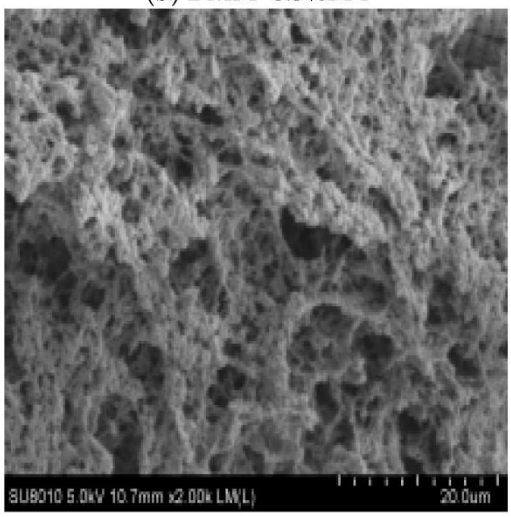
(**a**) DMPI–0%PPI



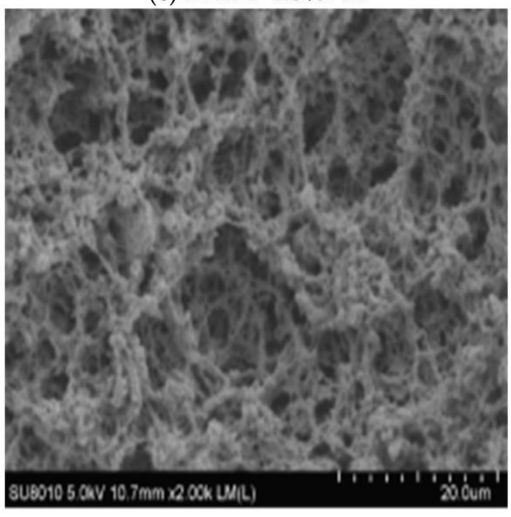
(**b**) DMPI–0.5%PPI



(**c**) DMPI–1.0%PPI



(**d**) DMPI–1.5%PPI



(**e**) DMPI–2.0%PPI

Figure 1-7 Effect of pea protein addition level (0%, 0.5%, 1%, 1.5%, and 2%) on the microstructure (SEM, 2000×) of DMPI–PPI mixed gel. Each treatment was performed

in triplicate. (**a**) DMPI–0%PPI; (**b**) DMPI–0.5%PPI; (**c**) DMPI–1.0%PPI; (**d**) DMPI–1.5%PPI; (**e**) DMPI–2.0%PPI (Zhu et al., 2022).

In previous Section 1.4.2.1, we found that the capacity of proteins to form three-dimensional networks underpinned their gelling characteristics. Gel properties of PPI were impacted by different gel treatments, Ben-Hard et al. (2018) summarized the different treatments to induce gel including acidic, thermal, and enzymic treatments regarding PPI and milk proteins. Schmitt et al. (2019) studied the gelation of dairy/plant protein by thermal or acidic treatment. The addition of plant proteins generally reduced gel strength when compared to milk protein alone, owing to the creation of distinct networks by the various protein sources, which seldom interacted substantially. The addition of a dispersed oil phase improved gel strength control by inducing a particular protein distribution at the interface and allowing the production of active oil droplets embedded in the protein network. In Table 1-7, heat-induced gelation was studied by Wong et al. (2013); Chihi, Sok, & Saurel (2018), Silva et al. (2018), Silva et al. (2019 a & b); Kornet et al. (2021b); acid-induced gelation was studied by Yousseef et al. (2016) and Mession, Roustel, & Saurel (2017b).

In addition, some of these researches have also concentrated on the interaction mechanisms and physicochemical factors that govern pea protein-milk protein interactions during gelation. For example, Wong et al. (2013) studied the thermal gelation of the mixture prepared by pea protein concentrate (PPC) and whey protein concentrate (WPC). They prepared the gels by heating up to 92 °C for 30 min and investigated the influence of pH and NaCl concentration on the rheological and textural features of thermal gels formed by combining proteins in various volume ratios and total protein concentrations. The addition of whey proteins improved the gelling properties of pea proteins by gradually enhancing the elasticity and stiffness of the produced thermal co-gels. Furthermore, the rheological and textural qualities created at pH 6 were superior to those formed at pH 4 and 8. This discrepancy was related to

electrostatic repulsions between convicilin (7S) subunits as the pH shifted away from their isoelectric pH, which was indicated close to 6. These researchers, on the other hand, discovered that the NaCl concentrations of 0.5-3.0 % (w/v) all demonstrated synergistic enhancement on gel hardness at the same PPC/WPC ratio of 1/4, with the lower NaCl concentrations producing larger degrees of synergistic enhancement. It should be highlighted that the processes of heat aggregation as well as the nature of the molecular connections that structure this mixed system were not explored in this study.

Silva et al. (2018, 2019 a & b) focused on the heat-induced gelation of the mixtures prepared by micellar casein (MC) and plant proteins, in which the former (2018) studied the gel prepared by MC and globular protein (GP: soy or pea or whey protein), while the latter (2019 a & b) analyzed the gel prepared by MC and SPI or PPI in aqueous solution or emulsions, respectively.

Silva et al. (2018) analyzed the impact of the addition of globule protein (GP: SPI, PPI, or WPI) with parallel concentration (0-6% w/w) at pH 5.6-6 on the heat-induced gelation of MC dispersion at 6 wt%. The critical gelation temperature (Tg) of MC suspensions increased with the addition of globular protein regardless of pH. Surprisingly, this rise varied by GP type and was strongest in the sequence Soy Proteins (SP) > Pea Proteins (P) > Whey Proteins (WP) (Figure 1-9 d). For example, at pH 5.8, adding 2 % w/w SP to 6 % w/w MC improved the Tg by 30 °C compared to 15 °C for PP and just 5 °C for WP. This impact was mostly explained by changes in calcium binding effectiveness across the various GP (SP > PP >> WP), as shown in Figure 1-8, and/or mineral makeup of the components. In other words, native globular protein influenced MC gelation indirectly by binding calcium, limiting the quantity available for micelles to bind. The addition of $CaCl_2$ counteracted this action, resulting in a decrease in Tg that was no longer dependent on the GP content and source in excess of $CaCl_2$. Tg of the suspensions dropped with lowering pH due to a decrease in MC net charge density as well as Ca^{2+} release, which favored heat-induced gelation of MC. The results suggested that globular proteins do not have the same capacity to bind calcium

as other proteins. Plant proteins bind more calcium than serum proteins, with soy proteins having a higher affinity than pea proteins. This differential in calcium affinity has a direct influence on the stiffness of acid gels and protein interactions.

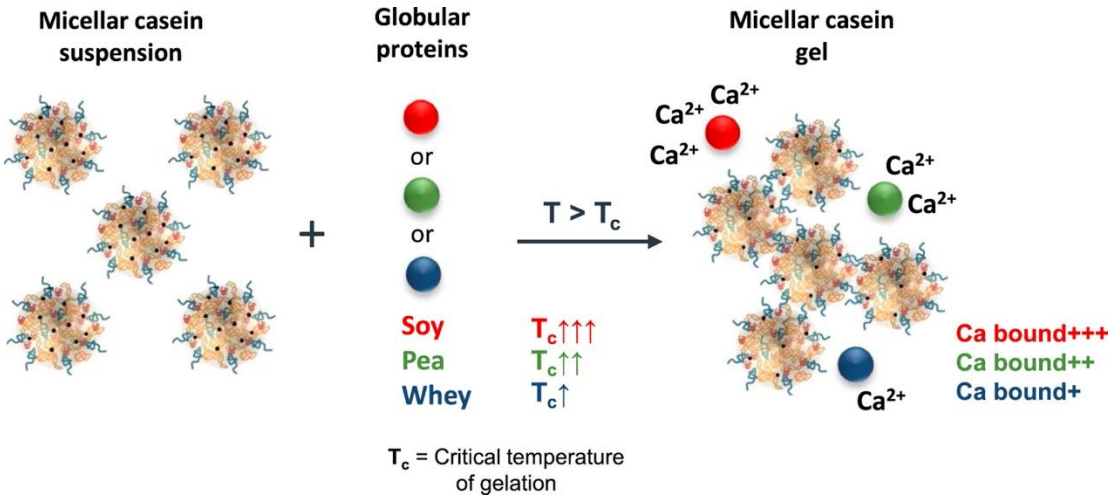


Figure 1-8 Increase in the critical gelling temperature (Tc) of casein micelles in the presence of globular proteins (whey, pea, or soy) explained by the difference in protein affinity for calcium ions (from Silva et al., 2018).

These findings indicate a considerable variation in the rheological behavior of co-gels generated at temperatures over 70 °C depending on the kind of plant proteins added. When whey proteins were added, the network was reinforced, and the gel's stiffness increases significantly. Whey proteins could form disulfide bonds with caseins on the surface of the micelles according to the work of Corredig & Dalgleish (1999). The inclusion of the two plant proteins, on the other hand, did not improve these features, demonstrating that, unlike the serum proteins, they did not interact with the casein micelles.

Silva et al. (2019b) conducted additional research to further understand how the protein composition influenced the heat-induced gelation of mixes of casein micelles and plant proteins. The samples were prepared by changing the MC/plant protein ratio from 0/100 to 100/0 for total protein concentrations of 4, 6, and 8 % (w/w) at pH 5.8

and 6.0. The critical temperature of MC gelation was shown to rise when MC was substituted with SPI or PPI due to calcium chelation by these plant proteins, and SPI bound Ca^{2+} more efficiently than PPI. This finding demonstrated that the amount of calcium required for casein micelle gelation varies depending on the type of plant proteins used, and that pea proteins allow for more casein replacement while complexing less calcium. Interestingly, it was shown that replacing the micelles with 40% soy proteins vs 70% pea proteins resulted in the lowest gel stiffness (Figure 1-9). As a result, the stiffness of mixed gels at a given total protein content reduced when MC was replaced by plant proteins or vice versa because the stiffness of both MC and plant protein gels reduced with decreasing concentration. Also, it was concluded that after heating, MC and plant proteins did not co-aggregate and that each kind of protein formed networks independently.

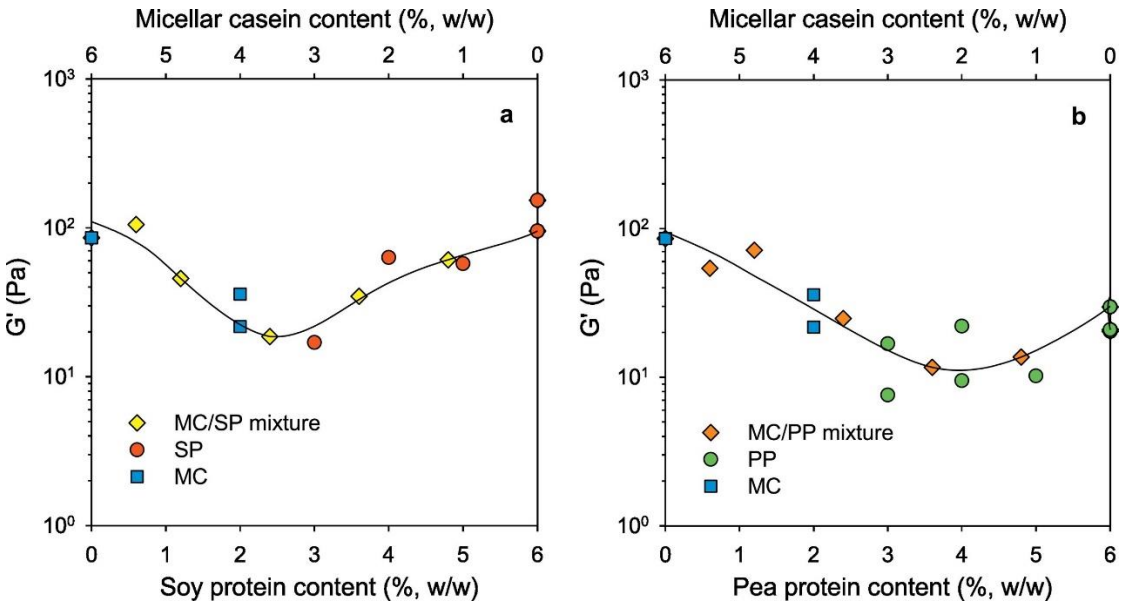


Figure 1-9 Storage modulus at 0.1 Hz after 1 h heating at 90 °C for (a) individual and mixed MC-SP and (b) individual and mixed MC-PP systems at 6% w/w total protein as a function of MC and plant protein content in the mixtures at pH 5.8 (Silva et al., 2019b).

Silva et al. (2019a) investigated the influence of the concentration of sunflower oil (0, 5, 10, and 15 % w/w) and total protein concentration (1-4 % w/w) on the heat-

induced gelation of MC and SPI or PPI. Samples were prepared at different protein compositions (MC/SPI or PPI = 100/0 to 0/100) and pH 5.8. The results showed that the gelation temperature (Tg) rose when an increasing proportion of MC was replaced by SP or PP, owing to calcium binding to plant protein, which agreed with the result of Silva et al. (2019b). Tg decreased with increasing oil content in the MC/plant protein emulsions, mostly up to 10 % w/w oil, which can be attributed to protein interactions at the oil-water interface. Interestingly, MC in emulsion gels could be effectively substituted by PLP while preserving the same gel stiffness, which was not the case for gels generated in the absence of oil. Gel stiffness rose as oil concentration increased, implying that oil droplets worked as active fillers. This study's findings may aid in determining the potential of plant protein to substitute milk proteins in food formulations such as yogurts, dessert creams, and ice creams.

Adal et al. (2017) discovered that mixing cationic lactoferrin (LF) with anionic pea protein isolate (PPI) resulted in complex formation and coacervate production under particular pH range circumstances, with maximal coacervate formation reported at charge neutrality by electrostatic interaction and soluble complexes maximized (pH 7). This electrostatic interaction agreed with the paper of Zheng et al. (2020), who discovered the effect of pH and NaCl on the mixtures of lysozyme (LYS) and soy protein isolate (SPI). It was found that the complex included not only electrostatic interactions but also hydrogen bonds. NaCl, on the other hand, can reduce the zeta potential of SPI and LYS and decrease the electrostatic connection between heteroproteins. The electrostatic assembly of SPI and LYS (1 mg/mL) may be inhibited by 200 mM NaCl. Furthermore, when NaCl concentration increased, the absolute levels of ΔG, ΔH, and ΔS fell dramatically.

1.5.2 Plant proteins mixed with egg white

According to studies of mixing systems on animal proteins and plant proteins, milk proteins are the most common animal proteins, whereas egg white protein was little

considered (Table 1-7). Recently, people have paid more attention to using egg white as a source of animal protein because of their unique functional properties (Campbell, Raikos, & Euston, 2003).

Zhang et al. (2020) prepared the composite protein microparticles by mixing soy protein isolate (SPI) and egg white (EW) at a total protein of 15 % w/w, in which, the proportion of SPI was 12 wt%, EW was 3 wt%, with different heating time (95 ℃， 5, 10, 15 min). The results showed that EW was engaged in the microparticulate of SPI leading to more intermolecular disulfide bonds. When the duration of heating during preparation was increased, the number of disulfide bonds in SPI/EWP composite particles increased by about 50 % compared to SPI-only. The flexibility of protein conformation decreased significantly as more covalent interactions engaged in creating the network inside the particles. Following heat treatment, the dispersions comprising SPI/EWP composite microparticles with a protein content of 12 wt% still showed poor viscosity and weak gelation ability. Meanwhile, their lubricating characteristics have been enhanced.

Su et al. (2015) demonstrated the gel properties of a mixed system of SPI and EW at pH 7 and with 0.05 g GDL. The mixtures were prepared by mixing EW in SPI at a weight ratio of 1:0, 9:1, 3:1, 1:1, 1:3, 1:9, 0:1 (SPI/EW, w/w) at a total protein concentration of 0.03, 0.06, 0.08, 0.10 g/mL, respectively. The results showed that the hardness, springiness, and water-holding capacity of composite gels all increased as protein concentrations rose. When total protein content was more than 0.03 g/mL, a 1:1 combination of SPI and EW improved springiness and water-holding capacity, suggesting that synergistic interactions were produced between SPI and EW. These results were confirmed by Figure 1-10, that the gel mixed in a 1:1 (SPI/EW) ratio was more homogeneous and included fewer big particles in the network, which was associated with the increased springiness and water-holding capacity. However, when the SPI/EW ratios were greater than 1:1, the homogeneity rose and the big particles reduced in the structures with the addition of EW; when the ratio was less than 1:1, the

network of gels became more uneven and rough, with more large particles as the EW content grew. The hardness and storage modulus of gels rose steadily as the egg white content of the composite gels increased. The gels' α-helical structure content was increased initially and subsequently dropped, but the β-sheet structure content was continuously raised with the rise in egg white ratio. The α-helical and β-sheet tendencies correlated with the springiness and hardness of gels, respectively.

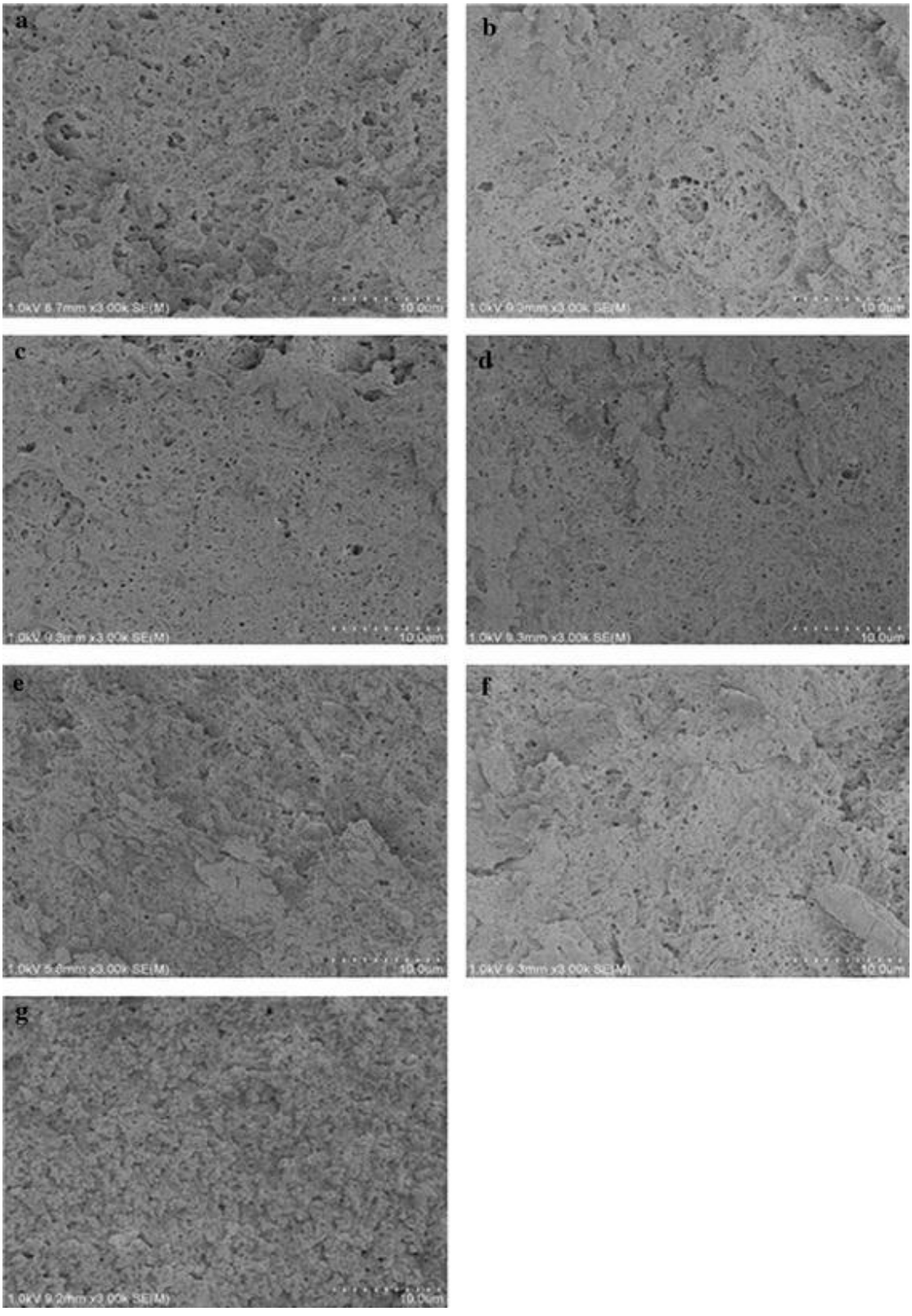


Figure 1-10 Scanning electron micrographs of the SPI/EW protein gels at 0.06 g mL^{-1} protein concentration. The pictures marked with a–g represent the gels at different SPI/EW ratios. **a** 1:0, **b** 9:1, **c** 3:1, **d** 1:1, **e** 1:3, **f** 1:9, **g** 0:1. (Su et al., 2015)

Alavi, Emam-Djomeh, & Chen (2020) investigated the thermal co-aggregation of egg white protein/hempseed protein (EWP/HPI) at different EWP/HPI weight ratios (0/100, 75/25, 50/50, 25/75 and 100/0) with a total protein concentration of 5 % (w/v) under alkaline pH, the GDL (0.115–0.185 g/g) and MTG (20 U/g) were used to induce acid-induced gels by thermal EW/HPI aggregates. The results revealed that thermal treatment at alkaline pH resulted in aggregates formed by both disulfide and non-disulfide covalent bonds, with low viscosity and high solubility. In the presence of GDL, EWP alone created gels of great mechanical strength, but a thermal aggregation of HPI alone produced no gels. Acid-induced self-supported gels were generated by EWP/EWP aggregates with varying EWP/HPI ratios. The elastic modulus, loss factor, creep performance, textural characteristics, fracture stress/strain, and microstructural homogeneity of the mixed EWP/HPI gels tended to decrease as HPI concentration increased. MTG treatment enhanced the mechanical characteristics of mixed gels somewhat, but not sufficient to compensate for the weakening effects of HPI.

1.6 Egg white manufacture

The chicken egg is referred to as a polyfunctional component because of its numerous techno-functional properties such as emulsifying, foaming, gelling, thickening, coloring, and fragrant capabilities that make it a staple in both home cooking and the food industry. More particular properties are desired for some preparations, which may necessitate the use of egg white and egg yolk separately: egg white is a standard in terms of whipping, while egg yolk is the emulsifying agent par excellence (Anton et al., 2011).

Egg products are eggs that have been taken from their shells for processing. Breaking eggs, filtering, mixing, stabilizing, blending, pasteurizing, chilling, freezing or drying, and packaging are all part of the egg product production process (Suman et al., 2018). Whole eggs, whites, yolks, and different processed and pasteurized mixes

with or without non-egg components are examples of egg products. These egg products may come in liquid, frozen, or dried form. The architecture of the manufacturing facilities must be flexible and efficient in order to handle the specialized procedures necessary for creating various egg products. Some factories additionally contain shell egg grading facilities and dual jurisdiction activities in the egg products facility, such as boiling and hard-boiled operations.

1.6.1 Processing of egg products

Products derived from the processing of the egg or its many components or mixes, which may be partially supplemented by other foodstuffs or additives, and can be liquid or concentrated, dried, crystallized, frozen, deep-frozen, or coagulated are referenced as egg products. The food service business commonly uses liquid, frozen, and dry egg products as ingredients in other dishes such as prepared mayonnaise, ice cream, salad dressings, frozen desserts, cream puff, cakes, confections, and so on or transformed egg products such as scrambled eggs or omelets (FSIS, 2013; Wu, 2014)

As a result, the egg products business now provides a variety of goods originating from diverse technical schemes, with the goal of reacting as closely as possible to the products' techno-functional applications (Figure 1-11). This business, like any other agri-food industry, is subject to a variety of regulatory restraints, one of which is the management of cleanliness. In the great majority of situations, the content of an egg deposited by a healthy hen is sterile, and if the shell is unbroken, it will remain so for a long time. To manage the microbiological quality of egg products, it is required to restrict their initial contamination, eradicate all or part of the contaminating flora, and limit or even prevent its growth using different stabilizing methods.

Regarding the washing step, because it is not possible to produce completely clean eggs, eggs are washed and cleaned to remove stains, dirt, and other surface contaminants in order to reduce bacterial contamination and prevent bacteria from penetrating the egg contents, as well as to improve the appearance to the consumer. To

minimize bacterial penetration of the shells, eggs should not be left to soak in water while washing. Modern egg washers blast the egg with water that contains a sanitizer as well as detergent (USDA, 2000) The temperature of the washing water should be kept at 32.2 ℃ or higher, and it should be at least 11 ℃ warmer than the internal temperature of the eggs to avoid the wash water from being sucked into the eggs. According to USDA (2000), eggs should be rinsed in water at a temperature equal to or hotter than wash water to remove any remaining dirt and chemicals, after washing. The rinsing water usually contains a sanitizer, and chlorine-based compounds such as sodium hypochlorite (not less than 50 ppm or more than 200 ppm of available chlorine or its equivalent) (USDA, 2000). To avoid the possibility of microbial penetration into the eggs, shell eggs must be thoroughly dried immediately after rinsing. Eggs are air-dried using electric fans before being moved to conveyors for the oiling process, where they are sprayed with mineral oil that should be tasteless and colorless (Wu, 2014).

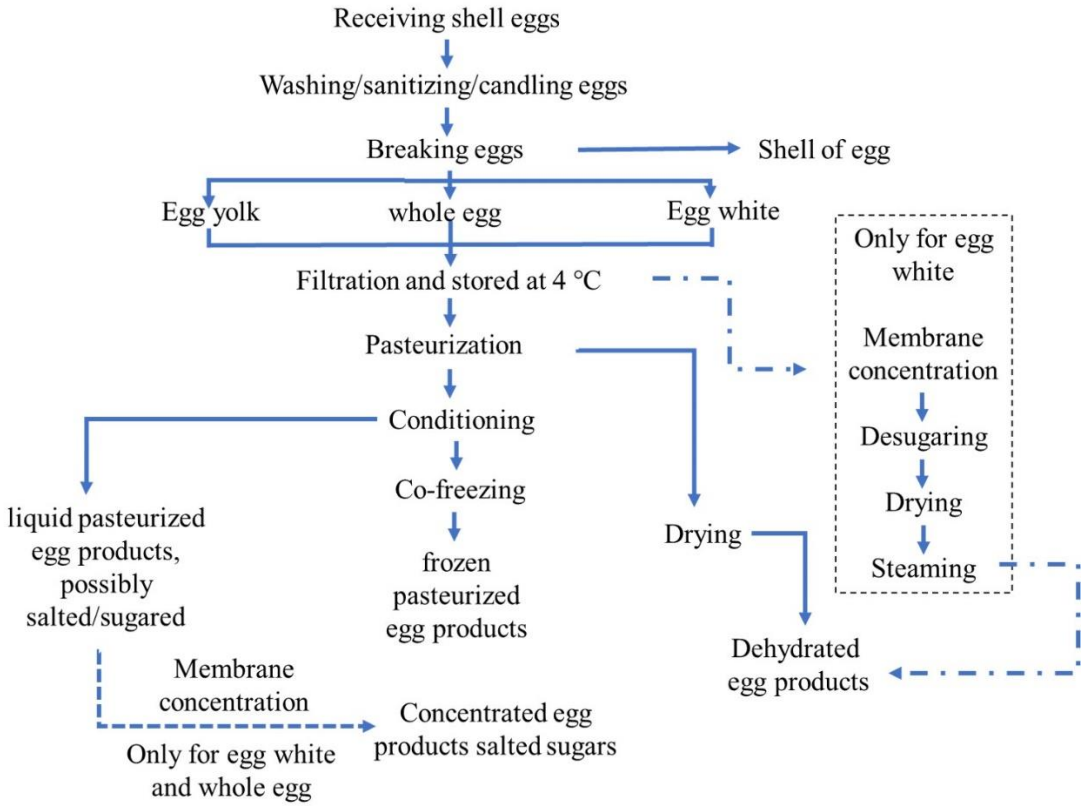


Figure 1-11 Production diagram of processing egg products (according to Anton et al., 2011; Suman et al., 2018).

After being oiled, the eggs are transported to the candling section, where faulty eggs are removed (Vaclavik & Christian, 2008). Candling is a useful technique to observe the shell and interior of eggs without shattering the shell; candling was previously used to examine arriving eggs for freshness by viewing their internal contents by candlelight, where egg contents may be seen when held up to a candle while being swiftly spun (Wu, 2014).

Individual eggs must be shattered, either automatically or manually (Anton et al., 2011). Specific equipment has therefore been designed (Figure 1-12), capable of producing up to 180,000 eggs per hour and enabling instantaneous or somewhat delayed separation of the white and yolk in time.

After filtering to remove shell pieces, the products are chilled to 4 °C before being delivered to storage tanks. In the storage tanks, dry extract adjustments, salt, sugar, or allowed additives (guar, xanthan in white egg, for example) can be added. After breaking and separation, the raw goods obtained include egg white with a dry extract of 10 to 11 % and a pH of 8.5 to 9.5, egg yolk with a pH of 6.5 and a dry extract of 42 to 45 %, and whole egg with a dry extract of 20 to 24 % and a pH of 7 to 7.5 (Anton et al., 2011). Variations in dry extract for the yolk and the whole egg are mostly linked to performance material (quality of white-yellow separation). The shells discarded at this stage are a rather high-humidity byproduct.

To limit the likelihood of food-borne pathogen contamination or proliferation, liquid eggs should be pasteurized as soon as possible after breaking. Pasteurization uses a combination of time and temperature to limit the number of live germs, particularly Salmonella. Pasteurization scales are often set around 65 °C for 2 to 6 minutes for the whole egg and yolk, and about 57 °C for 2 to 6 minutes for the egg white (Anton et al.,2011; Wu, 2014). There are also some other methods, such as ultra-heat treatment (UHT), which involves the utilization of high temperatures for a brief period. Typically, liquid eggs are heated at 70 °C for 1.5 mins and then packed in aseptic packages, extending the shelf life to up to 24 weeks, though the final product must still be

refrigerated (Zeidler, 2002; Wu, 2014). High-pressure processing (HPP) treatment typically preserves the food product's taste, look, and texture, as well as its nutrients (Cruz-Romero et al., 2004). When applied to liquid whole egg at 350 MPa and 50 °C at 2-min pulses for four cycles, HPP decreased Salmonella enteritidis, indicating that it can be employed as a pasteurization technique (Bari et al., 2008). Its potential for processing egg products has been thoroughly investigated (Juliano et al., 2012).

The products may be extended by freezing for up to 24 months. Freezing, however, has significant effects on the yolk and the liquid whole egg, resulting in irreversible gelation of the product and a modification of functional qualities (significant rise in viscosity product after thawing) (Anton et al., 2011).

Dried egg products are used in the manufacture of baked items, mayonnaise, salad dressing, pasta, and other products. Because dried eggs are easy to handle and prepare, they are ideal for storage and transportation. Spray drying, pan drying, and belt drying are the most common methods of drying, the most popular approach being spray drying. Liquid egg products can be concentrated before drying to improve thermal efficiency, increase dryer capacity, and change product properties such as lighter bulk density; liquid eggs can be concentrated using vacuum-type evaporation or ultrafiltration. The liquid egg is then atomized into a stream of heated air by high-pressure nozzles (500-6000 psi) in this procedure for fast water elimination (Wu, 2014). In the case of egg white, a "desugaring" step is required to avoid the development of the Maillard reaction throughout the drying process (Anton et al., 2011). This "desugaring" process involves the removal of glucose (about 0.5 g/L in a white liquid egg). It is produced either by controlled fermentation (yeasts or bacteria) or through an enzymatic method that employs the pair of glucose enzymes oxidase-catalase. This drying phase, as well as the subsequent steaming, can commonly increase specific properties functions of egg products (emulsifying properties, foaming properties).

Moreover, the concentration of the entire egg or egg white by ultrafiltration or reverse osmosis, followed by the addition of sugar and/or high concentration salt,

allows to produce goods that are stable for several months at room temperature.

1.7 Objective of the work

Through the discussion in the previous sections, we can find that: i: Pea proteins have a similar amino acid content to soybean proteins and are low allergenic proteins compared to soybean proteins. However, the extraction method, cultivation method, environment, and some other conditions may affect their protein and amino acid content. In addition, they have excellent functional qualities, are gluten-free, and are from a nongenetically modified organism. The techno-functional qualities and applications in food of pea proteins can be significantly influenced by protein concentration, concentration in 7S and 11S globulins, extraction procedure used, and processing conditions. Therefore, we should pay attention to the effect of the conditions of extraction and environment on pea proteins and their functional properties. ii: Eggs are an important source of animal proteins due to their high nutritional quality, excellent digestibility, and complete provision of essential amino acids. Egg white has better gelling properties than egg yolk which can be influenced by several conditions like pH, salt content, and protein content. iii: Although there are some researches focused on the mixtures of plant protein and animal proteins, they mainly concern soy and dairy proteins. There is a lack of research regarding the mixture of egg white as an animal protein source, especially on egg white and pea protein.

The context is the preparation of a new ingredient: so the scientific interests were as follows:

- The colloidal interactions in admixture of native proteins (solubility, complex formation…)
- Thermal behavior is very important regarding pasteurization treatment if a process similar to such ovoproduct is used to design a new ingredient
- The main functionality expected are foaming and gelling: a study of gelling

properties was preferred as it is the most exploited for plant proteins (meat analogues, eggs analogues): could we expect synergistic effects during the gelation of the mixed systems?

The originality of our work lies in the fact that it is a system derived from protein fractions of pea globulins and egg white which has not been studied so far. A complete study of heat-induced gelation and thermal properties will also be described. The different gels obtained will be characterized from a physicochemical point of view. The mechanisms of interactions between the different species present in solution and gels, as well as the gelation data will be confronted.

On the basis of the bibliography analysis and in order to achieve these objectives, the remainder of the document has been organized into five chapters:

Preparation and characterization of raw materials (Chapter 3). In this chapter, the focus will be on the production of raw materials as well as on their chemical and physico-chemical characterizations necessary for the study, such as sample composition (protein concentration, ash, water, etc.), protein composition (SDS-PAGE, SEC-HPLC), protein solubility.

Interactions between pea proteins and egg white proteins (Chapter 4, submitted to Food hydrocolloids). In this chapter, we will investigate the interactions between pea globulins and egg white proteins in aqueous mixtures at neutral and alkaline pH (pH 7.5 and 9), close to that of egg white. The potential interaction of whole pea globulins with purified LYS, OVA, or OVT was first examined by isothermal titration calorimetry (ITC) and ζ-potential measurements. Subsequently, the detected attractive interactions between LYS and pea globulins were further explored at different pH via characterization of formed structures by dynamic light scattering (DLS), laser granulometry, confocal laser scanning microscopy (CLSM), and optical microscopy.

Thermal behavior of pea and egg white protein mixtures (Chapter 5) (submitted to Food research international). In this section, we first prepare the protein mixtures at a

total concentration of 10 % (the concentration of a fresh liquid egg white manually broken from eggs), with different mass ratios (PPI-EW: 100/0, 75/25, 50/50, 25/75, and 0/100) at pH 7.5 and 9. Then, the Nitrogen solubility profile of the pure proteins and 50/50 mixture systems, and their polypeptide composition using electrophoresis are also characterized by Kjeldahl and SDS-PAGE respectively. Studies are followed by thermal properties obtained by MicroDSC from (25-105 °C), the gelling temperature (point) of the 10% protein PPI-EW mixtures was investigated by small amplitude rheology to anticipate possible pasteurization temperature adjustment in future manufacturing.

Nature of protein-protein interactions during gelation of mixtures between pea protein isolate and egg white proteins (Chapter 6) (to be submitted to Food Hydrocolloids). In this section, mixtures will be prepared at different mass ratios (PPI-EW: 100/0, 75/25, 50/50, 25/75, and 0/100) at pH 7.5 and 9 with a total protein content of 10 % (w/w). Gelation properties will be analyzed by small amplitude rheology (mesoscopic level), completed by a texture analysis (macroscopic level), and the observation of the microstructure of gels by confocal laser scanning microscopy (CLSM) (microscopic level). In addition, dissociation agents (Urea, Guanidine hydrochloride (GuHCl), Propylene glycol (PG), dithiothreitol (DTT), and a mixture of the 4 agents) will be used to have a better understanding of the interactions during gelation of the mixture. The dissociation agent is prepared in 100 mM Tris buffer at pH (7.5 and 9), and 100 mM Tris buffer is as a reference.

Conclusion and perspective (Chapter 7). In this section, we will collect the major conclusions on the interactions, thermal, and gelation properties of the mixture system between pea proteins (plant proteins) and egg white (animal proteins). Scientific perspectives are then proposed to broaden the scope of this work.

The objectives and strategy of this thesis have been represented in Figure 1-12.

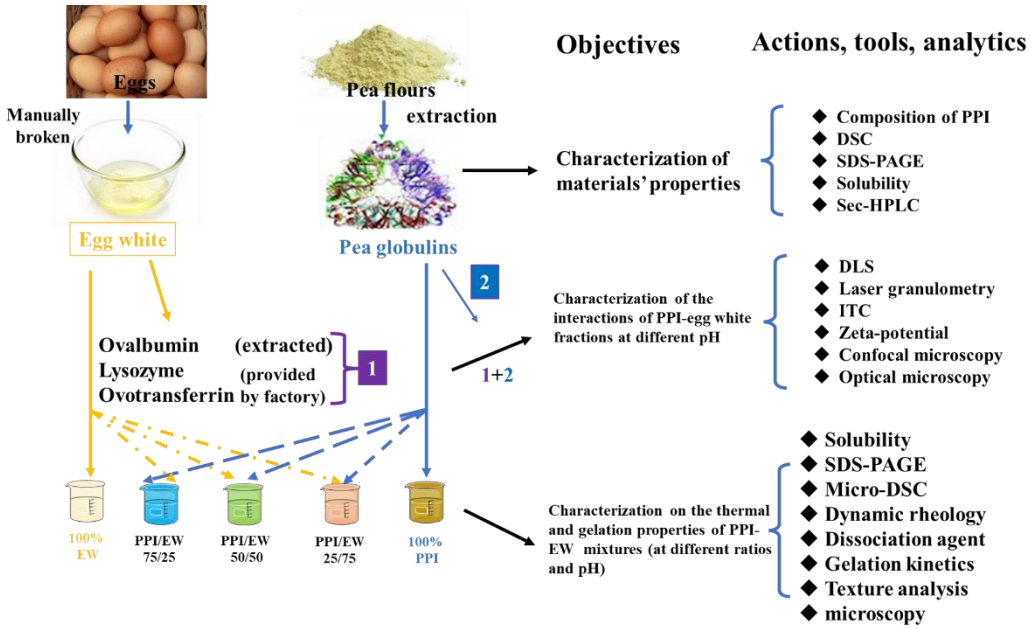


Figure 1-12 Synthesis of the thesis objectives and the sequence of the different phases of the project.

Chapter 2 Materials and methods

2.1 Materials

2.1.1 Pea flour

The yellow pea flour (P. sativum L.) used was supplied by the company Cosucra (Belgium). According to the supplier, the raw material behind these products is a mixture of several varieties of peas grown in France.

2.1.2 Egg, ovalbumin, ovotransferrin and lysozyme

Eggs were obtained from a local market in Dijon or Rennes (Label Rouge, France). The eggs were stored in a fridge at 4 °C and used 15 days before the expiration date. Ovalbumin (OVA) was extracted by the following methods. Ovotransferrin (OVT, 94 % protein content) and Lysozyme (LYS, 95 % protein content) were supplied from EUROVO (Annezin-les-Béthunes, France and Occhiobello, Italy, respectively).

2.1.3 Chemical reagents

All reagents and reagents used in the analysis were of analytical quality and obtained from Sigma Aldrich® (France) and VWR (France).

2.2 Methods

2.2.1 Preparation of purified protein fractions

2.2.1.1 Extraction of protein fractions from pea flours (pea globulins)

Pea globulins were extracted from smooth yellow pea flour (P. sativum L.), as supplied by Cosucra (Belgium). Isoelectric-precipitation technique was used to prepare pea protein isolate containing mainly globulins, based on the method of Chihi et al. (2016) and Mession et al (2012) with some modifications. Pea flour was mixed with

distilled water at 100 g/L, and the pH was adjusted to pH 8 with concentrated NaOH every two hours and stirred overnight at 4 °C. After adjusting the pH to 8, insoluble materials were removed by centrifugation (10 000 g, 30 min, 25 °C) and the recovered solution was adjusted to pH 4.8 by 0.5 M HCl. After acidification, the precipitated proteins were recovered by centrifugation (10000 g, 25 min, 4 °C). Afterwards, the pellets were dissolved in 5 L 0.1 M sodium phosphate buffer at pH 8 overnight at 4 °C for complete dissolution. The protein suspension was obtained by centrifugation (10000 g, 20 min, 20 °C) and then concentrated 5 times by ultrafiltration (from 5 L to 800-900 mL) and desalted by diafiltration against 10 L 5 mM ammonium buffer pH 7.2 and 0.05 % sodium azide on an 1115 cm^2 Kvick lab Cassette (UFELA0010010ST, GE Healthcare, Amersham Biosciences, Uppsala, Sweden) with a molecular weight cut-off of 10 kDa. The pressure of the device was lower than 2.5 bars. The pH of obtained protein solution was adjusted to pH 7.2 and then was frozen. The pea globulin powder was obtained after lyophilization and stored at -18 °C. The process for obtaining pea globulins is shown in Figure 2-1. The pea globulins prepared will be referred to as PPI.

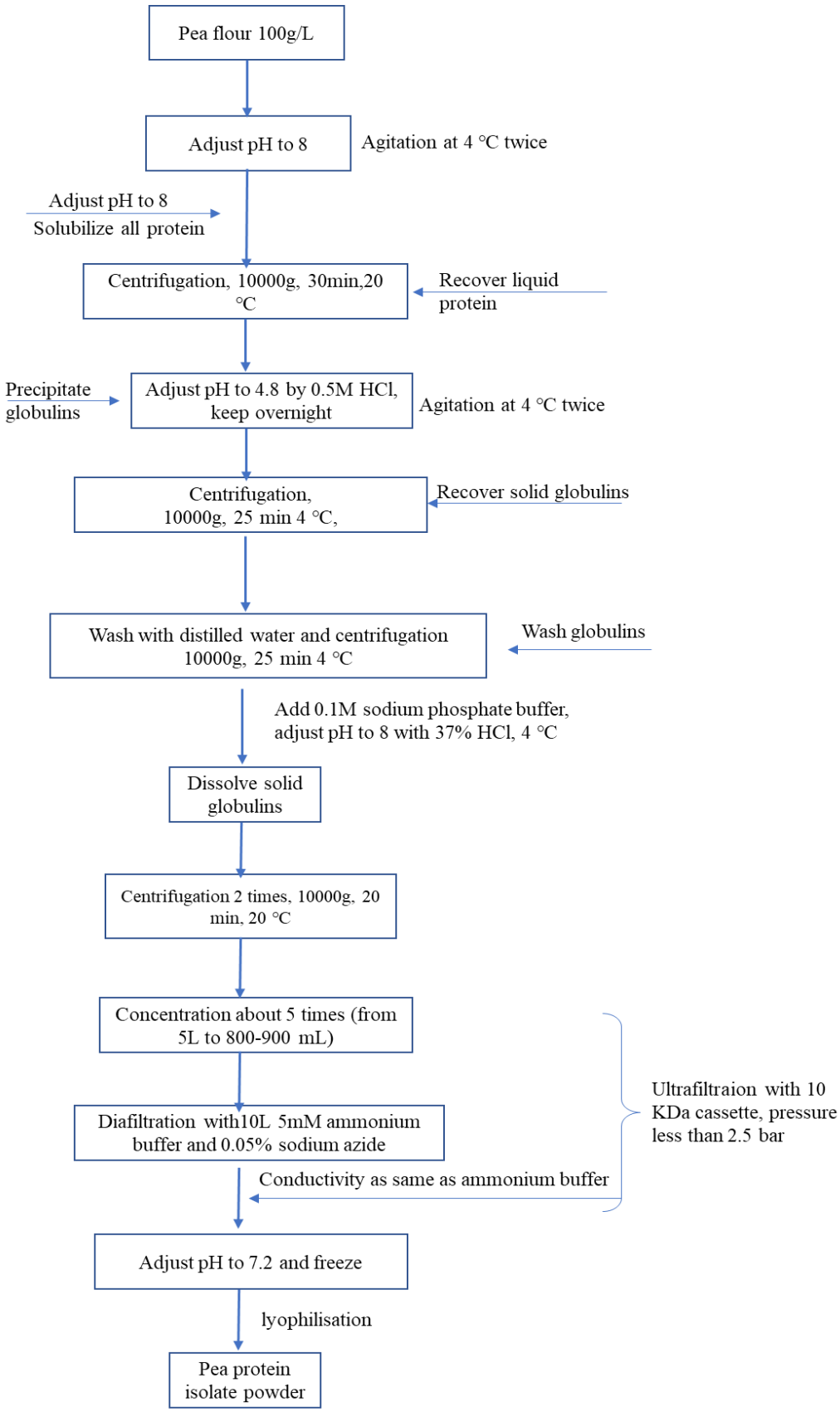


Figure 2-1 Pea globulin extraction procedure

2.2.1.2 Extraction of protein fractions (Ovalbumin) from fresh eggs

Ovalbumin was extracted from fresh eggs from the local market according to Croguennec, Nau, Pezennec, & Brule, (2000). Egg white recovered from 12 eggs was diluted with 2 volumes of distilled water, then the pH was adjusted to 6.0 with HCl 1 M to precipitate ovomucin. Subsequently, the solution was stirred at 4 ℃ overnight. Then the supernatant was recovered after centrifugation (10000 g, 4 ℃, 30 min) and adjusted to pH 8.4 with NaOH 5 M. After centrifugation (10000 g, 25 ℃, 25 min), the supernatant was filtered with a plastic strainer and injected to an anion exchange chromatography Q-Sepharose Fast flow column (Pharmacia Biotech AB, Saclay, France) to separate the OVA from the other egg white proteins. The collected ovalbumin was dialyzed by tube with a cut-off of 12000-14000 MWCO, at 4 ℃, around 3-5 days. Afterwards, the solutions were lyophilized, and the ovalbumin was obtained. The process for obtaining ovalbumin is shown in Figure 2-2.

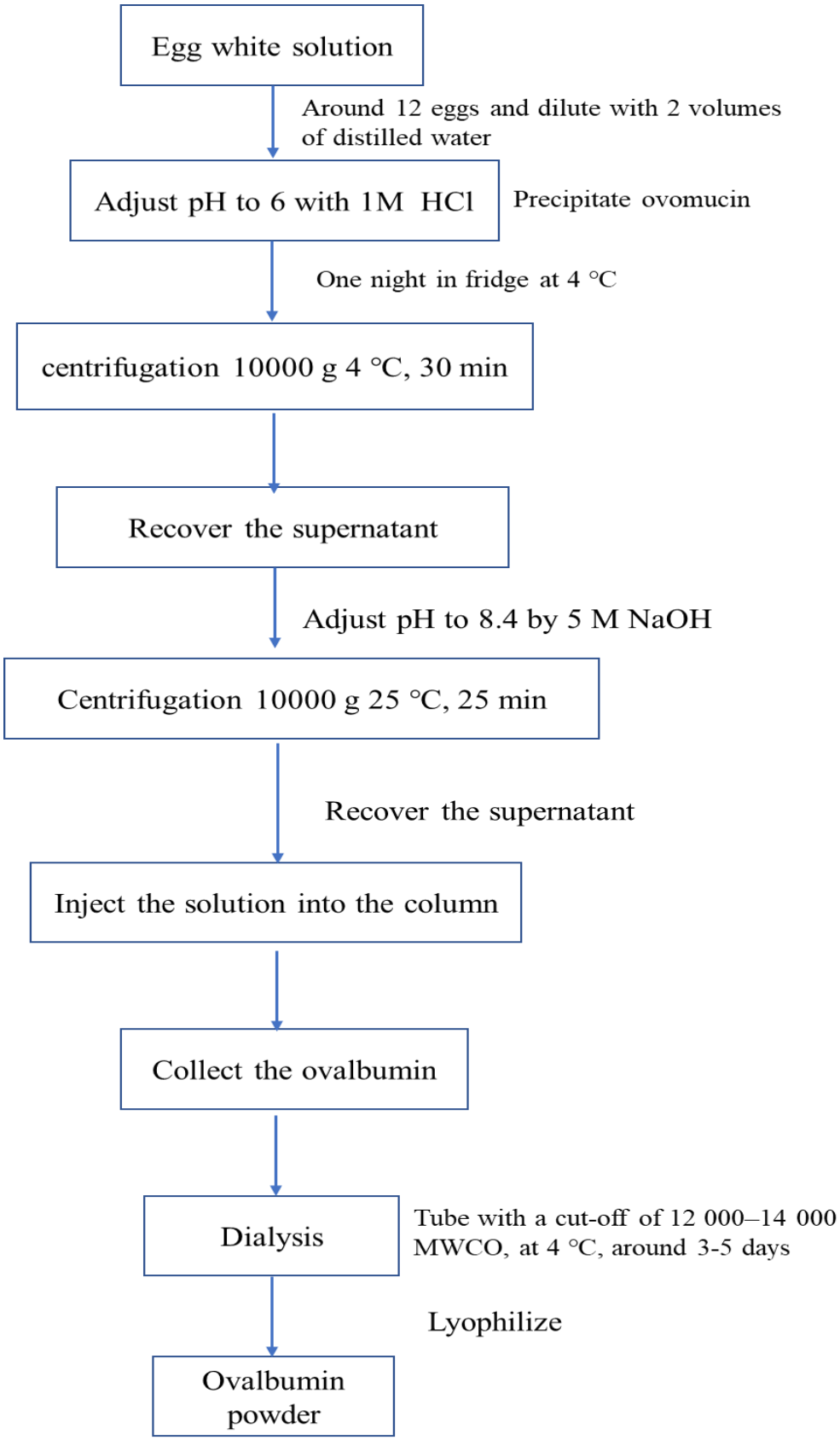


Figure 2-2 Ovalbumin extraction procedure

2.2.2 Preparation of protein solutions

2.2.2.1 Pea protein dispersions preparation

For thermal analysis and gelling properties evaluation, to obtain an initial dispersion at the necessary protein content (10 %), a quantity of extracted pea protein isolate (PPI) powder was mixed with distilled water. The dispersion was then agitated at 350-400 rpm for more than 3 hours at 4 °C to allow the proteins to completely hydrate. Then the pH was adjusted to 7.5 and 9 using 1 M NaOH or 1 M HCl without affecting the concentration of the dispersion.

To study the interactions between PPI and egg white fractions (ovalbumin, lysozyme, and ovotransferrin), a quantity of extracted pea protein isolate (PPI) powder was dissolved in either 10 mM HEPES at pH 7.5 or in 10 mM TRIS buffer at pH 7.5 or pH 9 and stirred mechanically at 400 rpm over 3 hours at room temperature to ensure complete hydration of the protein powders.

2.2.2.2 Preparations of egg white, ovalbumin, lysozyme and ovotransferrin stock solutions

Egg white was manually broken from fresh eggs (Label rouge, France) which were obtained from a local market in Dijon or Rennes (France), and carefully separated from egg yolks and chalaza. Then the fresh liquid egg white (EW) was agitated at 350-400 rpm for more than 3 hours at 4 °C. Then the pH was adjusted to 7.5 and 9 using 1 M NaOH or 1 M HCl without affecting the concentration of the dispersion. Lysozyme (LYS), ovalbumin (OVA), and ovotransferrin (OVT) stock solutions were dissolved either in 10 mM HEPES at pH 7.5 or in 10 mM TRIS buffer at pH 7.5 or pH 9 and stirred mechanically at 400 rpm over 3 hours at room temperature to ensure complete hydration of the protein powders. The insoluble protein part was estimated as negligible. The pH of the protein suspensions was then adjusted by 0.1 M HCl or NaOH before each test.

2.2.2.3 Preparations of mixture systems

2.2.2.3.1 Preparation of PPI-EW mixture systems

Extracted pea protein isolate powder (PPI) was dissolved in distilled water. The dispersion was then agitated at 350-400 rpm for more than 3 hours at 4°C to allow the proteins to completely hydrate. PPI-EW combinations were then made at various mass ratios of 100/0, 70/30, 50/50, 30/70, and 0/100 with the native pH of PPI and EW dispersions. Subsequently, the different combination systems were adjusted to pH 7.5 and 9 using 1 M NaOH or 1 M HCl without affecting the concentration of the dispersion.

2.2.2.3.2 preparation of gel

Protein combinations were prepared as mentioned in Section 2.2.2.3.2. Subsequently, the solutions were heated in a water bath from 25 to 95 °C, and then hold at 95 °C for around 30 min, then, all the samples were cooled down to room temperature with ice and were kept at 4 °C overnight.

2.2.3 Characterization of native protein fractions

2.2.3.1 Determination of moisture content

The moisture content was determined according to method 925.1 (AOAC 1990). A 1 g sample is weighed inside an aluminum capsule that has been pre-tared. It is then baked at 105 °C until it reaches a constant mass, after which it is chilled in a desiccator for 2 hours. The dry mass is calculated as the average of three observations. The proportion of moisture (MS) is calculated using the following equation (1):

$$\%\boldsymbol{MS} = \frac{\boldsymbol{M_{SEC}}}{\boldsymbol{M_i}} \times \mathbf{100} \text{..... Equation (1)}$$

Where: %*MS*: the moisture content (%)

M_i: mass of the initial sample (g)

M_{sec}: mass of the dry sample (g) after passing through the oven at 105 °C.

2.2.3.2 Determination of mineral (ash) content

The mineral content of protein isolates was determined according to Method 923.03 (AOAC, 1990). The dehydrated samples were placed in a muffle furnace (MR 260, Heraeus) at 600 °C for 24 hours. The percentage of mineral matter is calculated by equation (2):

$$\%MM = M_{cendres}/M_{sec} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots \textbf{Equation (2)}$$

Where: %*MM*: mineral matter content (%, w/w on a dry basis);

M_{sec}: mass of the sample after dehydration (g);

$M_{cendres}$: mass of ashes after calcination at 600°C (g).

Three measurements were performed for each sample.

2.2.3.3 Determination of protein content by determination of total nitrogen

The determination of total nitrogen was carried out according to method 920.87 (AOAC 1990), also known as the Kjeldahl method, in a digestion unit (Scrubber B414, Büchi, Rungis, France) and a distillation unit (K355, Büchi, Rungis, France). The protein content of the protein isolates and dispersions was then determined using the nitrogen-protein conversion coefficient K, which was found to be 5.44 for pea proteins (Mosse,1990) and 6.25 for egg white proteins (Stitcher, et al., 1969).

A precisely weighed amount of material was put into the matrass, resulting in 10 to 200 mg of protein per matrass. Each matrass received 10 ml of 96 % sulfuric acid, 2 glass balls, and 1 catalyst pellet (Kjeltabs NACT AA44, Thompson and Capper Ltd, Cheshire, England). Mineralization was carried out utilizing a BÜCHI K355 digesting equipment (Rungis, France). The samples were heated at 320°C for 20 mins, then 400 °C for an hour, allowing complete nitrogen conversion to ammonium ion. The acid vapors were eliminated by a B414 purifier from BÜCHI (Rungis, France). After cooling, each matrass received 25 mL of distilled water and 2 drops of phenolphthalein. The

matrass was installed on BÜCHI's K355 distillation machine (Rungis, France). 32% NaOH was added until the color changed to blue or light brown, indicating the transition from ammonium ion to ammonia. The ammonia condensates were distilled by trapping them in 50 mL of 2 % (m/v) boric acid. The assay was then carried out with a 0.1 M HCl solution until the initial pH of the 2 % (m/v) boric acid solution was obtained. The percentage of nitrogen was determined by the following equation (3):

$$\%\boldsymbol{Protein} = \frac{\boldsymbol{V \times N \times 14 \times 100}}{\boldsymbol{m \frac{\%MS}{100}}} \times \boldsymbol{K} \ldots\ldots\ldots\ldots\ldots\ldots \textbf{Equation (3)}$$

Where: *V*: volume of HCl used for sample titration (mL),

N: normal hydrochloric acid,

m: the mass of the sample,

% MS: dry matter percentage of the sample,

K: conversion factor for pea protein (5.44), egg white and pea flour (6.25).

2.2.3.4 Content of lipids extractable by petroleum ether

The lipid content was characterized by extraction on a Soxhlet apparatus with petroleum ether, based on the AOAC reference method n° 923.03. In cellulose cartridges, 5 g of product was precisely weighed (m2). These cartridges were placed in the Soxhlet apparatus, which was filled with enough petroleum ether to allow the solvent to reflux via the siphon. To compensate for anticipated solvent evaporation, an extra 25 mL of petroleum ether was added. The flasks were heated to reflux, and the extraction was carried out for 5 hours. At the end of the extraction, the solvent remaining in the flasks was evaporated using a rotary evaporator, under partial vacuum, with a water bath at 65 °C. The flasks were dried under a hood for one night, then for at least one hour in a desiccator to remove any remaining solvent and humidity before being weighed (m3). The lipid % was calculated using the following equation (4):

$$\%\boldsymbol{Lipid} = \frac{\boldsymbol{m3 - m1}}{\boldsymbol{m2}} \times \boldsymbol{100} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{Equation (4)}$$

Where: m1 the mass of the balloon and the glass beads alone,

m2 the mass of sample initially placed in the cellulose cartridge,

m3 the mass of the lipids, the balloon and the glass beads,

2.2.3.5 Content of carbohydrates

The mass fraction of carbohydrates was determined by the following formula (Ganzaroli et al., 2017) with some modifications:

$$\%\boldsymbol{M_C = 100 - (M_W + M_A + M_L + M_P)} \text{..... Equation (5)}$$

Where: M_C: carbohydrates content,

M_W: moisture content,

M_A: ash content,

M_L: lipid content,

M_P: protein content

2.2.3.6 Protein solubility as a function of pH

To study the effect of pH on the solubility of proteins in an aqueous medium, we used the experimental protocol described by Djoullah et al. (2015). Protein solutions (PPI and EW) and PPI-EW mixture at a weight ratio of 50/50 were prepared in distilled water to obtain the desired protein content (1 %, w/v). All the solutions were stirred for at least 3 hours to make sure completely hydrate, then were adjusted with a solution of NaOH (0.1 M) or HCl (0.1 M) to widen the range of pH studied varying from 2 to 10, also 0.1M NaCl was used to maintain the ionic strength in EW diluted condition. The mixtures were stirred for one hour at 4 °C. Protein suspensions were centrifuged at 10,000 g for 20 min at 4 °C. The protein content of the supernatants was determined by the Kjeldahl method. Solubility is expressed in grams of soluble protein per 100 grams of total protein or soluble nitrogen (NS) is calculated according to equation (6).

$$\boldsymbol{NS}(\%) = \frac{\boldsymbol{N\ dissolved\ in\ the\ supernatant}}{\boldsymbol{N\ initially\ in\ the\ isolate}} \times \boldsymbol{100\%} \text{..... Equation (6)}$$

where NS is nitrogen solubility and N is nitrogen amount.

Meanwhile, the expected solubility at each pH was used as a reference for the solubility of PPI-EW (at a weight ratio of 50/50) and calculated by equation 7.

$$\boldsymbol{solubility_{expected} = solubility_{ew} \times 0.5 + solubility_{PPI} \times 0.5} \quad \textbf{……Equation (7)}$$

2.2.4 Polypeptide composition by electrophoresis (SDS-PAGE)

Electrophoresis is an analytical technique that allows for the separation of proteins on gel under the influence of an electric field, resulting in protein migration based on the difference in mobility of the polypeptides and their molecular weights. The electrophoresis gel is prepared by cross-linking acrylamide monomers, which form a three-dimensional network. This kind of method makes it possible to identify and quantify the protein composition of a sample. It also allows for the estimation of denaturation effects and the clarification of relationships amongst aggregated proteins. The pore size of the gel is determined by the concentration of acrylamide. In a low-mesh gel, proteins migrate at a constant rate, whereas in a high-mesh gel (separating gel), the migration rate varies depending on the protein species; small particles will reach the migration front (low molecular weight) quickly, while large particles will be trapped away from this migration front (high molecular weights). Sodium dodecyl sulfate (SDS) functions as an anionic surfactant during gel formation to raise the total negative charge of proteins. Furthermore, adding SDS to the sample solution dissociates the subunits from the oligomeric protein, allowing the polypeptide chain to completely stretch. Reducing chemicals, such as dithiothreitol (DTT), decrease all S-S covalently bonded proteins, whether native or aggregated. The addition of SDS and DTT demonstrates the entire movement of protein components.

The different samples (pea protein isolate, egg white, and PPI-EW mixtures at a weight ratio 50/50) were treated and prepared at least half in sample buffer: 187.5 mM Tris-HCl, pH 8.9, 10 % (m/v) glycerol, 2 % (m/v) SDS and 0.05 % (m/v) of

bromophenol blue, in the presence (reducing conditions) or in the absence (non-reducing conditions) of 2 % (m/v) of dithiothreitol (DTT). Novex™ electrophoresis gels (ThermoFisher, Dardilly, France) at 10 % to 20 % Tris-Glycine were used. It should be noted that samples prepared in reducing conditions should be heated at 90 °C for 10 min to reduce disulfide covalent bonds. Therefore, the samples under reducing conditions were heated in a water bath for 10 min at 95 °C. All the samples were prepared and then 10 µg of protein was deposited. Molecular weight protein markers from Sigma–Aldrich® (SigmaMarker™ S8445, wide range, Mw 6.5 to 200 kDa) or Thermo Scientific™ (PageRuler™ Unstained Broad Range Protein Ladder, Mw 5 to 250 kDa) were used. The migration was carried out at 35 mA per gel, with the following migration buffer: 0.3 % (w/v) trizma base, 1.45 % (w/v) glycine, and 0.1 % (w/v) SDS, in a Scientific® Mini Gel Tank of Migration (Thermo Fischer). The gels were then rinsed with distilled water, and the fixation was performed in four successive distilled water baths heated for 1 min in a microwave at 550 W. The staining of the gels was performed with Coomassie blue, Thermo Scientific™ PageBlue™ Protein Staining Solution, overnight. The discoloring was then achieved in several baths of distilled water, until the desired color. The gels were imaged by the ChemiDoc™ XRS+ System from Bio-Rad Lab. To know the difference between the polypeptide of the PPI-EW mixture with or without centrifugation, a centrifugation step (10000 g, 20 min, 4 °C) was performed on protein suspensions before analysis.

2.2.5 Thermal properties by differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique for measuring the absorption and/or release of heat during the thermo-denaturation of a molecule. In other words, the device can determine the temperature and heat flow associated with material transitions as a function of time and temperature. What is known to us is that intense heating causes proteins to lose their spatial conformation permanently. Therefore, this research determines the temperature of thermal denaturation of a molecule as well as

the amount of energy required for the latter to change states.

The sample is heated simultaneously with an inert reference (solution in which the protein is solubilized) during the experiment, and the differential heat flow between the sample and the reference is monitored over time. When an exothermic or endothermic reaction occurs, a positive or negative peak with respect to the baseline develops. If a sample does not change state, the flow exchanged with the reference is restricted to the baseline. The average denaturation temperature is indicated at the peak apex (Td). As a result of the DSC analysis, it is feasible to determine not only the temperatures of the transitions of state but also their associated enthalpies, which are represented by the areas beneath their respective peaks (ΔH_d). Meanwhile, deconvolution is a method of separating the overlapping peaks, and it can be used in DSC peak separation (Miyagawa, & Adachi, 2019). The values at beginning of the endothermic peak (50 ℃) and the end of the heating ramp (105 ℃) were connected by a straight line to draw the baseline. Deconvoluted curves were generated by subtracting baselines from DSC curves. Differentiating the original DSC curve with time determined the number of peaks to be separated. The function that represents each peak was chosen from the functions built into the software so that the curve obtained by summing the individual peaks could accurately represent the original curve, i.e., the asymmetric double sigmoid and Lorentz functions shown in equations (8) and (9), respectively, were chosen to represent the peaks.

$$y = H_1 \frac{1}{1+exp\left(-\frac{\theta-\theta_c+w_1/2}{w_2}\right)}\left[1 - \frac{1}{1+exp\left(-\frac{\theta-\theta_c+w_1/2}{w_3}\right)}\right] \quad \text{Equation (8)}$$

$$y = \frac{2H_2}{\pi}\frac{w}{4(\theta-\theta_c)+w} \quad \text{Equation (9)}$$

where y is the heat flux, θ is the Celsius temperature, θ_c is the Celsius temperature at the center of the distribution, w, w_1, w_2, and w_3 are constants related to peak width, and H_1 and H_2 are constants.

The study of the thermodynamic properties of the initial dispersions of PPI, EW, and PPI-EW mixtures at different mass ratios (25/75, 50/50, 75/25) at pH 7.5 and 9 with

a protein concentration of 10 % (w/w) was carried out using a Micro DSC III differential scanning calorimeter (Setaram, Caluire, France). Approximately 0.5 g of sample was weighed in an aluminum pan, hermetically sealed, and heated from 25 to 105 °C at 0.5 °C/min. A pan with distilled water at room temperature was used as a reference. All experiments were conducted in triplicate. One replicate of each sample was re-heated after cooling to check that denaturation was irreversible. The evolution of the heat flux as a function of temperature (thermogram) was processed using the thermal analysis SETSOFT 2000 software (Setaram) and the following parameters were evaluated for each peak: the denaturation temperature (Td) and the associated denaturation enthalpy (ΔH_d). Deconvolution of thermograms was performed by Origin 2019 Pro.

2.2.6 Determination of particle surface charge or Zeta potential (ζ)

The zeta potential is an essential and easily measured indication of the stability of colloidal dispersions that may be used to assess protein-protein and protein-solvent electrostatic interactions. From a theoretical standpoint, zeta potential is the electric potential in the interfacial double layer of a dispersed particle or droplet against a point in the continuous phase distant from the interface（Lu & Gao, 2010). The amount of the zeta potential in a dispersion shows the degree of electrostatic repulsion between nearby, similarly charged particles. A large zeta potential will impart stability on molecules and particles of sufficient size, i.e., the solution or dispersion will resist aggregation as shown in Figure 2-3 (Selvamani, 2019). When the potential is low, attractive forces may outweigh repulsion, causing the dispersion to break apart and flocculate. Colloids with a high zeta potential (either negative or positive) are electrically stabilized, whereas colloids with a low zeta potential coagulate or flocculate. The pH of the medium is the most critical element affecting zeta potential. Other parameters include ionic strength, additive concentration, and temperature. The zeta

potential of particles can be calculated by the following equation (10):

$$\zeta = \frac{3\eta f(ka)}{2\varepsilon \frac{V}{E}} \text{..... Equation (10)}$$

Where: ε: the medium permittivity,

η: the viscosity of the dispersion medium,

f (ka): a function related to the proportion between the size (a) characteristic of the system and the Debye length (1/κ),

V: particle velocity ($\mu m.s^{-1}$),

E: the electric field applied per unit length ($V.cm^{-1}$).

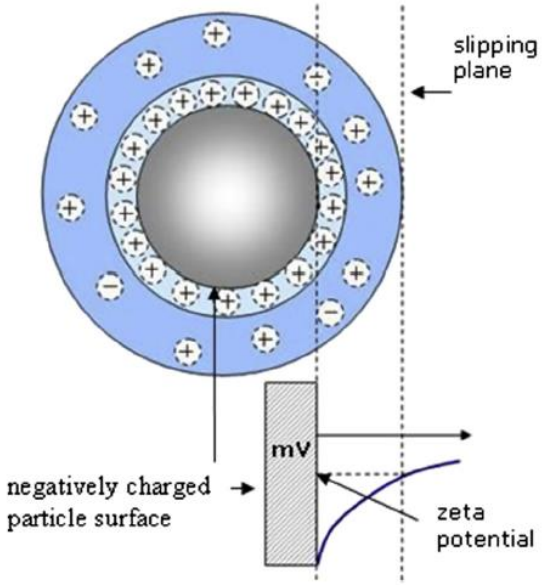


Figure 2-3: Schematic diagram of the electric double layer on the surface of liquid nanoparticles (Selvamani, 2019)

The measurements were made on 1mL of PPI (2.4 g/L), LYS (14.3 g/L), and their mixtures at different molar ratios (see the ratios in section 4) prepared in TRIS buffer at pH 7.5 and 9 using a Malvern Zetasizer Nano ZS (Nanosizer, Malvern Instruments, UK). The ζ-potential was measured at 25 °C using a laser Doppler velocimetry and phase analysis light scattering (M3-PALS0) using disposable electrophoretic mobility cells (DTS1070). The equilibration time was set at 120 s, and at least 11 runs were performed for each measurement. The measurements were carried out at a voltage of

150 volts and were repeated three times for each sample (PPI, LYS, and PPI-LYS mixtures at pH 7.5 and 9). To identify the ζ-potential of PPI and LYS as a function of pH, PPI and LYS stock solutions were prepared as follows. PPI (2.4 g/L) and LYS (14.3 g/L) were dissolved in distilled water until complete protein hydration. Then, 0.1-1 M HCl or NaOH was used to adjust pH from 2-12.

2.2.7 Determination of particle size by dynamic light scattering (DLS) and laser granulometry

2.2.7.1 Study of particle size by DLS

DLS was performed to have a better understanding of the changes in size and distribution of the particles present in various protein mixtures. DLS is a technique for determining the polydispersity of particles in protein suspensions as well as the size of the populations produced by these particles. The basic principle of this equipment is based on measuring the intensity of light dispersed by particles agitated by a Brownian motion and with no interactions between them at a specific detection angle. Small particles disperse more quickly in the medium, resulting in a fast-fluctuating intensity signal when compared to big particles, which diffuse more slowly (Hassan, Rana, & Verma, 2015). The primary results of DLS are particle size (hydrodynamic radius or hydrodynamic diameter (Dh)) which is governed by the Stokes-Einstein equation, which is based on Brownian motion of particles as a function of aqueous phase viscosity and temperature. Individually moving things, such as single particles and particle aggregates or agglomerates, are referred to by the hydrodynamic diameter. It usually represents the outside dimensions, but it has nothing to do with the size of the constituent particles within an aggregate or agglomeration. To measure the particle size, it is necessary to calculate the speed of movement of these particles in their solvent by measuring the diffusivity of light (coefficient of translation).

Variations in the intensity of scattered light as a function of time are attributed to

the scattering of the entities in solution when the solvent is known, and the temperature is constant. A correlator calculates the adjustment of the cumulative intensity of scattered light following an exponential decline in the case of spherical particles during the measurement. This allows the translational diffusion coefficient to be calculated, which is connected to a single apparent (spherical) particle diameter. The Stokes-Einstein equation (11) was used to calculate the distribution of apparent hydrodynamic diameter (Dh) from the mean translational diffusion coefficient distribution:

$$\boldsymbol{Dh = \frac{kT}{3\pi\eta D}} \quad \textbf{..... Equation (11)}$$

Where: *Dh*: the particle's hydrodynamic diameter (m) ;

T: The absolute temperature (K) ;

K: The Boltzmann constant ($J.K^{-1}$) ;

η: The dispersing medium's viscosity ($m^{-1}.Kg.s^{-1}$) ;

D: The diffusion coefficient ($m^2.s^{-1}$).

The size distribution of PPI and LYS was determined by DLS (Nanosizer, Malvern Instruments, UK). The progress was measured according to the previous paper by Schmitt, et al. (2007) with some modifications. PPI and LYS stock solutions were prepared as mentioned before in section 2.2.2, and first diluted 5 times in 10 mM Tris-HCl buffer at pH 7.5 or 9, before measurement. Solutions were filtered with a 0.22 μm cellulose membrane (Millipore Corp.) as well. 1 mL of the samples was measured at least 3 times at 25 °C.

2.2.7.2 Study of particle size by laser granulometry

Laser granulometry as known as laser diffraction is based on the principle of laser diffraction, in which particles diffract light at a specific angle. The diffraction angle is inversely proportional to particle size, and the intensity of the diffracted beam at any angle is a measure of the number of particles in the optical path with a certain cross-sectional area (Di Stefano, Ferro, & Mirabile, 2010).

A parallel beam of monochromatic light is directed onto detectors after passing through a suspension housed in a sample cell. Two diffraction theories are often employed to calculate particle sizes from light intensity perceived by detectors: the Fraunhofer diffraction (Franuhofer, 1817) and the Mie theory (Mie, 1908). Both theories imply that the particles have a spherical form; in other words, the particle dimension is the optical spherical diameter, which is the diameter of the sphere with the same cross-section area as determined by laser diffraction (Di Stefano et al., 2010; Magno et al., 2018). It evaluates grain size as a proportion of volume. The volumetric diameter d [4;3] was calculated by equation (12)

$$\boldsymbol{d_{[4;3]} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}} \quad \textbf{.....Equation (12)}$$

Where: n_i, d_i: The total number of particles with particle size d_i is n_i

The size distribution of PPI-LYS mixtures at different ratios was determined by laser granulometry (Mastersizer 2000, Malvern Instruments, UK). PPI and LYS stock solutions were prepared as mentioned before in section 2.2.2. 1 mL of the samples was measured at least 3 times at 25 °C. The stirring speed was set at 1100 rpm. The refractive index of the continuous phase was set at 1.33, which corresponds to that of water, and at 1.45 for the dispersed phase.

2.2.8 Determination of thermal properties by isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) is not only advantageous in that it provides thermodynamic parameters, namely variations in Gibbs energy (ΔG), changes in entropy (ΔS), and enthalpy (ΔH), as well as binding constant and stoichiometry (n) from a single titration. What is most important is that ITC is label-free, so it doesn't add artifacts (Rajarathnam & Rösgen, 2014; Loh, Brinatti, & Tam, 2016). Typical equipment and the progress of ITC are shown in Figure 2-4. In brief, the sample cell and the other reactant are inserted in the syringe as a ligand. An adiabatic device

separates the sample cell and the reference cell (Figure 2-4 A). The ligand is continually dripped into the sample pool by the syringe, which also has a stirring function. After a specific amount of time, the instrument monitors the sample cell's heat change and compares it to the reference cell, displaying an endothermic or exothermic peak. To maintain the temperature constant, the exothermic reaction causes the negative feedback of constant temperature power, whereas the endothermic reaction causes the positive feedback of constant temperature power. Observations are shown versus time as the power required to keep the reference and sample cells at the same temperature (Figure 2-4 B). As a result, the experimental raw data is a series of heat flow (power) spikes, with each spike corresponding to one ligand injection. These heat flow spikes/pulses are time-integrated to give the total heat exchanged after each injection. The pattern of these heat effects as a function of the molar ratio [ligand]/[macromolecule] may then be examined to determine the thermodynamic characteristics of the interaction under consideration. Degassing samples is frequently required in order to achieve accurate measurements since the presence of gas bubbles within the sample cell causes incorrect data plots in the recorded findings. The entire experiment is computer-controlled.

The study provides stoichiometry (n), affinity constant (K), and the binding reaction's enthalpy (ΔH). Therefore, the free energy of binding (ΔG) and the entropy (ΔS) are then calculated using the equation (13) and (14), respectively.

$$\Delta G = -RT \ln K \quad \textbf{Equation (13)}$$

$$\Delta G = \Delta H - T\Delta S \quad \textbf{Equation (14)}$$

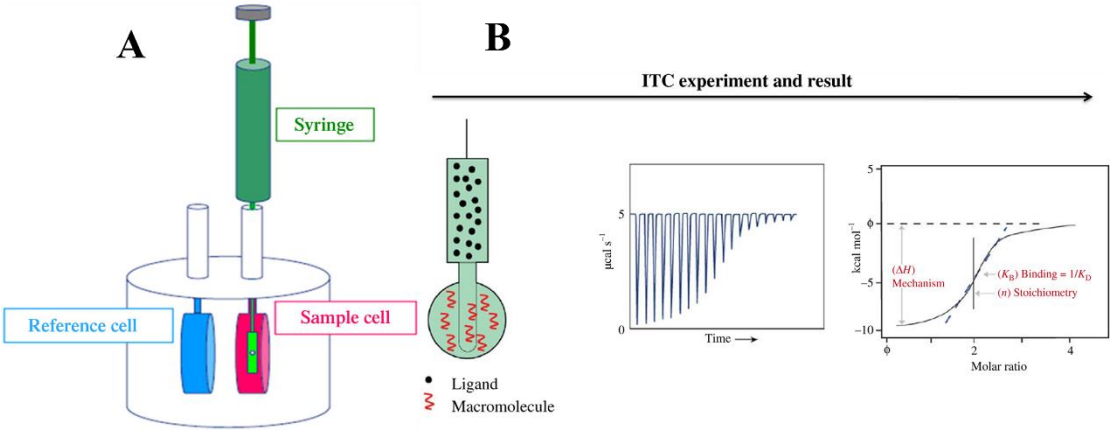


Figure 2-4: Introduction of ITC (Srivastava & Yadav 2019). A: equipment of typical ITC, B: presentation of the progress and result of ITC

ITC experiments were carried out using an ITC calorimeter (Microcal, Northampton, MA) with a standard volume of 1.4255 mL at 25 °C. Stock solutions prepared as mentioned before (Section 2.2.2) were filtered through 0.2 µm filters and degassed under vacuum several times to guarantee no bubbles inside the solutions. The solutions of PPI, egg white proteins (LYS, OVA, OVT), and buffer were placed in the reaction cell, syringe, and reference cell respectively. A total number of 29 injections of egg white protein stock solutions (10 µL of each) were performed after the calorimeter finalized the primary equilibration, with a 200 s interval between the injections, leaving 60 s at the beginning of the experiment before the first injection. These conditions are according to the methods of Nigen, Croguennec, Renard, & Bouhallab (2007). The stirring rate was set at 300 rpm. Data resulting from the subtraction of reference values (dilution heat) from the sample values were analyzed by Micro ORIGIN version 7.0 (Microcal, Northampton, MA). Control experiments were performed in each case by titrating the egg white protein into the buffer and were subtracted from raw data to determine corrected enthalpy changes. Each ITC data were collected by at least two independent measurements and reproducible data was employed.

To analyze ITC results, the mean molecular weight of globulins in PPI (Mw PPI) was approximated by the following equation (15):

Mw PPI = (Mw PPI-11S) × (11S-to-(7S+11S) ratio) + Mw PPI-7S (7S-to-(7S+11S) ratio) **Equation (15)**

with Mw PPI-11S = 360 kDa, Mw PPI-7S = 150 kDa, and 11S-to-(7S+11S) ratio = 0.59 and 7S-to-(7S+11S) ratio = 0.41 deduced from enthalpy area deconvolution from DSC spectra (chapter).

The Mw PPI value was thus estimated at 273,9 kDa. Then, the molar concentration of PPI (C_{ppi}) was obtained by dividing the protein concentration in the solution by the mean PPI Mw.

2.2.9 High-performance liquid chromatography *(*HPLC)

2.2.9.1 Size exclusion chromatography for PPI

High performance size exclusion chromatography (SEC-HPLC) is a type of liquid-phase chromatography that is also known as gel chromatography. The basic theory behind this technology is to move molecules or macromolecules at a constant pace through an elution buffer (mobile phase) in a column packed with a material comprising porous polymers (stationary phase). Macromolecules with a high molecular weight are quickly eluted, but those with a low molecular weight have a prolonged residence time and travel a more convoluted course through the stationary phase.

The particle size distributions of the species present in the different samples studied (Shimadzu SPD-20AV) were carried out using a high-pressure liquid chromatography system (HPLC Shimadzu Corporation, Kyoto, Japan) equipped with an isocratic pump (Shimadzu LC-20AT) and a UV-Vis) with a Yarra 3 µ-SEC-S3000 phenomenex size exclusion column (4.6 mm id x 30 cm long, Phenomenex industry, France). The column was equilibrated at 25 °C with a mobile phase consisting of 50 mM phosphate buffer (Na_2HPO4) and 0.2 M NaCl, respectively, for samples prepared at low and high ionic strength, pH 7.2, pre-degassed and filtered through a 0.2 µm filter The column is pre-calibrated using SIGMA ALDRICH and GE Healthcare protein standards. The markers used were glucan blue (MW: 2000 kDa), thyroglobulin (MW:

669 kDa), Ig A (MW: 300 kDa), Ig G (MW: 150 kDa), ovalbumin (MW: 44 kDa), Myoglobulin (MW: 17 kDa), Cytochrome (MW: 12 kDa).

The protein solutions (20 g/L) were prepared in sodium phosphate 50 mM + NaCl 0.2 M buffer solution and adjusted to pH 7.5 and 9, subsequently filtered through a 0.2 µm membrane (Millipore Corp.) according to the procedure cited by Roesch & Corredig, (2005). Samples (25 µL) were manually injected into the system using a 100 µL injection volume sample loop. The speed and total elution time were set at 0.8 ml/min and 20 min respectively. The analyzes were recorded at a wavelength of 214 nm and 280 nm and the elution peaks were analyzed with the LC solution software (V. 1.25, Labsolutions, Shimadzu). All samples were measured in triplicate.

2.2.9.2 RP-HPLC for ovalbumin

The determination of extracted ovalbumin was carried out using a Reverse phase high-pressure liquid chromatography system (HPLC Shimadzu Corporation, Kyoto, Japan) equipped with an isocratic pump (Shimadzu LC-20AT) and a UV-Vis) with a Vydac® 214TP C4 HPLC Columns (50 x 4.6 mm, Avantor®, France). The column was stored in a 70% acetonitrile solution without trifluoroacetic acid (TFA). Two buffer solutions were prepared: buffer A) Mili Q water with 0.02655 TFA and filtered through 0.2 membranes (Millipore Corp.); buffer B) 100% acetonitrile and 0.025% TFA. For samples prepared at low and high ionic strength, pH 7.2, pre-degassed and filtered through a 0.2 µm filter. The column is pre-calibrated using SIGMA ALDRICH and GE Healthcare protein standards. The markers used were lysozyme (MW: 14 kDa), ovalbumin (MW: 44 kDa), and Ovotransferrin (MW: 77 kDa).

The protein solutions (100 g/L) were prepared and made a 1/200 dilution with buffer A, subsequently filtered through a 0.2 µm membrane (Millipore Corp.) According to the procedure cited by Roesch & Corredig, (2005). Samples (100 µL) were manually injected into the system using a 100 µL injection volume sample loop. The procedure of injection and elution of samples was shown in table 2-1. 100 µL of

buffer A was used as a baseline. The speed and total elution time were set at 0.8 mL/min and 32 min respectively. The analyzes were recorded at a wavelength of 280 nm and the elution peaks were analyzed with the LC solution software (V. 1.25, Labsolutions, Shimadzu). Gradients 2 and 3 are used to rinse the column because highly hydrophobic ovalbumin tends to adsorb onto the column. All samples were measured in triplicate.

Table 2-1 Procedures of injection and elution of samples.

Time	Buffer A	Buffer B	Rate
0 min.	95 %	5 %	0,8 ml/min.
17 min.	30 %	70 %	0,8 ml/min.
19 min.	30 %	70 %	0,8 ml/min.
20 min.	95 %	5 %	0,8 ml/min.
22 min.	95 %	5 %	0,8 ml/min.
24 min.	30 %	70 %	0,8 ml/min.
25 min.	95 %	5 %	0,8 ml/min.
27 min.	30 %	70 %	0,8 ml/min.
28 min.	95 %	5 %	0,8 ml/min.
32 min.	95 %	5 %	0,8 ml/min.

0-20 min, using gradient 1, 22-25 min using gradient 2, and 25-32 min using gradient 3.

2.2.10 Gel properties determined by small-strain dynamic rheology

The objective of this manipulation is to identify the gelling properties (gel point, loss factor, linear viscoelastic region) of PPI, EW, and PPI-EW at different weight ratios (25/75, 50/50, 75/25) by small-strain dynamic rheology.

Samples were prepared as described in section 2.2.2. Subsequently, the sample was loaded into a rheometer MCR 302e (Anton Paar, Graz, Austria) equipped with a cone-plate geometry (50 mm diameter, 2 angles). Approximately 1 mL of the protein suspension was transferred to the lower plate of the rheometer. The upper cone was lowered to give a gap width of 1.0 mm. A thin layer of light mineral oil was added to the well of the upper cone geometry and a solvent trap cover was used to prevent sample

drying during heating. In this way, a water-saturated atmosphere was maintained at the surface of the sample. The following heating protocol was used. The sample was first equilibrated at 25 °C for around 3 min, then heated and then cooled under 1 % of shear strain and 1 Hz of frequency over a temperature range of 25–95-25 °C at a rate of 2 °C/min (heating ramp) and 5 °C/min (cooling ramp) respectively. Rheological data were collected for every degree change during heating and cooling. Subsequently, a frequency sweep at 1 % strain and a strain sweep at 1 Hz were performed at 25 °C. In detail, frequency sweep and strain sweep of the gel were conducted over a range of 0.01-40 Hz and 0.01-100 %, respectively. Values of G' (storage modulus) and G'' (loss modulus) were recorded for temperature, strain and frequency sweeps as has been done previously (Arntfield et al., 1990; Cai & Arntfield, 1997). The loss factor or tangent delta (tan δ = G''/G') was also calculated, as well as the linear viscoelastic region (LVR). The gelling point temperatures of protein suspensions were determined as the intersection of two linear parts of curves on either side of the marked inflection point of the elastic modulus (G′). The thermal gelation profiles of EW, i.e., the storage modulus (G') as a function of temperature, obtained at pH 9 was chosen as a typical curve to illustrate how the data were analyzed (Figure 2-3). In this case, we observed two inflection points at ~58 °C and ~75 °C respectively where the G' values rose sharply and diverge significantly from the loss modulus values (G'>>G'', data not shown). The points on both sides of one inflection point were fitted by linear models and the intersection of the respective straight lines was considered as the gelling temperature or gelling point as represented in Figure 2-5. LVR was calculated from Figure 2-6 by using an example of PPI-EW mixtures at a weight ratio of 25/75. The intersection of the two lines on both sides of the inflection point is the maximum strain without causing permanent deformation or called the yield point. Samples were run at least in triplicate.

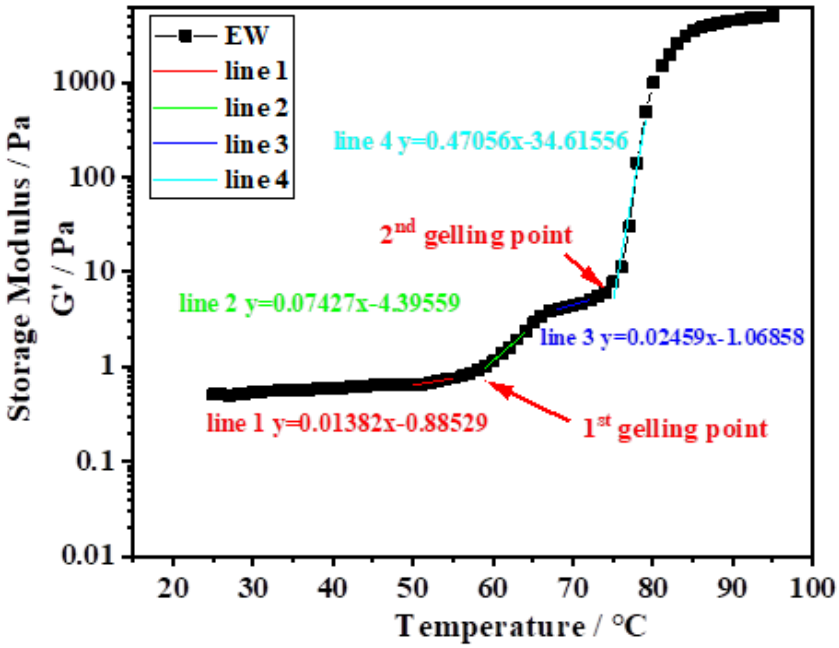


Figure 2-5: Temperature sweep for EW at pH 9 showing the determination of gelling point.

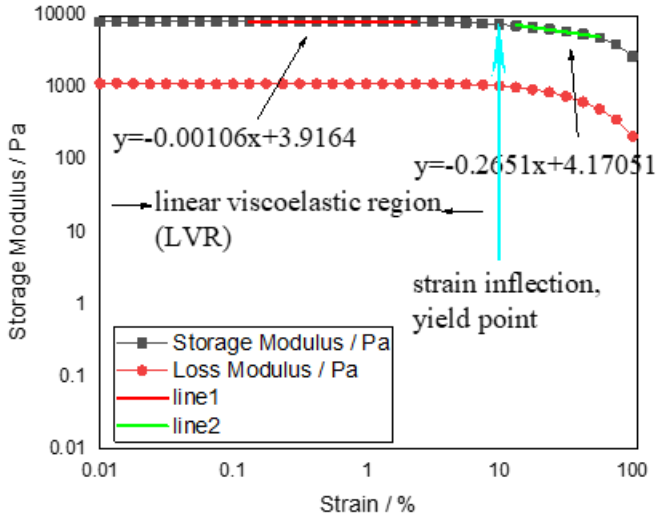


Figure 2-6: Strain intersection (yield point) of PPI-EW mixtures at weight ratio of 25/75 at pH 7.5.

2.2.11 Gel solubility by dissociation agents

The goal of this manipulation is to investigate the effect of different dissociating agents (urea, dithiothreitol, guanidine hydrochloride, and propylene glycol) on protein solubilization in order to identify the nature of the interaction forces that stabilize the three-dimensional networks formed by heat-treated protein mixtures after gelation. Control samples were tested in parallel.

The experimental approach was applied according to the previous methods of Liu & Hsieh (2008) and Chen et al., (2021) with some modification. Four different extracting reagents were used to analyze protein-protein interactions contributing to gelation. In brief, a 100 mM Tris buffer solution (Tris) (pH 7.5 and pH 9) was used as control (i). Tris containing 8 M Urea (ii) or 2 M Guanidine hydrochloride (GuHCl) (iii) or 20% Propylene glycol (PG) (iv) was used to extract proteins by affecting noncovalent interactions. Urea is more efficient in breaking hydrogen bonds, while its substituted form is mainly targeted for hydrophobic interactions. Tris containing 100 mM dithiothreitol (DTT) was used to extract proteins by reducing disulfide bonds (v). Tris containing 6 M urea, 100 mM DTT, 2 M Guanidine hydrochloride (GuHCl) and 20% Propylene glcol (PG) was used to extract proteins by dissociating all disulfide and non-covalent bonds as a second control (vi).

The different gel samples (~2.5 g) were added into individual extractant (~40 mL), stirred at 25 °C (1 h), homogenized at 10000 rpm for 1 min with a T 25 digital ULTRA-TURRAX® (IKA, USA), then centrifugated (16000 rpm, 30 min, 4 °C). The supernatants were collected, filtered (0.45 μm filter), weighed, and diluted around 6 times with the same extractant. The protein content in the dilutes was measured with a commercial Coomassie protein assay kit (660 nm) using BSA as the standard. The solubilized protein was then calculated by the ratio of the protein content in the supernatant to that of total proteins in gels and expressed as percentages, as shown in the following equations. At least three extractions were conducted and analyzed for each sample, and the average results were shown.

$$\textbf{total protein solubility}\ (\%) = \frac{\textbf{protein concent in gel supernatant solution}}{\textbf{protein content in gels}} \times$$

100...Equation (16)

$$\textbf{Net protein solubility in dissociating buffer}\ (\%) =$$

$$\textbf{total protein solubility} - \textbf{protein solubility in Tris}$$ **.....Equation (17)**

2.2.12 Texture analysis of gels

Texture profile analysis (TPA) was developed to link mechanical testing to sensory evaluations of food texture. A sample of standard size and form is put on a base plate and compressed and decompressed twice by an upper plate connected to the drive system in such a test. The force vs. deformation curve is recorded, and seven textural characteristics may be derived from it: hardness, elasticity, adhesiveness, cohesiveness, brittleness, chewiness, and gumminess (Bourne, 2002). Figure 2-7 gave a typical TPA curve. Hardness is determined by the maximum force exerted during the first compression cycle. The springiness of a compressed sample is the amount to which it recovers to its original size when the force of the initial compression cycle is eliminated. It is determined by dividing the original sample height by the distance compressed during the second compression to the peak force. The ratio of the effort required to compress the sample on the second bite (positive force area A2) to the work necessary to compress the sample on the first compression is defined as cohesiveness (positive force area A1).

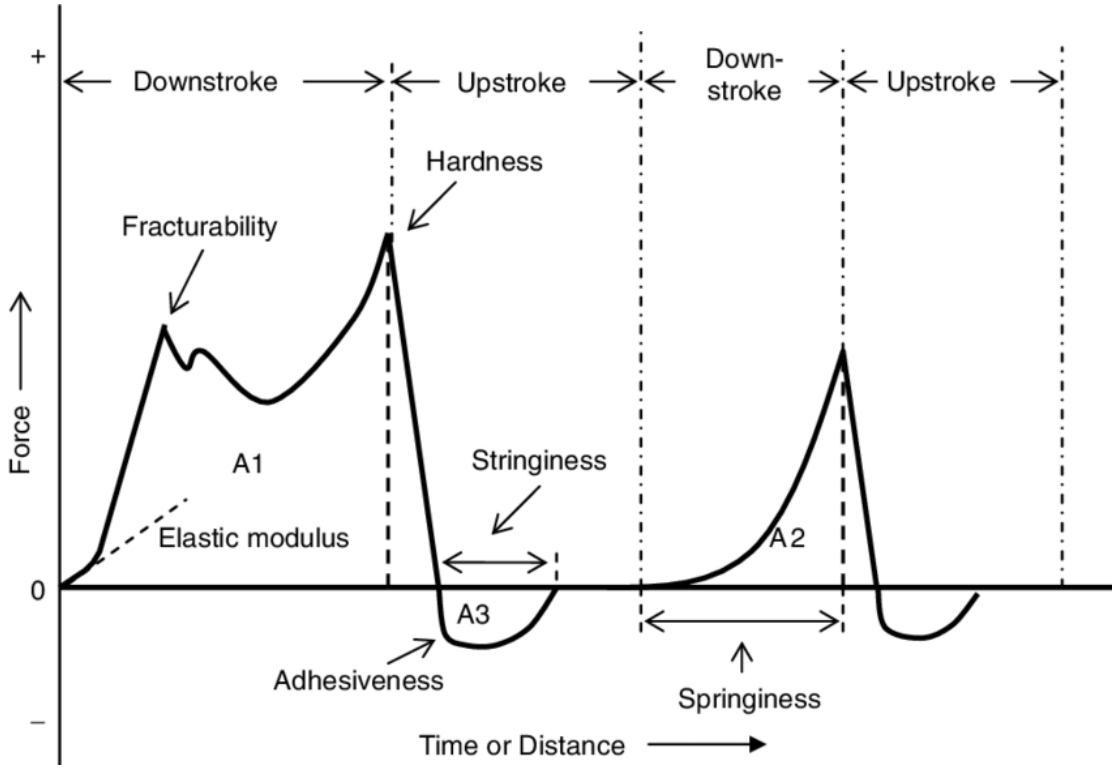


Figure 2-7: Typical profile for a texture profile analysis for a gel (Adapted from Wang & Cui, 2005).

All samples were prepared as noted above (section 2.2.2) in plastic tubes (Krchalm, Dercula, Netherlands). 40g of sample suspensions were heated from 25 °C to 95 °C (to induce denaturation of proteins and allow interactions between PPI and EW) in a water bath and kept at this temperature for 30 min, and then cooled down with ice to room temperature, and kept at 4 °C overnight. Textural analyzer (TA1 with Hand Held Remote, LLOYD INSTRUMENTS, AMETEK company, UK) was used to analyze hardness, springiness, and cohesiveness of PPI-EW formed gels by measuring its textural property according to the method of Bourne (Bourne, 1978). The parameters were set as follows: pre-test speed: 0.5 mm/s; test speed: 0.5 mm/s; post-test speed: 0.5 mm/s; compression deformation: 37.5 %. A time of 10 s was allowed to elapse between the two compression cycles. All the samples (height of 20 mm and diameter of 40 mm) were placed on the platform of the TA-XT Plus, fitted with a 5 N load cell and a cylindrical plunger 12 mm in diameter (SMS-P/35). All samples were prepared in

duplicate and tested at least 2 times. Hardness was defined as the maximum peak force during the first compression cycle. Springiness was defined as the recovery degree of gels after decompression to their initial shape. Cohesiveness was measured as the ratio of the work of penetration of the second penetration on one of the first penetration as well. Data were calculated using the Texture Expert software version 1.22 (Stable Micro Systems).

2.2.13 Confocal laser scanning microscopy (CLSM)

A confocal microscope's principal purposes are to generate a point source of light and reject out-of-focus light, allowing it to scan deep into tissues with high resolution, and optical sectioning enabling 3D reconstructions of imaging materials. The illumination and detection optics are focused on the same diffraction-limited spot, which is moved across the sample to form the entire picture on the detector, according to the basic idea of confocal microscopy. While the whole field of vision is illuminated during confocal imaging, anything outside the focus plane adds nothing to the picture, reducing haze and enabling optical sectioning. Figure 2-8 depicts a schematic of the core optics of a contemporary confocal microscope.

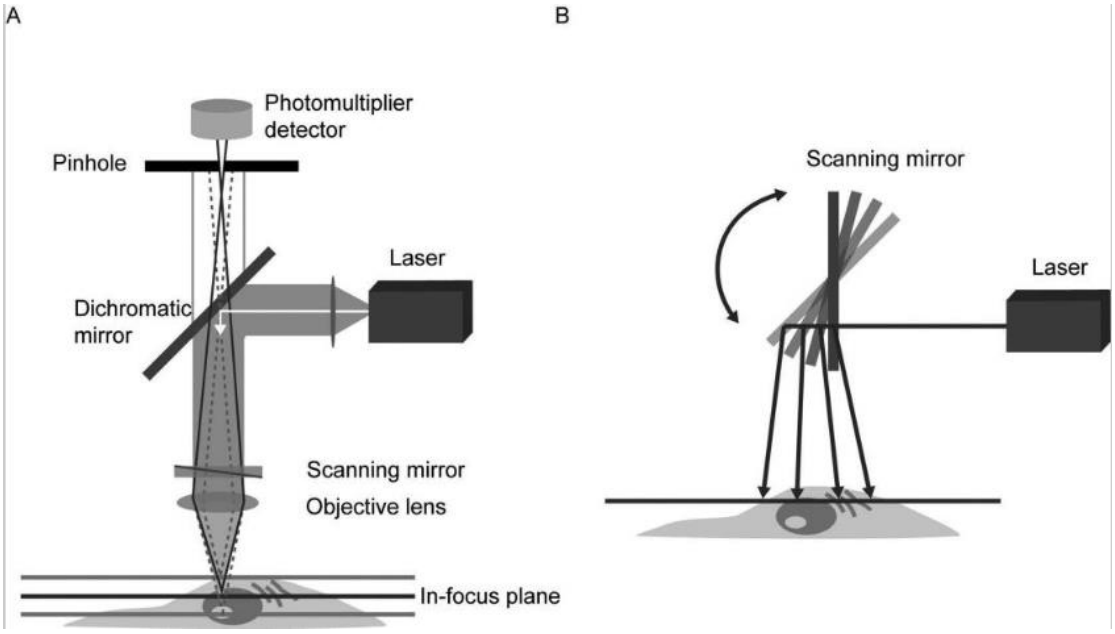


Figure 2-8: Principle of confocal microscopy (Adapt from Elliott, 2020). A: component of confocal microscopy; B: the scanning mirrors used by confocal microscopes to sweep the excitation light across the sample are seen schematically.

2.2.13.1 Protein particle formation

To obtain the structure information prepared by PPI-LYS mixtures in TRIS buffer at pH 7.5 at 20 °C, confocal laser scanning microscopy (CLSM) is used a ZEISS LSM 880 inverted confocal microscopy (Carl Zeiss AG, Oberkochen, Germany) using the Airyscan detection unit as previously developed by Halabi et al. (2022) and Somaratne et al. (2020a). PPI and lysozyme were prepared as mentioned in the previous section 2.2.2.2. PPI-LYS mixtures are prepared as mentioned in section 4. Then, PPI, LYS, and PPI-LYS mixtures at a different molar ratio at pH 7.5 at 20 °C were gently mixed with Fast Green aqueous solution (1 % w/v, 6 µL). Subsequently, the solutions were kept in dark at 20 °C for at least 10 mins. 20 µL of the mixture was deposited on a glass slide in a spacer and a coverslip was placed on top of all samples. Fast green was excited using a He–Ne laser system at a wavelength of 633 nm at a 1.72 µs pixel dwell scanning rate and detected using a PMT between 635 and 735 nm.

Images were observed inside the channel slide system using the high-resolution

mode of the confocal microscope equipped with the Airyscan detection unit and a Plan Apochromat 63x with a high numerical aperture (NA = 1.40) oil objective. Airyscan images were acquired with a main beam splitter MBS488/561/633, no additional emission filter, again setting of 700–780, a pixel dwells time of 1.54 µs and no averaging. The zoom was automatically set to 1.8 as requested by the system. Images were processed using confocal acquisition software Zen Black 2.1 (Version 13.0.0.0) to process the acquired datasets using the 2D mode at the default setting of the Airyscan processing function.

2.2.13.2 Gel structure preparation

The device used in the study of the microstructure of the gels formed is a confocal laser scanning microscopy (CLSM) using a ZEISS LSM 880 (Carl Zeiss AG, Oberkochen, Germany) with the Airyscan detection unit. Samples were prepared as in the previous section 2.2.2. Then, around 400 µL of the pH 7.5 or 9 mixture solution was poured into 1mL Eppendorf tubes. They were mixed with a 12 µL aliquot of 1% (w/v) of Fast Green. Then all the solution was slowly injected into the chamber of an IBIDI µ-Slide 8 well Uncoated system (IBIDI GmBH, Grafelfing, Germany). The system was then covered using an included lid and wrapped tightly with Parafilm (Dispense Parafilm Through This Opening, USA) at the gap of the lid, meanwhile, aluminum foils were used to prevent photo-bleaching of fluorescent molecules. Finally, the systems were put into the IBIDI systems and were heated as the previous gel preparation 2.2.2.3.2. To optimum resolution improvement, a Plan Apochromat 63x with a high numerical aperture (NA = 1.40) oil objective was used. A He/Ne laser with a wavelength of 633 nm was used to excite the Fast Green dye, with appropriate emission in each system. Zen Black 2.1 (version 13.0.0.0) software was used to process the acquired datasets using the 2D mode at the default settings of the Airyscan processing function.

2.2.14 Optical microscopy

The objective of the test is to understand what happened at a small molar ratio of PPI-LYS mixtures.

An optical microscope (Olympus BX51TF, Olympus Andre ORVAIN) equipped with an Olympus DP11 camera was used to obtain the images of the PPI-LYS systems. Samples were prepared as described before in section 2.2.2. Structures were immediately observed at a magnification of 10× at room temperature.

2.2.15 Statistical analysis

Values were expressed as means ± standard deviations of triplicate determinations. Statistical analyzes of the experimental data were carried out by analysis of variance (ANOVA). Significant differences ($p < 0.05$) between samples were determined by Tukey's test using STATISTICA 12 (64 BIT) software. Regarding the linear regressions and the associated statistical parameters, they were obtained with the Excel® software (2020.5.1.1040).

Chapter 3 Preparation and characterization of protein materials

The primary objective of this section will be to describe the extraction of adequate amounts of the most "native" fractions of pea protein isolate (mainly globulins which are simplified as PPI) and ovalbumin. Characterizing and preparing samples demands a substantial number of raw materials (PPI), and most pea protein isolates on the market are denatured globulins that cannot be utilized as substrates for this study, thus enough protein must be obtained at the beginning of the experiment.

This section is followed by an evaluation of the physical chemical properties of pea protein isolate, liquid egg white, and ovalbumin, including the SDS-PAGE, thermal properties, the solubility of pea protein isolate and egg white. In addition, the result of SEC-HPLC regarding PPI and ovalbumin was added to complete the properties of extract samples.

3.1 Preparation and characterization of pea protein isolate, egg white and ovalbumin

3.1.1 Extraction method

We collected enough experimental samples using the procedure described in section 2 and pea powder supplied by the industry. To produce "natural" pea protein powder, the approach employs combined processes: isoelectric-precipitation extraction and ultrafiltration as well as diafiltration. The extracted materials are fully combined before undergoing characterization investigations.

3.1.2 Physicochemical composition

3.1.2.1 Physicochemical composition of pea protein isolate and pea flour

The composition of the pea flour and purified pea protein isolate (pea globulin) fraction are given in Table 3-1. The concentration of protein, ash, moisture, lipid,

carbohydrate, and endosperm color are the quality parameters of a yellow pea. In particular, protein content is the most critical factor in these parameters. Thus, around 24.3 % protein (based on a dry basis) was present in yellow pea flour, which was consistent with the previous value reported to be in the range of 23.1 %-30.9 % (Boye, Zare & Pletch, 2010).

Table 3-1 Physicochemical composition of flour and the globulin fraction

Samples	Protein content / %*	Moisture / %	Lipid / %*	Ash / %*	Carbohydrate / %**
Pea flour	24.3±0.12	13.32±0.15	4.55±0.27	2.85±0.15	54.98
Pea globulin	89.74±0.02	3.61±0.01	0.95±0.04	1.89±0.07	3.81

* %: calculated on a dry matter basis,

**: Calculated by the difference of 100 %.

Regarding the pea globulin extraction, the protein and lipid concentration (based on a dry basis) was approximately 89.74 % (N factor of 5.44) and 0.95 %, respectively. According to Shand et al. (2007), it is critical to note that the presence of a small amount of fat in the globulin isolate reduces lipid-protein interaction, otherwise, large amounts of lipid in fractions would change the solubility in water and increase the turbidity of protein suspensions, as seen in soy protein solution (Li et al., 2007). Similar protein concentrations (higher than 80 %) have been reported by others for laboratory-produced pea protein isolates (Barać et al., 2010; Boye, Aksay et al., 2010; Stone et al., 2015b). Some differences in the protein content of extracted isolates may be due to the difference in protein content of cultivars, which was confirmed by Boye, Aksay et al. (2010), who highlighted that the protein concentration of six pea types studied ranged from 22.3 to 31.8 % and that in isolates ranged from 84-89 %.

3.1.2.3 Physicochemical composition of egg white and ovalbumin

The protein content of egg white was 10.4±0.25 % calculated by the Kjeldahl method (N x 6.25) mentioned in section 2.2.3.3, which agreed with the literature that mentioned that egg white consists of 11% of protein (section 1.2).

The protein concentration of ovalbumin was analyzed by the Bradford method (Bradford, 1976) using BSA as a standard. So, the protein content of ovalbumin extract was around 96.6 %.

3.1.3 Polypeptide composition of PPI and EW

The protein isolate was separated into distinct fractions using an SDS-PAGE gel under denaturing (SDS) and reducing (DTT+SDS) conditions. The composition of the PPI and EW at pH 7.5 (left part) and 9 (right part) was shown in Figure 3-1. In general, the electrophoretic profile of the PPI and EW prepared at pH 7.5 and 9 showed great similarities (Figure 3-1).

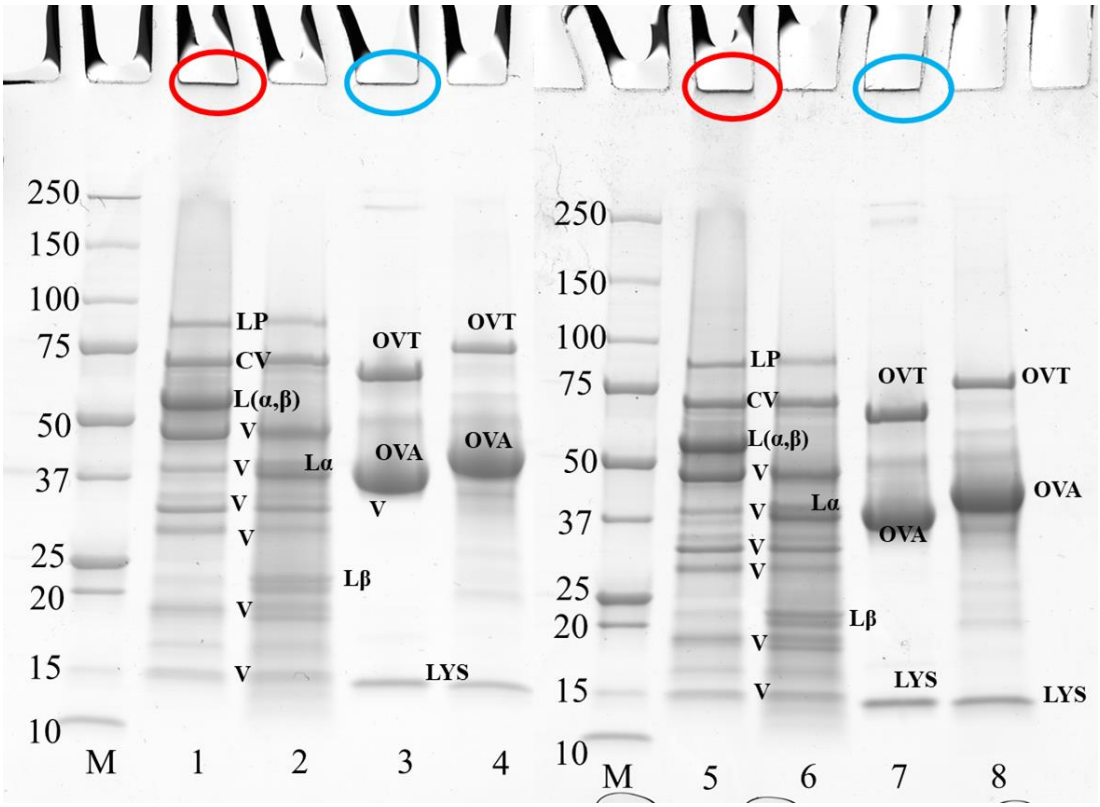


Figure 3-1 SDS-PAGE profile of extracted pea protein isolate and egg white when

prepared at pH 7.5 and 9. The samples on lanes 2, 4, 6, and 8 were treated under reducing conditions with SDS+DTT reagents. Circles in red and blue were the aggregates of PPI and egg white, respectively at the top of the bands. Lane 1-4 at pH 7.5, Lane 5-8 at pH 9; Lane M: molecular weight (Mw) markers; lanes 1-2, and 5-6: PPI; lanes 3-4, and 7-8: EW; LP, lipoxygenase; L (α, β), legume; CV, convicilin; Lα, legume acid polypeptide; Lβ, legume basic polypeptide; V, vicilin; OVA, ovalbumin; OVT, ovotransferrin; LYS, lysozyme.

Regarding PPI, a high number of component polypeptides of pea globulins is noticeable at first look. Focusing on Lanes 1 and 5 non-reducing part at pH 7.5 and 9, Lanes 2 and 6, the reducing part at pH 7.5 and 9, respectively, a molecular weight of around 88 kDa, was probably lipoxygenase (Sun & Arntfield, 2010; Mession, Sok, Assifaoui & Saurel, 2013). A strong band at around 60 kDa corresponded to legumin 11S main subunits (Lαβ), which were dissociated into acidic subunits Lα (~38-40 kDa) and basic subunits Lβ (~20-22 kDa) under reducing conditions as also observed elsewhere (Gueguen, & Barbot, 1988; Shand, et al. 2007; Liang & Tang 2013; Mession, Chihi, Sok, & Saure, 2015). The band around 15 kDa could be vicilin fragments γ (12-16 kDa) (Gatehouse et al., 1981; Mession et al., 2013). Polypeptides of vicilin were considered to be the majority pea globulin polypeptides with different bands around 16-50 kDa (Figure 3-1), in detail, i: polypeptides in the range of 20-37 could respond to fragments α (≈20 kDa), α:β (~30-36 kDa), and β:γ (~25-30 kDa), ii: the presence of a minority fraction of vicilin of 19 kDa (Gatehouse, Lycett, Croy, & Boulter, 1982), iii: large band around 50 kDa could be assigned to the vicilin monomer (Mession et al., 2013). The convicilin fraction, which was considered to be the third storage polypeptide, was present at 71 kDa as expected (Croy, Gatehouse, Tyler, & Boulter, 1980; Liang & Tang 2013). Aggregates with molecular weights of more than 250 kDa (Figure 3-1, lanes 1 and 5) were found (indicated by a red solid circle), which were probably produced during the extraction process regarding the study of Karaca, Low &

Nickerson (2011).

Three main polypeptide components were shown in egg white samples (Figure 3-1, lanes 3 and 7 at pH 7.5 and 9, respectively): ovotransferrin (76 kDa), ovalbumin (44 kDa), and lysozyme (14.6 kDa), in agreement with previous works (Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995, Raikos, Hansen, Campbell, & Euston, 2006). No band corresponding to ovomucoid (~28 kDa, 11 %) could be visualized on the gel. As expected, ovalbumin was the largest band on the gel, because it is the most abundant protein in egg white (Li-Chan et al., 1995). A fraction of protein aggregates that did not enter the electrophoresis gel (indicated by blue solid circles in Figures 3 lanes 3 and 7) was presumably formed via covalent interactions such as disulfide bonds, as they disappeared under reducing conditions (Figure 3-1, lanes 4 and 8), as already mentioned by Alavi et al. (2019). It can be noticed that the bands of ovotransferrin and ovalbumin appeared with a higher molecular weight under reducing conditions (Figure 3-1, lanes 3 vs 4 at pH 7.5, lanes 7 vs 8 at pH 9), probably due to the rupture of their internal disulfide bonds that expand their structure and thus increase their apparent molecular weight (Katekhong & Charoenrein, 2016; Chaiyasit, Brannan, Chareonsuk, & Chanasattru, 2019).

3.1.4 Solubility of PPI and EW as a function of pH

In the food sector, good protein solubility is a highly desired physicochemical feature. It provides the benefit of homogenous protein distribution in the food matrix, which affects the texture and organoleptic qualities of the complete product. Protein-protein and protein-solvent interactions influence protein solubility, resulting in either precipitation or protein solubility (Damodaran, 1996). The environment, such as pH and ionic strength (Deng et al., 2011), the extraction method (Boye, Aksay et al., 2010; Papalamprou et al., 2009), the drying process (Gueguen, 1983), the amino acid composition (hydrophilic and hydrophobic residues), and their distribution on the protein's surface (Kimura et al., 2008) all influence both types of interaction. The

solubility of PPI and EW (0.1 M NaCl) was examined as a function of pH in this study.

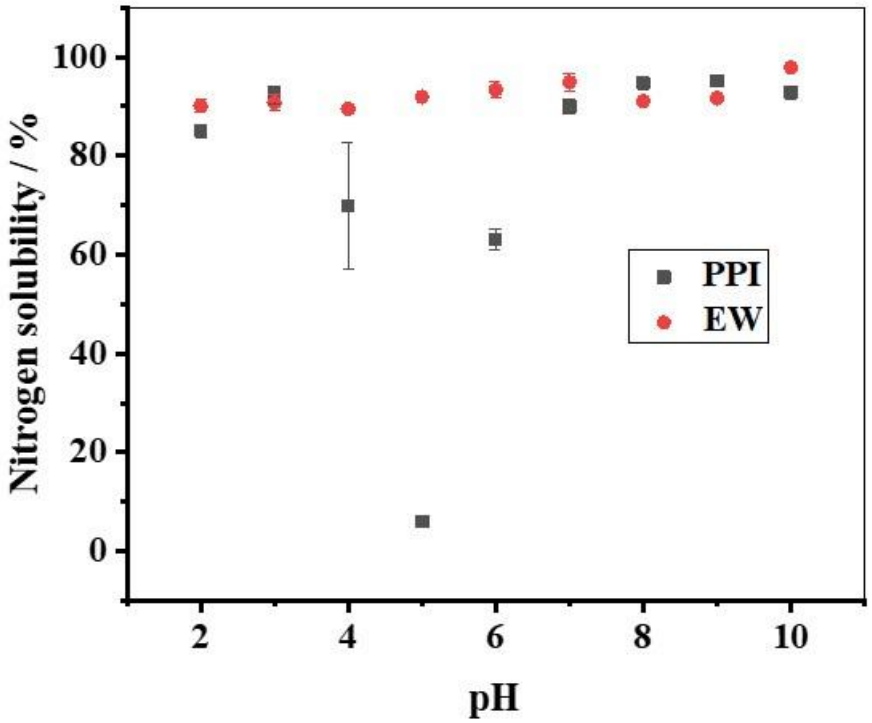


Figure 3-2 Nitrogen solubility of PPI and EW (0.1 M NaCl) as a function of pH in distilled water.

The change in the solubility of PPI and EW as a function of pH is shown in Figure 3-2. The globulin's solubility reaches a minimum of around pH5, corresponding to the region of the isoelectric point (pI~4.8) (Gueguen et al., 1988). Previous paper of Shevkani, Singh, Kaur, & Rana. (2015) reported the solubility of tested pea protein isolate was only 2–4 % at pH 5. Outside this range, the solubility increased and reached values of around 85 % and 89 % for pH values below 3 and above 7, respectively. On the other hand, the solubility of PPI showed a U-shaped solubility profile. Similar pH-dependent protein solubility profiles of pea proteins have been observed for commercial or native PPI (Adebiyi & Aluko, 2011; Burger & Zhang, 2019; Shand et al., 2007; Taherian et al., 2011; Liang & Tang, 2013) or pea protein concentrate (Boye et al., 2010). This solubility behaviour of globulins is attributed to electrostatic repulsion and

hydration of charged residues (Damodaran, 2008). At pI, the protein has as many positive as negative charges, which promotes the affinity and interaction between protein-protein at the expense of protein-solvent, thus minimizing solubility. For acid and basic pH, the protein is positively and negatively charged, respectively. This large net charge increases the repulsion forces, thereby promoting the protein-solvent interaction, which results in better solubility. Furthermore, in an acidic condition, the quaternary structure of the globulins is ruptured by the dissociation of the legumin subunits caused by the protonation of the carboxylic groups (Lakemond et al. 2000).

When focusing on the solubility of egg white, it was always over 88%. However, a little lower solubility of EW was observed around pH 4, which is close to the isoelectric point of ovalbumin (pH 4.5), the major egg white protein. This result was in good agreement with the previously reported solubility profiles of egg white and attested to the high hydrophilicity of egg white proteins (Machado et al., 2007). Abdo et al. (2021) reported that the solubility of egg white declined at a pH range of 4-6 due to isoelectric point of egg white protein.

3.1.5 Thermal properties of PPI and EW

3.1.5.1 Thermal properties of PPI

Thermal characteristics of the acquired pea protein isolate, and egg white were investigated. The DSC measurements allowed us i: to confirm that the extraction and purification steps had limited effect on the protein structure, ii: to identify the ideal temperature for denaturing the proteins alone, and therefore, iii: to pick the proper temperature for creating the mixed soluble aggregates. Thermograms, denaturation temperature (T_d), and change of enthalpy (ΔH) acquired by differential scanning calorimetry (DSC) at pH 7.5 and 9 were shown in Figure 3-3, Table 3-1 for pea protein isolate, Figure 3-4, Table 3-2 for egg white, respectively.

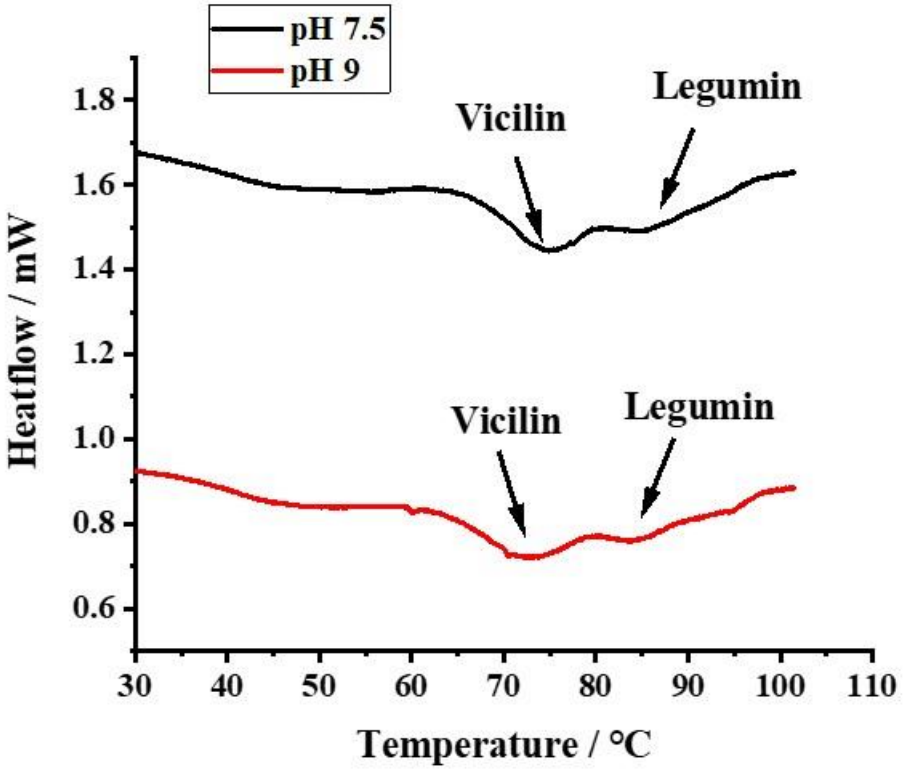


Figure 3-3 Thermogram of 10% pea protein isolate (m/m) heated at 0.5 °C/min at pH 7.5 and 9.

Table 3-1 Thermal denaturation temperature (Td) and enthalpy (ΔH) of PPI at pH 7.5 and 9.

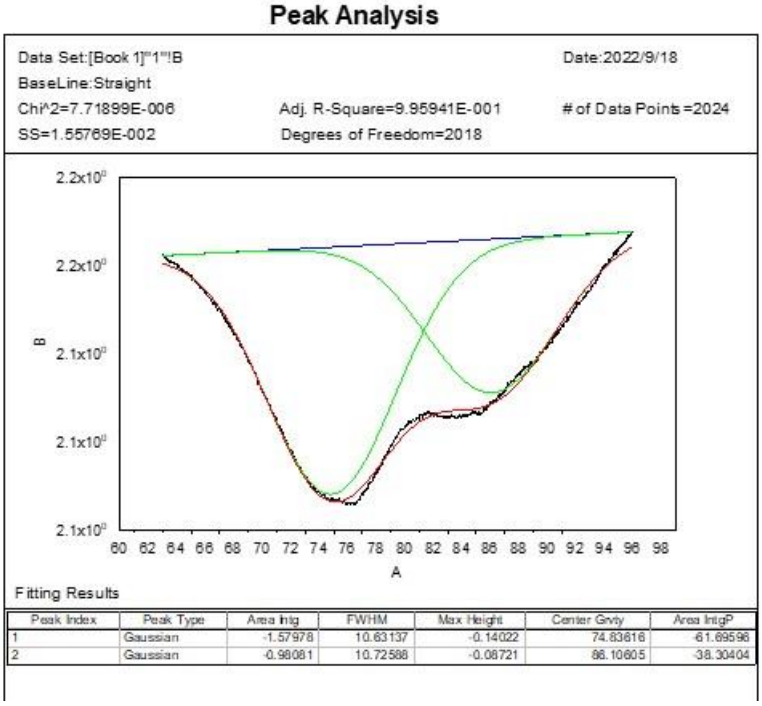
samples	T_d vicilin (°C)	T_d legumin (°C)	ΔH（J/g）
pH 7.5	75.8±0.4*	87.4±0.5*	10.8±0.1*
pH 9	71.3±0.4**	84.5±0.2**	3.6±0.2**

Means followed by different numbers of * for the same column are significantly different.

According to Emkani, Oiete, & Saurel (2021), PPI thermal curves showed the two characteristic denaturation endothermic peaks for 7S and 11S globulins. Regarding pea protein isolate (Figure 3-3, Table 3-1), the first peak at around 75.8 °C and 71 °C corresponded to the denaturation of the lower molecular weight fraction (7S vicilin) at

pH 7.5 and 9, respectively, and the second one at around 87 °C and 84 °C was related to the high molecular fraction (11S legumin) at pH 7.5 and 9, respectively. These results are consistent with data previously obtained by other authors on vicilin and legumin denaturation. For instance, O'Kane et al. (2004a) reported that the denatured temperature of purified vicilin from pea protein at pH 7.6 was around 69.9-71.8 °C. O'Kane et al. (2004b) illustrated that 11S legumin prepared at pH 7.6 had a denaturation temperature of around 87 °C at 0.5 °C/ min heating rate. In addition, the denaturation temperatures of legumin and vicilin were significantly decreased with increasing pH, which agreed with previous studies, where the Td values of salt-extracted pea protein isolate (with no salt) were maximum at pH 5-6 and declined at both acid and alkaline pH (Sun & Arntfield, 2011a). T_d and ΔH of faba bean proteins also declined on each side of the isoelectric point (Arntfield & Murray, 1981); enthalpy and Td of red bean globulin decreased under the impact of excessively acidic and alkaline pHs (Meng & Ma, 2001). This kind of decreased phenomenon on T_d suggested that a decrease in thermal stability was caused by growing repulsive negative charges at pH which was far away from pI (Arntfield & Murray, 1981; Meng & Ma, 2001). Table 3-1 shows the enthalpy (ΔH) values for suspensions PPI at pH 7.5 and 9, respectively The denaturation enthalpy (ΔH) of pea globulins was in the same order of magnitude as those obtained by Sun & Arntfield (2011a), i.e., around 8.3 J/g of salt-extracted pea protein isolate at an increasing temperature rate of 0.5 ℃/ min, or by Mession, Assifaoui, Cayot & Saurel (2012), i.e., 11.4 J/g of acidic precipitated pea protein isolate at 10 ℃/ min at pH 7.5, but these values were higher than those obtained by Shand et al. (2007) (from 0.725 J/g to 0.992 J/g of native pea protein isolate under NaCl concentration from 0% to 2%). The ΔH value of the PPI sample at pH 7.5 in our study is an indication that the globulin fractions produced were low-denatured. However, the enthalpy at pH 9 decreased compared to pH 7.5. This could be explained by the high pH, far from pI, causing the partial unfolding of protein molecules due to the increasing intramolecular net charges and repulsive forces, as demonstrated by Meng & Ma (2001) on red bean globulins.

Pea globulin denaturation was found to be irreversible, as evidenced by the absence of transition following a re-heating of the same sample (results not shown).



Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1	Gaussian	-1.57978	10.63137	-0.14022	74.83616	-61.69596
2	Gaussian	-0.98081	10.72588	-0.08721	86.10605	-38.30404

Figure 3-4 Samples of Deconvolution of pea protein isolate 10%.

It is necessary to know the vicilin/ legumin ratio (V/L) in pea as pea proteins can be used in a variety of dietary applications due to the diversity of the V/L ratio (Gallardo, Thompson, & Burstin, 2008; Mertens et al., 2012). Indeed, this ratio influences both the nutritional value of pea proteins (such as accessible lysine concentration) and their functionality (such as solubility, interfacial, textural, sensory, foaming, and emulsifying capabilities) (Dagorn-Scaviner, Gueguen, & Lefebvre, 1986; Mujoo, Trinh, & Ng, 2003; Rangel et al., 2003; Martínez-Villaluenga et al., 2008). Vicilin exhibits superior interfacial characteristics than legumin, according to Dagorn-Scaviner et al. (1986). It also makes gels stiffer and improves the stability of foams and emulsions (Mujoo et al., 2003). According to the previous papers of Mertens et al. (2012), Lam et al. (2018), different analysis methods can change the variability of the V/L ratio, such as ultracentrifugation, rocket immunoelectrophoretic, sodium dodecyl sulfate-polyacrylamide gel electrophoresis/densitometry, reverse-phase high-performance

liquid chromatography (RP-HPLC), differential scanning calorimetry, and ion exchange chromatography. In this paper, the respective areas of the 2 peaks were calculated from the differential scanning calorimetry curve through deconvolution. Figure 3-4 shows one of the results of the deconvolution calculation, and the area assigned to 7S (~58,8 %) was much higher compared to the area assigned to 11S (~41,2 %) confirming that 7S was the major fraction of globulins in our PPI sample. The rest of the deconvolution result is shown in Figure Annex 1.

3.1.5.2 Thermal properties of EW

Figure 3-5 shows typical DSC thermograms for EW with 4 main peaks. In agreement with literature data (Ferreira, Hofer & Raemy, 1997; Barhut & Findlay, 1990), the peaks at ~63, ~69, ~76 and ~83 °C could be assigned to ovotransferrin, lysozyme, ovalbumin, and S-ovalbumin (the more heat-stable form of ovalbumin (Smith & Back, 1965), respectively. However, at pH 7.5, for the egg white sample, the first peak showed a shoulder probably corresponding to lysozyme, indicating the peak of lysozyme was overlayed by ovotransferrin one (Figure 3-5), maybe due to co-aggregation and heteroprotein formation between ovotransferrin and lysozyme as suggested by Wei et al. (2019) and Iwashita et al. (2019). Many studies performed on liquid egg white around neutral pH indeed mentioned two main denaturation peaks around 65 and 80 °C attributed to ovotransferrin and ovalbumin, respectively (Ibanoglu & Erçelebi, 2007, Tóth et al, 2017; Renzetti, van den Hoek, & van der Sman, 2020).

Table 3-2 shows the enthalpy (ΔH) values for suspensions EW at pH 7.5 and 9, respectively. The ΔH value of pure protein suspensions was influenced by pH. The ΔH value of the EW sample showed a slight but significant increase from pH 7.5 to 9. The denaturation enthalpy (ΔH) of the egg white agreed with previous data of 20.6 J/g at pH 7 obtained by Ferreira et al. (1997) and 21.0 J/g at pH 8.58 obtained by Rossi & Schiraldi (1992). Some differences in denaturation enthalpy compared to published values can be explained by the sensitivity of the apparatus (which is higher in our

experiments using Micro DSC) and by variable baseline fitting (Ferreira et al., 1997).

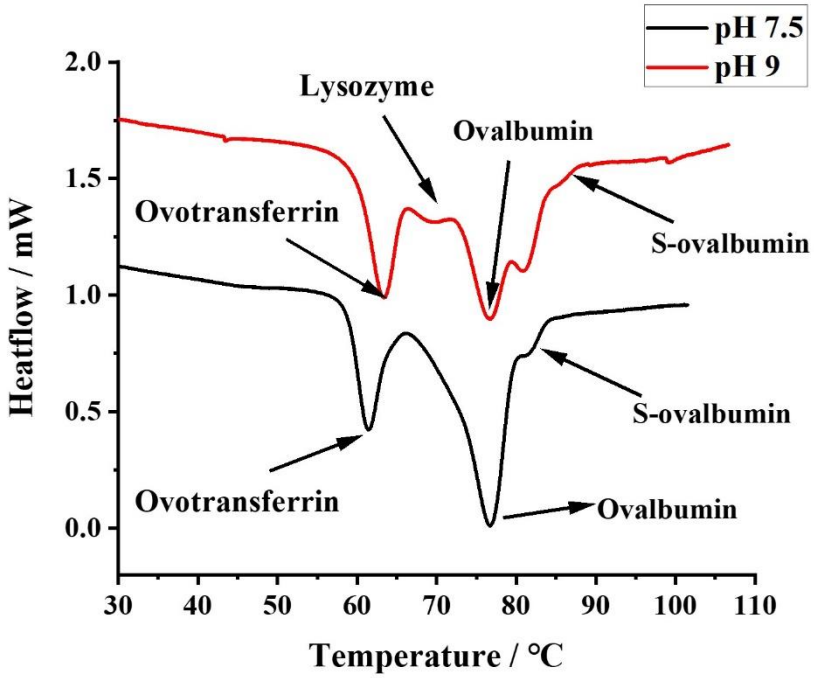


Figure 3-5 Thermogram of 10% egg white (m/m) heated at 0.5 °C/min at pH 7.5 and 9.

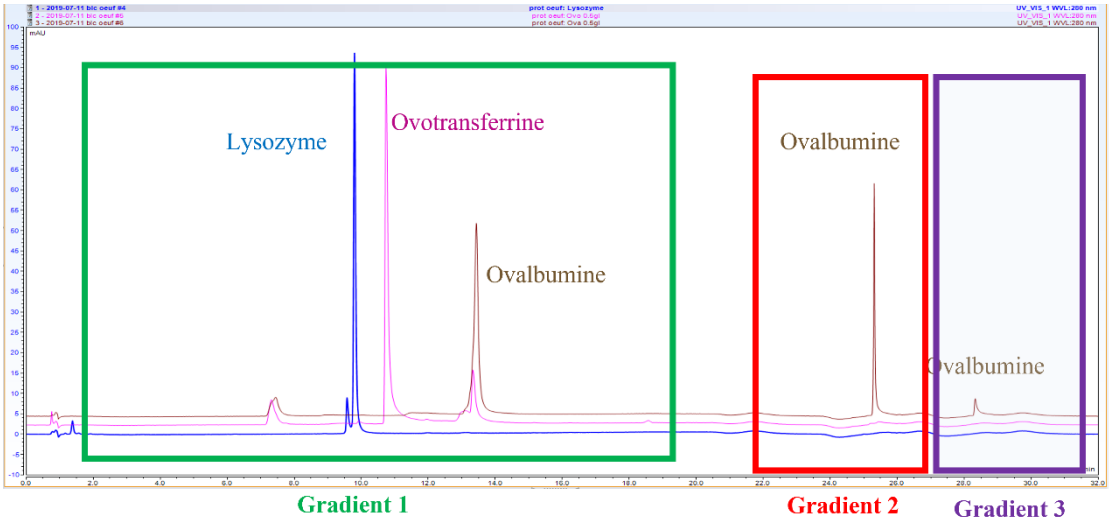
Table 3-2 Thermal denaturation temperature (Td) and enthalpy (ΔH) of EW at pH 7.5 and 9

samples	T_d1(°C)	T_d 2(°C)	T_d 3(°C)	T_d 4(°C)	ΔH（J/g）
pH 7.5	61.1±0.1*	-	76.7±0.1*	83.5±0.8*	22.3±0.5*
pH 9	63.2±0.1**	69.5±0.1**	76.4±0.1**	83.1±0.7*	23.8±0.2**

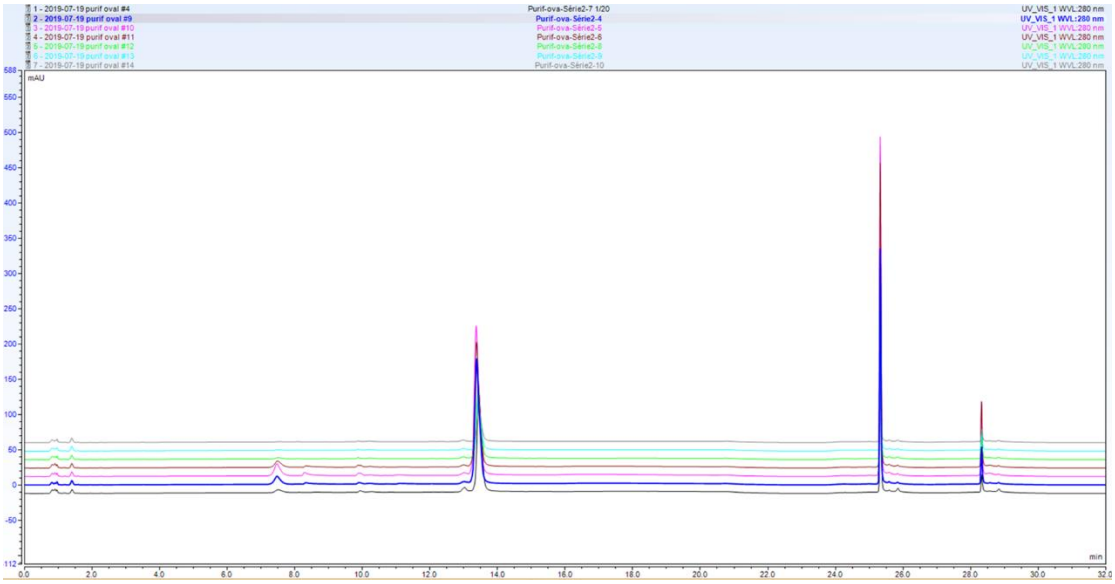
All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same numbers of * are not significantly different. T_d1: ovotransferrin; T_d2: lysozyme; T_d3: ovalbumin; T_d4: s-ovalbumin.

3.1.6 RP-HPLC of EW and SEC-HPLC of PPI

Several batches of purification of ovalbumin were performed, and then the purity was analyzed by RP HPLC as shown in Figure 3-6. Then, ovalbumin fractions with high purity were kept for the following experiments.



A



B

Figure 3-6 several batches of extracted ovalbumin fractions analyzed by RP-HPLC. (A): markers of different molecular weights: ovotansferrin (75 kDa), lysozyme (14 kDa), ovalbumin (44 kDa); (B): one sample of several batches of extracted ovalbumin.

Figure 3-6 B showed several batches of purification, the purity of extracted ovalbumin, that different batches of extracted ovalbumin of 2-4, 2-5, and 2-6 were not pure compared to the rest of extracted ovalbumin. The rest batches of extracted ovalbumin analyzed by RP-HPLC were in Figure Annex 2. After all the extracted

ovalbumin has been identified, the purest ones were collected and mixed to be used for further investigations.

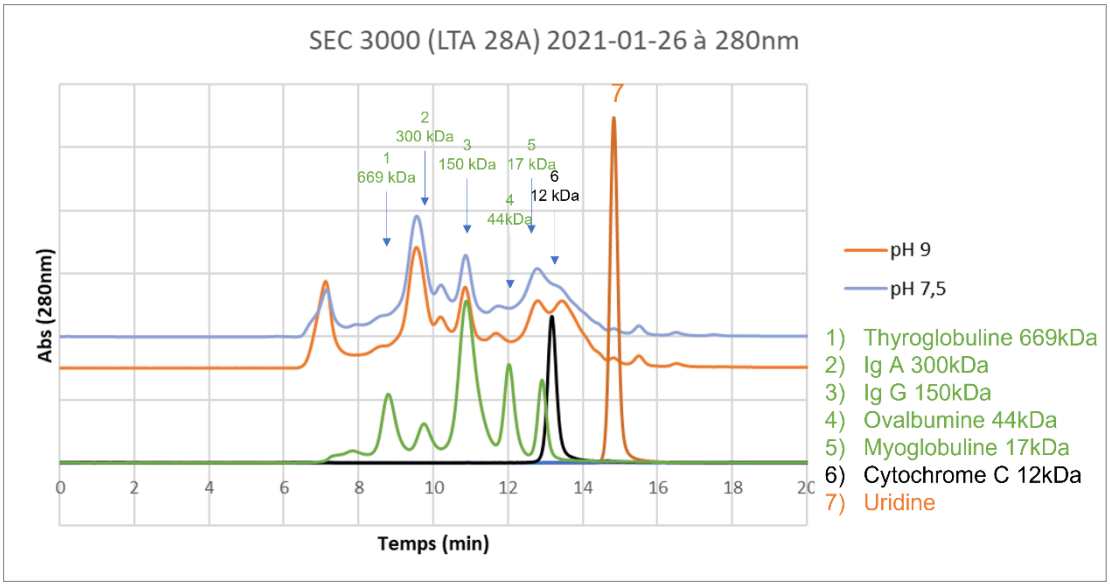


Figure 3-7 SEC-HPLC profile of pea protein isolate studied with UV detection at 280 nm as a function of time.

Figure 3-7 shows the SEC-HPLC profile of extracted pea protein isolate at pH 7.5 and 9, respectively. To our knowledge, there was no big difference between pea protein isolates prepared at pH 7.5 and 9. The globulin fraction represented by two peaks was obtained around 9-11 min, which agreed with the results of Mession et al. (2013). In detail, the two main peaks were attributed to legumin 11S and vicilin, with a molecular weight of around 360 and 150 kDa. To be noted that between the two main peaks, it could be the peak of 7S convicilin, which has a molecular weight of around 210-280 kDa (Barać et al., 2010; Boye, Zare & Pletch, 2010; Tzitzikas et al., 2006). There are also some minor peaks around 44, 17, or 12 kDa, which could be the vicilin fragments in small amounts. Mession et al. (2013) also found this kind of vicilin fragments at a molecular weight of around 54, 30, or over 30 kDa. The SEC-HPCL results also agreed with SDS-PAGE.

3.2 Conclusion

In our study, pea globulin fractions were obtained from extraction from delipidated pea flour. These fractions are rich in legumin (41.2 %) and vicilins and convicilins (58.8 %) subunits. Delipidation made it possible to have protein fractions richer in protein and purer.

The solubility of extracted pea protein isolate was over 89 % when pH is over 7, for egg white, the solubility was higher at an alkaline solution, which could help us to have a better soluble protein at selected pH of 7.5 and 9.

The thermograms obtained by MicroDSC confirmed that pea globulins were low-denatured proteins by the chemical and physico-chemical treatments during the extraction phases and therefore usable for the rest of our study. For egg white, it showed four thermal peaks regarding ovotransferrin, ovalbumin, lysozyme, and s-ovalbumin at pH 9, while at pH 7.5, the peak of lysozyme was overlapped, maybe due to co-aggregation and heteroprotein formation between ovotransferrin and lysozyme.

Chapter 4 Interactions between isolated pea globulins and purified egg white proteins in solution

4.1 Abstract

In the present work, the interactions and associations between low-denatured pea globulins (PPI) and purified main egg white proteins (ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (LYS)) were studied at pH 7.5 and 9 by using isothermal titration calorimetry (ITC), dynamic light scattering (DLS), laser granulometry and confocal laser scanning microscopy (CLSM). From ITC, we detected strong exothermic interactions between PPI and LYS at both pHs, which led to aggregation. At these pH values, the net positive charge of lysozyme favored electrostatic interactions with negative charges of pea proteins, and oligomers were formed during titration experiments. Furthermore, DLS, laser granulometry, and CLSM data showed that the particle size of the mixture increased with increasing LYS to PPI ratio. Large irregular aggregates up to 20-25 µm were formed at high molar ratios and no complex coacervate was observed. No or very weak interactions were detected between OVT or OVA and PPI whatever the pH. These results suggest a selective association of PPI with LYS when added to egg protein mixtures.

4.2 Introduction

With the increase in world population and food transition in emerging countries, the demand for protein is expected to increase by 40 % until 2030 (Ozanne, 2015). If the demand for animal proteins increases in emerging countries, developed countries have initiated their second food transition to diversify their protein supply to benefit plant proteins (Mottet, 2015). However, plant proteins may raise concerns for organoleptic acceptability, functional properties, and nutritional quality compared to animal proteins.

Therefore, interest in food-based design protein mixtures between animal and plant proteins has been increasing over the years (Ersch, ter Laak, van der Linden, Venema, & Martin, 2015; Ainis, Ersch & Ipsen, 2017; Hinderink, Sagis, Schroën, & Berton-Carabin, 2020; Guyomarc'h et al., 2021). One of the issues of mixing animal and plant proteins is to at least maintain, or better increase, their functional properties such as foaming, gelling, or emulsifying. Several studies obtained value-added functionalities of the resulting product (Kebary, 1993; Alu'datt, Alli & Nagadi, 2012; Kristensen et al., 2020; Jarpa-Parra et al., 2017).

Alves & Tavares (2019) recently compile the impact of technological treatment on protein structure and techno-functional properties of animal and plant proteins in mixed systems. They noticed that mixing animal and plant proteins enable to obtain gels and /or films to display various microstructure and by a consequence displaying different rheological behavior. All the studies on gelling properties mentioned used milk proteins as animal proteins and either soy or pea proteins as plant proteins.

Few studies concerned foaming and emulsifying properties of mixed systems and among them, very few dealt with egg proteins as animal proteins (Wang, Troendle, Reitmeier, & Wang, 2012; Jarpa-Parra et al, 2017; Wouters et al. 2018). Wouters et al (2018) pointed out that in combination with hydrolyzed gluten, egg white proteins formed a second layer by electrostatic and hydrophobic interactions after adsorption of hydrolyzed gluten at the interface, thus increasing the resistance of the foam bubbles against coalescence.

Some other studies focused on protein interactions at a molecular level in the mixed systems (Roesch & Corredig, 2005; Anuradha & Prakash, 2009; Chihi, Mession, Sok, & Saurel, 2016, Mession, Roustel, & Saurel, 2017a). Globular proteins denature when heated and form mixed aggregates depending on the initial protein mixes and the respective properties of the mixed proteins, such as the presence of free sulfhydryl groups and disulfide bonds (Guyomarc'h et al, 2021). The presence of milk proteins drives the heat-induced co-aggregation of all disulfide-containing proteins and yields

new aggregate forms, while soybean and pea proteins mostly aggregate through hydrophobic and non-covalent interactions.

Egg white is a proteinic solution used in many food applications for its gelling or foaming properties. It is a good candidate for mixing with plant proteins, especially because its basic pH (from 7.5 just after laying to 9.5 a few days later) may help their solubilization. However, the literature is very scarce on the interactions between egg white and plant proteins. The aim of this study is thus to highlight the interactions that may occur between egg white proteins as a model of animal proteins, and pea proteins, as a model of plant proteins.

Egg white usually contains about 11 % proteins which consist of more than 40 different kinds of proteins. Ovalbumin (OVA) is the major protein and represents about 54 % of the total egg white proteins, while ovotransferrin (OVT) and lysozyme (LYS) constitute about 1 2% and 3.4 %, respectively (Belitz, Grosch & Schieberle, 2009; Burley & Vadehra, 1989). OVA consists of a peptide chain containing 385 amino acid residues and its isoelectric point is estimated at 4.5. It has a molecular weight of 44.5 kDa and contains four thiols and one disulfide group. OVT, unlike OVA, is not denatured at the interface but coagulates at lower temperatures. It consists of one peptide chain of 686 amino acids and contains one oligosaccharide unit made of four mannose and eight N-acetylglucosamine residues. Its molecular weight is around 77.7 kDa. The isoelectric point is 6.1. OVT has 15 disulfide bonds and about 55 % reactive residues (Zabik, 1992). LYS is a relatively small secretory glycoprotein, consisting of 129 amino acids linked by four disulfide bonds, and is a 14.4 kDa protein with an isoelectric point of 10.7. LYS is relatively stable, its denaturation temperature being 74 °C (Shih & Kirsch, 1995, Shih, Holland & Kirsch, 1995, Ueda, Masumoto, Ishibashi, So, & Imoto, 2000).

Recently, there is a growing interest in the consumption of pulse proteins, such as yellow pea (*Pisum sativum* L.) protein isolate (PPI), as a preferable alternative to animal proteins due to its cheaper price, more sustainable source of proteins with a lower

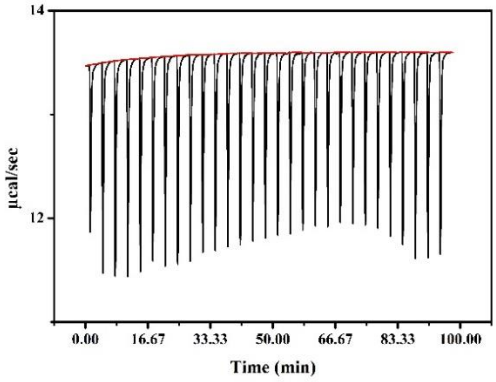
carbon footprint, allergen-free and gluten-free claims that can be made on food labels (Adebiyi & Aluko, 2011; Havemeier, Erickson, & Slavin, 2017). Protein accounts for 20-30 % of pea seed, which mainly consists of globulins and albumins. Globulins, known as salt-soluble proteins, represent around 50-60 % of total pea proteins while water-soluble albumins accounted for 15-25 %, according to a study by Gueguen (1983). Meanwhile, legumin (11S) and vicilin/convicilin (7S) constitute pea globulins. Legumin is a hexameric homo-oligomer with a molecular weight (Mw) of 360-400 kDa. Each subunit is around 60 kDa and consists of an acidic (~ 40 kDa) and a basic polypeptide (~20 kDa) linked by a disulfide bond. The acidic chain also has one free thiol (Gatehouse, Croy, Morton, Tyler, & Boulter, 1981; O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004a). Vicilin is a trimeric protein with a molecular weight of around 150 kDa, where the main vicilin subunit (~50 kDa) can undergo in vivo proteolysis at two potential cleavage sites. The vicilin-associated protein, convicilin, is a 210-290 kDa protein, consisting of subunits (~71 kDa) associated in trimeric or tetrameric form (O'Kane et al., 2004a).

To better understand the behavior of the two types of proteins in association with food systems, this article proposes a first approach to investigate the interactions between pea globulins and egg white proteins in aqueous mixtures at neutral and alkaline pH (pH 7.5 and 9), close to that of egg white. The potential interaction of whole pea globulins with purified LYS, OVA, or OVT was first examined by isothermal titration calorimetry (ITC) and ζ-potential measurements. The detected attractive interactions between LYS and pea globulins were further explored at different pH via the characterization of formed structures by dynamic light scattering (DLS), laser granulometry, and confocal laser scanning microscopy (CLSM).

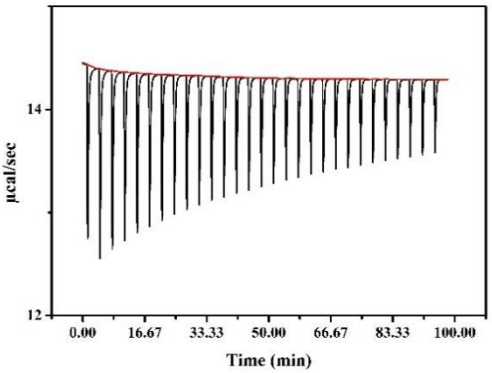
4.3 Results and discussions

4.3.1 LYS interacts with PPI through electrostatic interactions

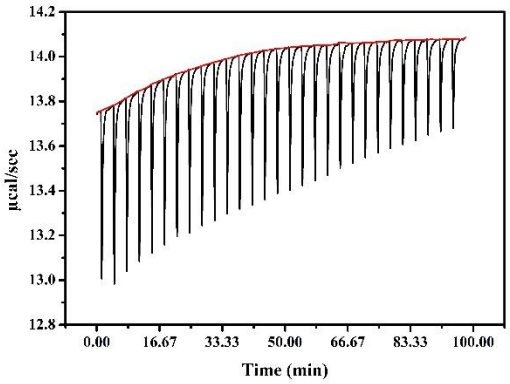
The ITC experiment was used to provide a detailed thermodynamic description and a better understanding of the mechanism of interactions of PPI and egg white proteins in solution. The ITC profiles for PPI with OVA (as acidic protein), OVT (as neutral protein), and LYS (as basic protein) were measured. The heat flow versus time profiles resulting from the titration of the PPI with the three egg proteins at various conditions are shown in Figure 4-1.



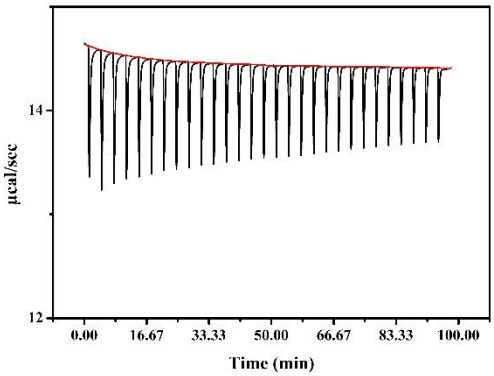
(A1)



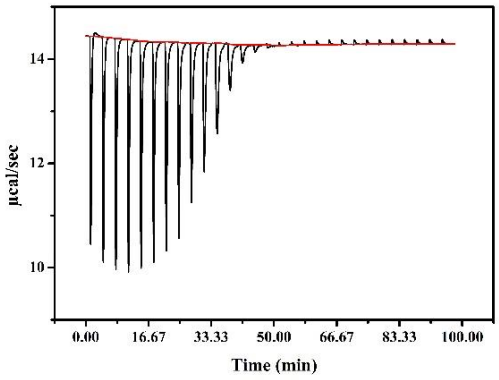
(A2)



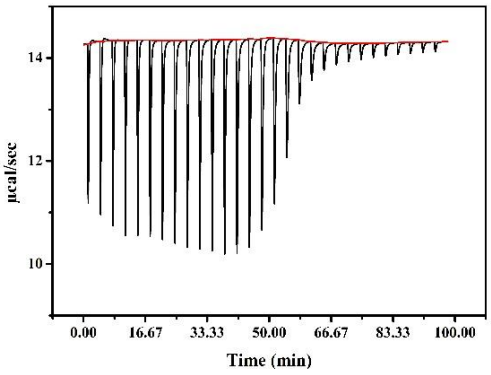
(B1)



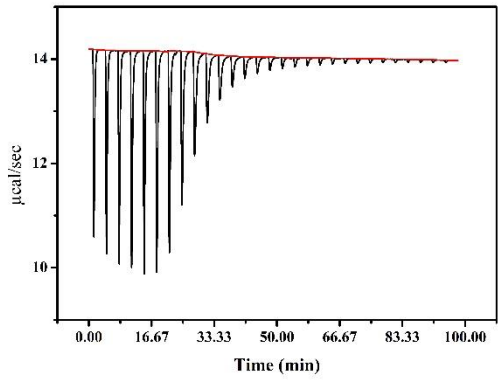
(B2)



(C1)

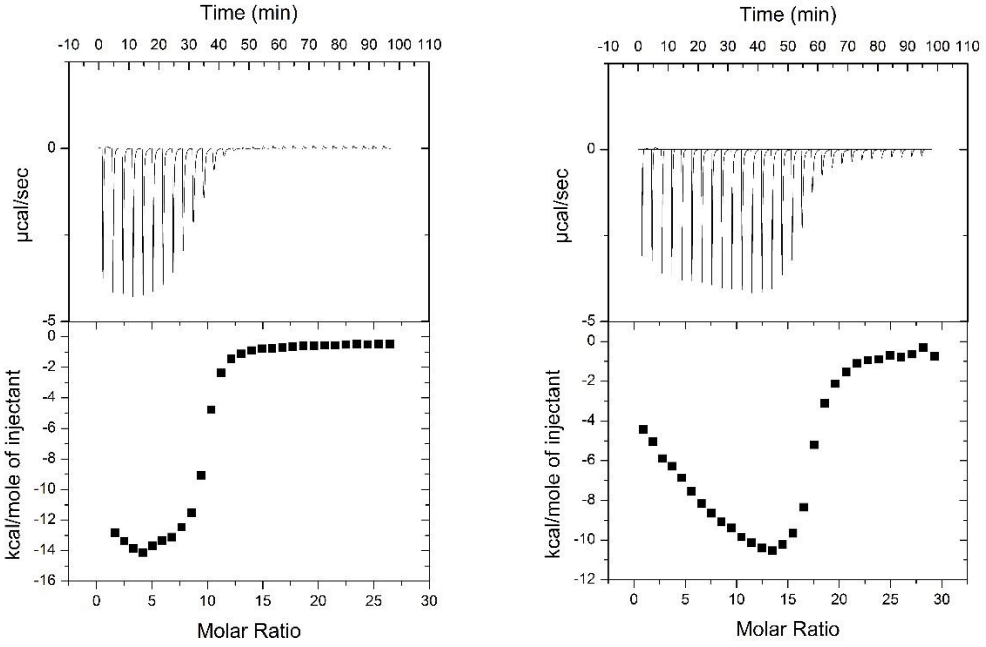


(C2)

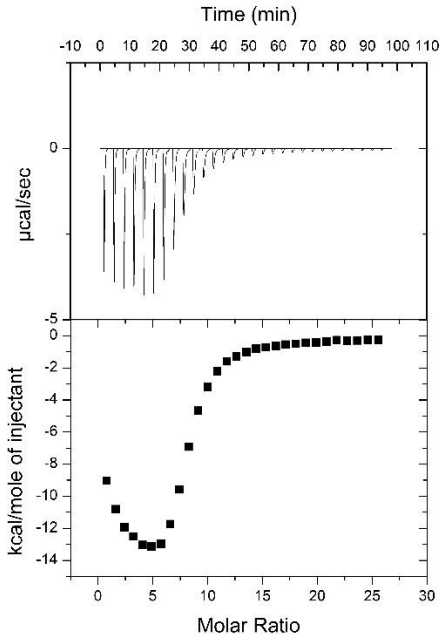


(C3)

Figure 4-1: Thermograms for the titration of PPI (0.0078 mM) with OVA (1.6 mM) in HEPES buffer pH 7.5 (A1), with OVA (1.7 mM) in Tris-HCl buffer pH 9 (A2), with OVT (0.64 mM) in HEPES buffer pH 7.5 (B1), with OVT (0.68 mM) in Tris-HCl buffer pH 9 (B2), with LYS (0.92 mM) in HEPES buffer pH 7.5 (C1), with LYS (1.02 mM) in Tris-HCl buffer pH 9 (C2), with LYS (0.89 mM) in Tris buffer pH 7.5 (C3). All the titration experiments were performed at 25 °C.



(A) (B)



(C)

Figure 4-2: Thermograms (top panels) and binding isotherms (bottom panels) for the titration of PPI (0.0078 mM) with LYS (0.92 mM) in HEPES buffer pH 7.5 (A), with LYS (1.02 mM) in Tris-HCl buffer pH 9 (B), with LYS (0.89 mM) in Tris-HCl buffer

pH 7.5 (C). All the titration experiments were performed at 25°C.

Whatever the egg protein studied, the ITC signal exhibited an exothermic profile. However, the signal intensity depended on the protein injected and the pH value (Figure 4-1). Weak interactions were observed between OVA or OVT and PPI at both pHs (7.5 and 9). The observed interactions in these mixed systems exhibited a saturating behavior but the signals are too weak to allow access to the thermodynamic parameters (Figure 4-1A, B). These results suggested that when mixed with PPI, OVA or OVT co-exist in solution without co-aggregation or complexation at neutral to basic pH values and low ionic strength. In contrast, when LYS was injected into PPI, a large exothermic signal was obtained at pH 7.5 but also at pH 9 (Figure 4-1 C). Meanwhile, to be consistent with the same buffer at both pH, and to avoid the potential buffer/protein interaction already reported by Rabiller-Baudry & Chaufer (2001), LYS in TRIS-HCl buffer at pH 7.5 was kept for further analyses.

The strong interaction between LYS and PPI was further explored. Figure 4-2 shows the ITC profiles and corresponding binding isotherms of the injection of LYS into PPI solution at two different pHs. The isotherms resulting from titrating PPI with LYS exhibited a visually obvious biphasic profile. The initially integrated heats of injection show a trend toward increasingly negative enthalpy, while later data trend positively until saturation was reached.

The area under each peak represented the heat exchange within the ITC cell after each injection, after subtraction of the heat of dilution of LYS into the buffer solution. While the overall ITC profiles were similar at both pH values, the enthalpy of the interaction was higher at pH 7.5 than at pH 9. The observed difference does not seem to be linked to the buffer nature as observed in other protein systems (Nigen, Le Tilly, Croguennec, Drouin-Kucma, & Bouhallab, 2009). Indeed, the same ITC signal was recovered at pH 7.5 when HEPES buffer was substituted by Tris-HCl (Figure 4-2 A, C).

At both pHs studied, a strong biphasic exothermic signal was obtained, underlying

at least two distinct events. During the first phase, the height of the exothermic peaks continuously increased with the addition of LYS until a critical value of LYS/PPI molar ratio beyond which the trend was reversed; further addition of LYS decreased the exothermic intensity of the signal (phase 2) until saturation. By comparing the general appearance of the two signals, two major linked differences can be noticed: i) the slope of the two phases is steeper at pH 7.5 than at pH 9; ii) the critical inversion LYS/PPI molar ratio is shifted to a higher value at pH 9, i.e., around 13 against 5 at pH 7.5. Similar biphasic ITC profiles were reported for other heteroprotein systems involving LYS such as LYS/bovine lactalbumin at 45 °C (Nigen, Croguennec, Renard, & Bouhallab, 2007) and LYS/conglycinin (Zheng et al., 2022). Such results were explained by ionic complexation between oppositely charged polymers forming supramolecular structures.

The shift of the molar ratio can be explained by the change of the negative-positive charge balance at the surface of the proteins, in particular, LYS given its high isoelectric point (Ip). At pH 9, a value approaching its Ip (i.e., 10.7), the LYS is less positively charged than at pH 7.5. Consequently, more LYS molecules are required to neutralize the actual number of negative charges on one PPI molecule, which do not vary significantly from pH 7.5 to pH 9. Charge compensation is the main parameter driving electrostatic complexation between oppositely charged proteins (Croguennec, Tavares, & Bouhallab, 2017).

The explanation of what happens during the two phases is not simple since each thermodynamic signal is a result of the contribution of several phenomena: classical interaction, protein conformational change, the release of water, protons, and other ions, complexation, reorganizations, aggregation, etc (Doyle & Hensley, 2005). The measured signal, therefore, comes from endothermic and exothermic reactions whose final absolute value is the result of the dominant energy.

To go further in the exploration of the thermodynamic changes occurring during titration, we tried to fit the binding isotherms using different binding models offered by

Microcal Origin software. The ‘two sets of sites’ model seems to better match the experimental titration profiles (data not shown). However, as already pointed out by other authors relating to other macromolecular systems (Girard, Turgeon, & Gauthier, 2003; Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010), we are convinced that the existence of two independent sets of binding sites has no physical meaning when dealing with interactions involving two macromolecules. In particular because of the simultaneous occurrence of several complex events as mentioned above. Hence, the use of the “2-stages structuring model” expression, underlying the presence of two distinct structuring phases instead of the “2-sites model” is more appropriate.

When using the “2-binding site model” as an approximation to extract the thermodynamic parameters of the interaction (namely *Ka* and $\triangle H$) between LYS and PPI at the three experimental conditions, erroneous values with large errors were obtained (data not shown). Consequently, we were unable to quantify the binding parameters using the ITC Microcal-associated origin software because the curves were complex and difficult to fit.

Although the appropriate thermodynamic parameters for the interaction between LYS and PPI could not be calculated, it is clear that the overall process leading to particle formation is enthalpically driven. A contrary situation occurred with the two other egg proteins tested, with no or only small negative heats detected by ITC. From the literature data (Leavitt & Freire, 2001; Klebe, 2015), enthalpy (ΔH) is related to the energy involved in molecular interactions and reflects the contribution of hydrogen bonds, electrostatic interactions, and van der Waals forces, while the change in entropy ($T\Delta S$) reflects a change in the order of the system and is related to hydrophobic interactions.

As possible particle formation between PPI and LYS was supposed from ITC data, the aqueous mixture of both proteins was further analyzed in terms of particle size, ζ-potential, and microstructure.

4.3.2 LYS-PPI aggregates size depends on protein molar ratio

From the previous study of ITC, two steps in aggregation between PPI and LYS happened. To have a better understanding of the aggregation, DLS and laser granulometry were performed. Due to large particles scattering more light than small particles (according to Rayleigh approximation, the scattering light intensity of particles is proportional to the sixth power of its diameter), intensity distribution and volume distribution were listed together to understand the relative amounts of the proteins with different particle sizes.

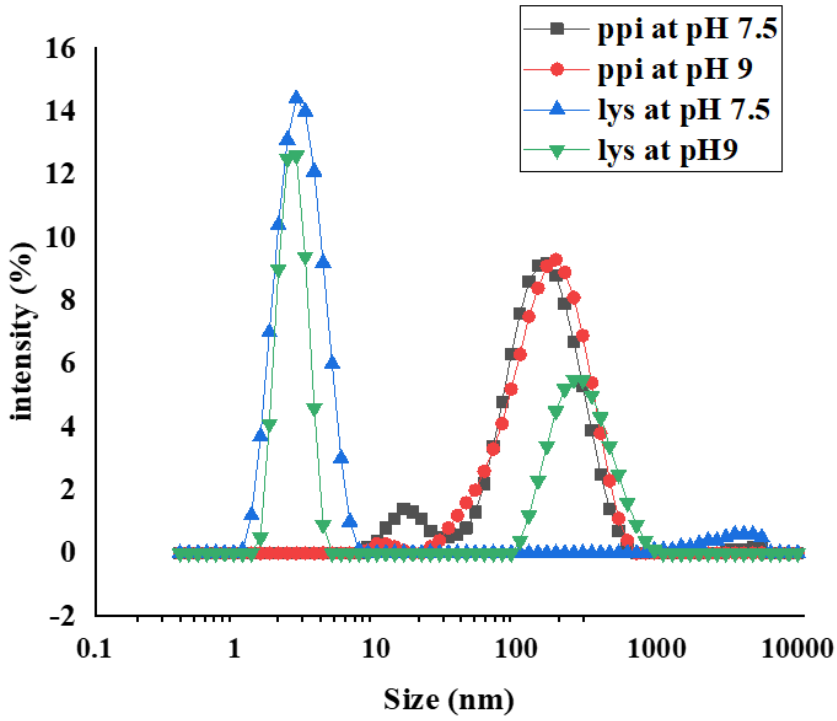
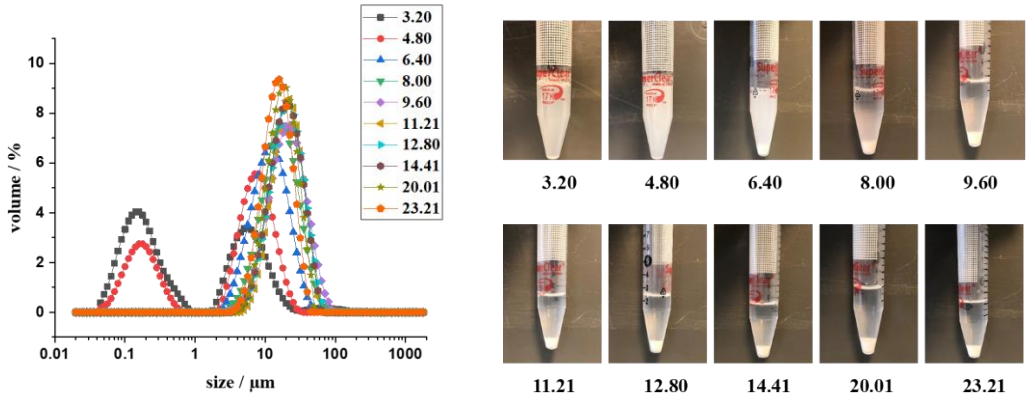


Figure 4-3: Particle size distribution measured by DLS of PPI (2.4 g/L) and LYS (14.3 g/L) suspensions in TRIS buffer at pH 7.5 and 9.

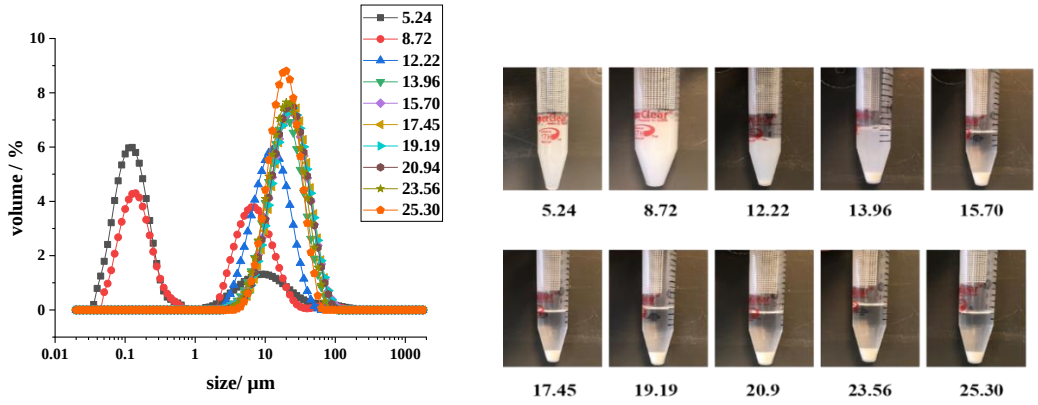
Figure 4-3 showed that the size distribution of pea globulins evidenced a bimodal distribution at pH 7.5 and 9. Particles around 19 nm and 11 nm at pH 7.5 and 9, respectively, may correspond to 7S and 11S oligomers, whereas those around 180 nm and 189 nm at pH 7.5 and 9, respectively, could be aggregated protein particles formed

during PPI preparation or initially present (Li et al., 2007; Chihi et al., 2016). The mean size of LYS at pH 7.5 and pH 9 was in the range of 2.5 to 3.0 nm, in line with the LYS monomer (Zheng et al., 2021). At pH 9 results also showed a double distribution where particles around 314 nm could originate from the aggregation of LYS resulting from less electrostatic repulsion between protein molecules at this pH closer to the Ip of LYS. To characterize aggregation for the mixture in a larger range of particle sizes, laser granulometry was used.



(A) (B)

Figure 4-4: Particle size distribution by laser granulometry (A) and pictures (B) of PPI-LYS suspensions at different LYS/PPI molar ratio in TRIS buffer pH 7.5



(A) (B)

Figure 4-5: Particle size distribution by laser granulometry (A) and pictures (B) of PPI-LYS suspensions at different LYS/PPI molar ratios in TRIS buffer at pH 9.

Table 4-1: The D (4,3) results of PPI-LYS mixtures in TRIS buffer at pH 7.5 and 9.

pH 7.5		pH 9	
Samples LYS/PPI molar ratio	D [4, 3] - Volume weighted mean (µm)	Samples LYS/PPI molar ratio	D [4, 3] - Volume weighted mean (µm)
3.2	5.2±0.6a	5.2	4.9±0.2a
4.8	6.2±0.005a	8.7	5.5±0.5a
6.4	12.8±0.04b	12.2	11.8±0.1b
8.0	21.7±0.5de	14.0	21.9±0.2c
9.6	22.7±0.1df	15.7	27.2±0.4e
11.2	**25.3**±0.1g	**17.5**	**28.1**±0.2e
12.8	23.8±0.2f	19.2	27.9±0.2e
14.4	23.1±0.2df	20.9	27.0±0.5e
20.0	20.5±0.1d	23.6	23.9±0.2d
23.2	17.2±0.1c	25.3	22.0±0.2c

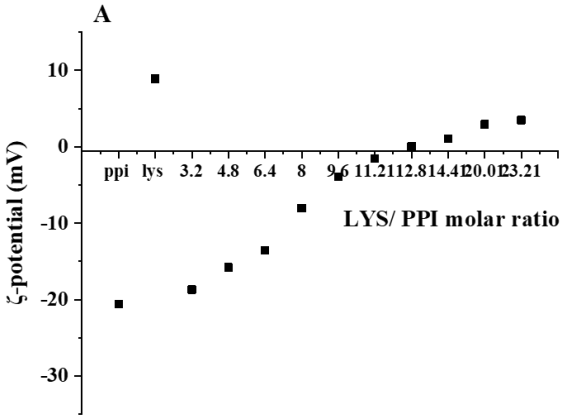
Means followed by a different small letter for the same column are significantly different ($P<0.05$)

Figures 4-4 and 4-5 demonstrated the particle size distribution by laser granulometry (A) and visual appearance (B) of PPI-LYS mixtures at pH 7.5 and 9, respectively. The particle size of the mixtures formed by PPI and LYS at different LYS/PPI molar ratios were reported in table 1 for the respective pH. As shown in Table 4-1, the size particle in the PPI-LYS mixture at pH 7.5 showed two distinct situations. First, it increased with the increasing proportion of LYS, then decreased when the LYS/PPI molar ratio was more than 11.21. Table 4-1 also gives the mean particle size for the pH 9 counterparts, showing similar behavior to the results at pH 7.5 with maximum particle size for an LYS/PPI molar ratio of 17.45. As the particle size decreased from an LYS/PPI molar ratio of ~11 at pH 7.5 and 17 at pH 9, respectively (Table 4-1), it could be hypothesized that mixed aggregates became more and more compact from this threshold, as repulsive forces between aggregates increased with the

addition of LYS. This increased the density of the aggregates which led to more precipitation of the aggregates as suggested by the lower quantity of the material on the CLSM picture (Figure 4-7 G and H). Furthermore, Figures 4 B and 5 B showed the visual appearance of PPI-LYS mixtures at different molar ratios at pH 7.5 and 9.0, respectively. Precipitates were observed directly after mixing PPI and LYS as the molar ratio exceeded the inflection point previously revealed for ITC binding isotherms, i.e., > 5 and > 12 at pH 7.5 and 9.0 respectively.

4.3.3 The maximal size of aggregates was obtained for neutral charge

The ζ-Potential of PPI, LYS, and their mixtures were measured in TRIS buffer at pH 7.5 and 9 (Figure 4-6 A-B). PPI and LYS solutions alone as a function of pH were also presented in Figure 4-6 C, which indicated the Ip of PPI and LYS were around 4.9 and 10.7, respectively. These values are in good agreement with the previously reported Ip values of these proteins (Klassen & Nickerson, 2012; Helmick, Hartanto, Bhunia, Liceaga, & Kokini, 2021; Rezwan, Studart, Vörös, & Gauckler, 2005; Yadav, Kumar, Aswal, & Kohlbrecher, 2017). Therefore, LYS showed a positive charge at pH 7.5 and 9, whereas PPI showed a negative charge respectively.



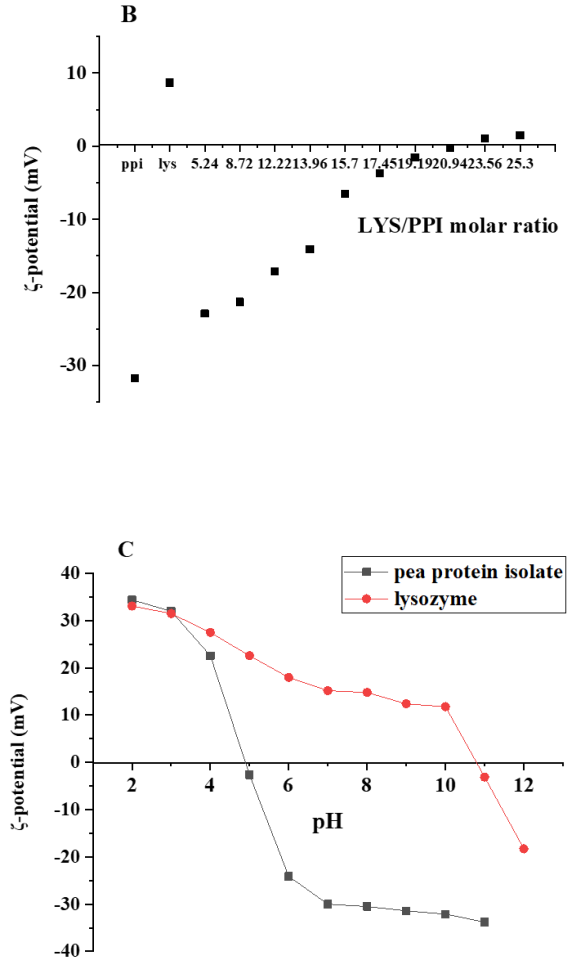


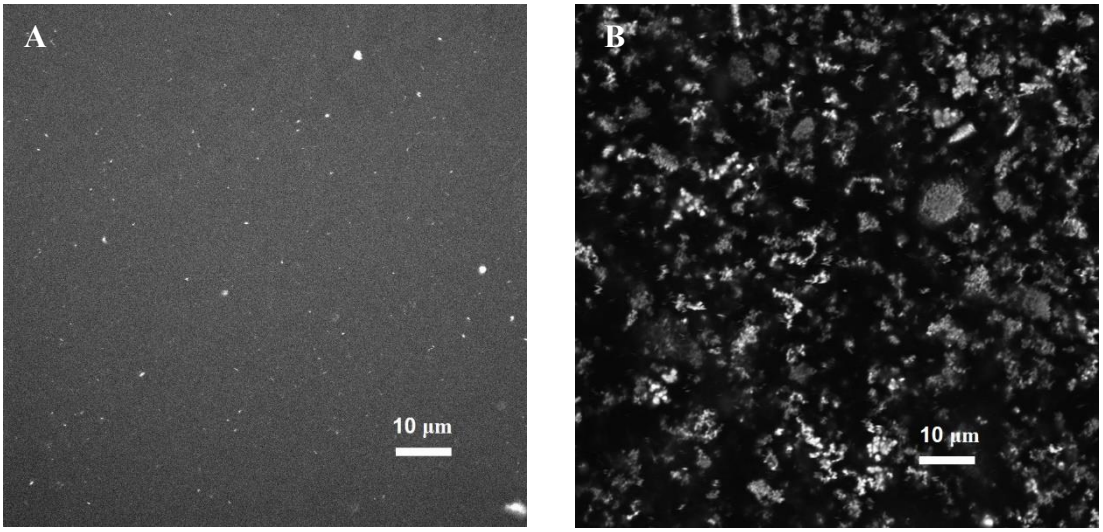
Figure 4-6: The ζ-potential of PPI-LYS mixtures at different LYS/PPI molar ratios in TRIS buffer at pH 7.5 (A) and pH 9 (B), and PPI and LYS solutions alone as a function of pH (C).

At both pHs, the PPI-LYS mixture's charge increases with LYS content, ranging from a negative charge at the smaller LYS/PPI ratio in the mixture to a positive charge at a higher LYS ratio in the mixture. The variation of the ζ-potential showed a typical charge inversion from positive ζ-values when the polycation was in excess ($Z < 1$) to negative ones when the polyanion was in excess ($Z > 1$) (Figure 4-6) in line with the recent work of Rodriguez, Binks, & Sekine, (2018). We can hypothesize that a positive charge on the LYS interacted with negatively charged segments of pea protein isolate,

leading to the formation of electrostatic complexes. This behavior indicated the presence of an interaction between the carboxyl groups of pea protein isolate and the amino group of LYS, featuring electrostatic binding. The charge was null for molar ratios close to 12 and 21 at pH 7.5 and 9, respectively. These results agreed with the previous results of ITC where the enthalpy didn't change anymore with the increasing proportion of LYS from these molar ratios (Figure 4-2). It could indicate that at these concentrations, LYS molecules had completely counteracted pea globulin charges.

4.3.4 Particle aggregates rather than coacervates were observed by microscopy

In order to better understand the microstructural properties and aggregation phenomena in PPI-LYS mixture systems, PPI and LYS stock solution and six suspensions at different PPI/LYS molar ratios (0.8, 1.6, 3.2, 4.8, 11.2, and 20) were analyzed by CLSM (Figure 4-7).



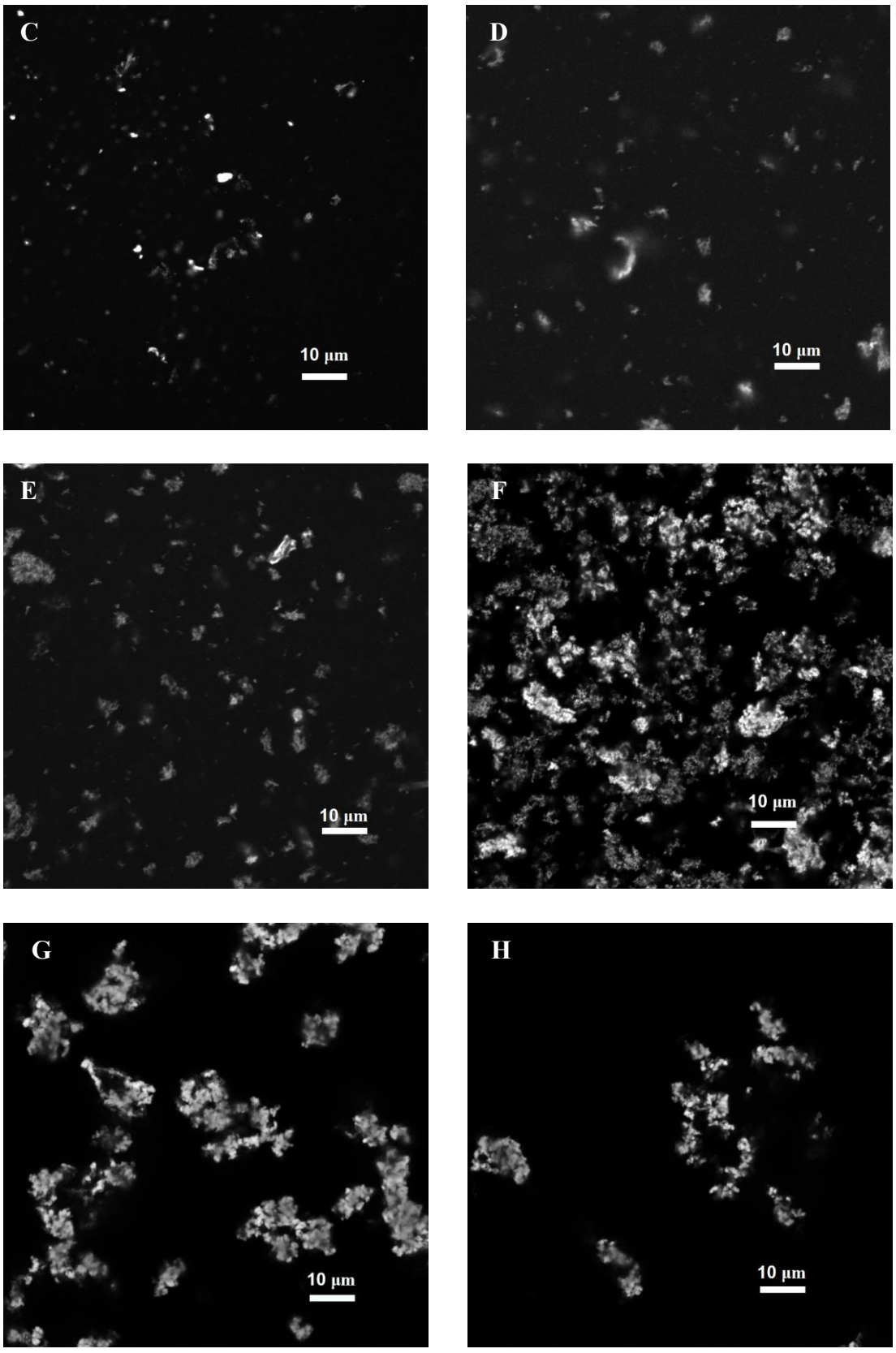


Figure 4-7: Microscopic observations by CLSM of mixed PPI-LYS suspensions at 20 °C in TRIS buffer at pH 7.5: PPI (2.4 g/L) (A), LYS (B), and LYS/PPI molar ratio

of 0.8 (C), 1.6 (D), 3.2(E), 4.8 (F), 11.2 (G), 20 (H).

The white color indicated the protein particles stained by Fast Green. From Figure 4-7 A, the PPI solution showed homogeneous distribution of tiny particles. A similar microstructure was previously reported for soluble PPI (Lan, Ohm, Chen, & Rao, 2020). LYS showed aggregates from Figure 4-7 B, as the solution is very concentrated (14.3 g/L), however, when mixed in the PPI solution, the aggregates dissociated with dilution, and no more aggregates are observed as suggested by DLS results (Figure 4-3). As the concentration of LYS increased, large aggregates with increased size were observed (Figure 4-7 C to H), in agreement with the previous particle size results (Figure 4-5). These protein aggregates had heterogeneous forms with irregular shapes. This increased size of protein particles could be attributed to strong attractive interactions between the two oppositely charged proteins (i.e., PPI and LYS) and contributed to forming of larger aggregated complexes which increased with LYS addition. As the particle size decreased from an LYS/PPI molar ratio of ~11 at pH 7.5 (Table 4-1), it could be hypothesized that mixed aggregates became more and more compact and more and more individualized from this threshold. Similar CLSM images of complex aggregation were also previously reported in a PPI-low-methoxyl pectin mixture (Lan et al., 2020), whey protein–beet pectin (Chen, Li, Ding, & Suo, 2012), and soybean protein-chitosan (Yuan, Wan, Yang, & Yin, 2014). Obviously, the present results showed that no spherical-shaped aggregates between PPI and LYS were formed excluding the possibility of complex coacervation in the studied conditions.

4.3.5 Optical microscopy

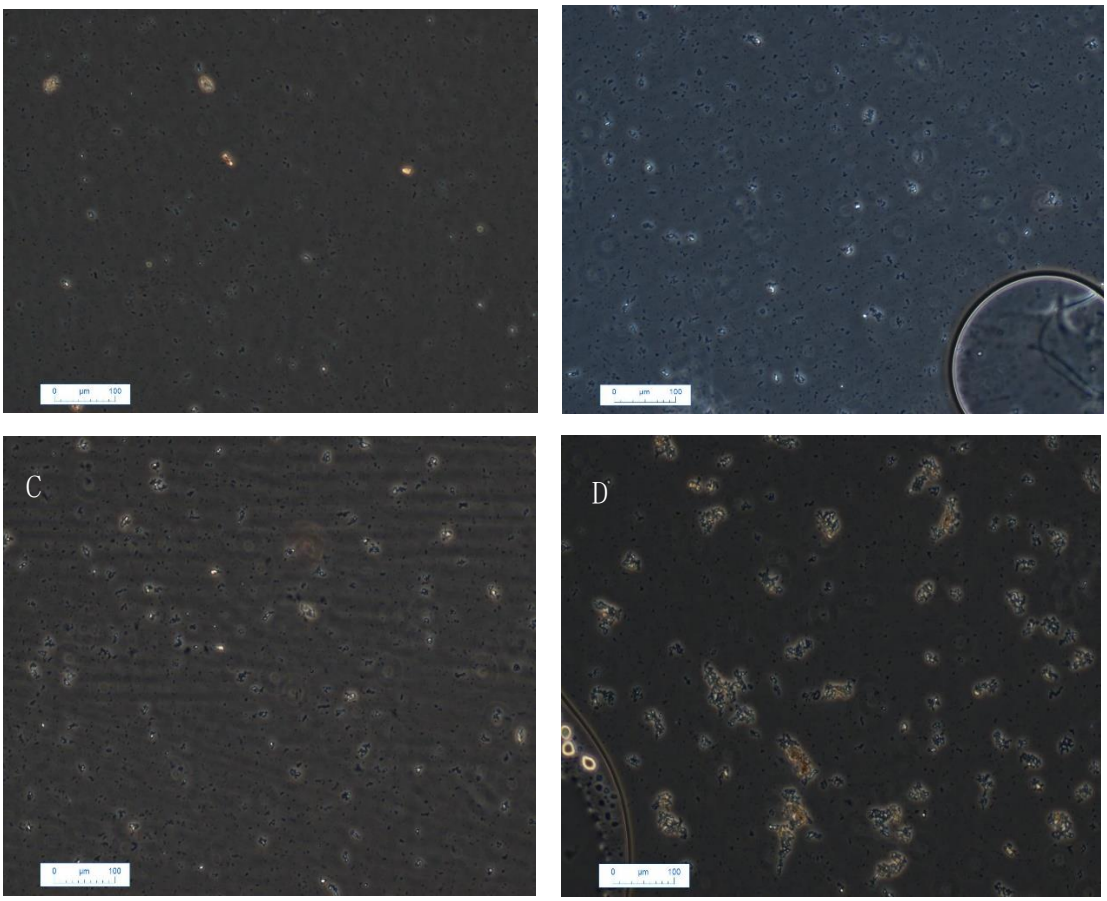


Figure 4-8: Phase contrast micrographs obtained by optical microscopy of mixed PPI-LYS systems taken at room temperature in TRIS buffer at pH 7.5 as a function of the ratio between PPI and LYS: LYS to PPI at a ratio of 0.8 (A), 1.6 (B), 3.2(C), and 4.8 (D) respectively

To complete the microstructure observations, phase contrast micrographs obtained at pH 7.5 in Tris-buffer (10 mM) at room temperature for different LYS/PPI molar ratios, ranging from 0.8 to 4.8, are shown in Figure 4-8. At a low LYS/PPI molar ratio, the formed complexes seemed to be no different. Small aggregated structures were observed against numerous larger aggregates obtained for higher molar ratios discussed above. Moreover, the number and size of formed particles seemed to increase with increasing LYS/PPI molar ratios confirming the results of particle size measurements. Interestingly, the overall shape of the particles obtained at a low molar ratio was more

spherical (Figure 4-8 A-C) than at higher ratios. The spherical nature of these particles corresponds probably to heteroprotein complex coacervation between positively charged LYS and negatively charged PPI. Complex coacervation in heteroprotein mixtures, corresponding to liquid-liquid phase separation that occurs in specific physico-chemical conditions starts to be well documented (Croguennec et al., 2017). The formation of complex coacervates between LYS and a mix of globulins in PPI deserves to be studied in depth.

4.4 Conclusion

The interactions and aggregation phenomena of pea proteins with three different egg white proteins were investigated. Only weak interaction was detected between PPI and acidic or neutral proteins from egg-like OVA and OVT, respectively. Special attention was paid to the mixture of PPI and LYS which showed specific interaction–aggregation behavior. It was evidenced that non-spherical aggregates were formed from low LYS/PPI molar ratio growing into large irregular aggregated structures that insolubilized at high molar ratio excluding the formation of pure complex coacervates. By combining the results obtained by the different techniques implemented here, we proposed a simple mechanism for the interaction–aggregation that occurs when LYS is mixed with PPI (Figure 4-9). At low ionic strength, LYS interacts with PPI at pH 7.5 and pH 9 according to two major structuring step processes: (i) the first step leads to the spontaneous formation of soluble complexes, and (ii) the second step involves the aggregation of these structures to form large separated aggregates with higher size centered around 20-25 µm. The transition from step 1 to step 2 is governed by pH-dependent protein stoichiometry needed to achieve opposite charge compensation. This transition occurs at a lower LYS/PPI ratio at pH 7.5 thanks to the higher surface positive charge of LYS as compared to pH 9. These results suggest that LYS, as an egg basic protein, will play a key interacting role when PPI is mixed with egg white for application purpose that deserves to be studied in depth in such a complex system.

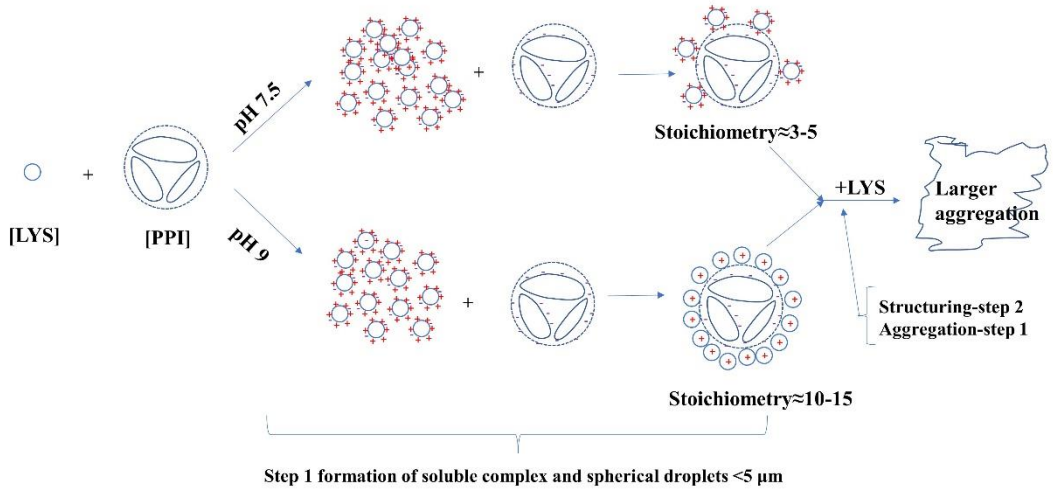


Figure 4-9 mechanism of the aggregate process between lysozyme and pea protein isolate.

Chapter 5 Thermal behaviour of pea and egg white protein mixtures

5.1 Abstract

In this work, a laboratory-prepared pea protein isolate (PPI) was used to partially substitute raw egg white (EW), and the thermal behaviour and sol-gel transition of the mixed systems submitted to heat treatment (<100 °C) were studied. The protein systems were investigated at pH 7.5 and 9 for different PPI/EW weight ratios (100/0, 75/25, 50/50, 25/75, 0/100) mainly by using Differential Scanning Calorimetry (DSC) and dynamic rheology. The denaturation enthalpy (ΔH) of the PPI-EW mixtures at each pH reflected the sum of ΔH of each protein by considering the respective content in PPI and EW and the loss (<10%) in total solubility of proteins in admixture while the thermal denaturation temperature (Td) of individual EW and PPI proteins were slightly (+/- 3 °C) or no affected depending on the pH. In particular, interactions between proteins were supposed to change the Td of ovotransferrin, lysozyme, and legumin. Upon temperature sweeps (25-95 °C), rheological data indicated two thermal sol-gel transitions at around 60 °C and 75 °C in EW-containing systems coinciding with Td of ovotransferrin and ovalbumin/S-ovalbumin/7S globulins, respectively. As the elastic modulus (G') of the mixtures decreased with the EW content, it was deduced that the gel formation was governed by the EW proteins. The first transition shifted by about + 2-3 °C at pH 9 probably by a hindering effect due to the presence of unfolded and non-associated pea globulins at this pH. These results will serve to develop an adapted heat treatment for this protein mixture used as a food ingredient.

5.2 Introduction

Owing to the population growth and diet-related socioeconomic changes over the coming decades, humans are increasingly recognizing that greater consumption of

plant-based foods and less dependence on meat and other animal-based products will contribute to improving the sustainability of the food system (Aiking & de Boer, 2020; Alexandratos & Bruinsma, 2012; Clark & Tilman, 2017; Godfray et al., 2018; Poore & Nemecek, 2018; Röös et al., 2017; Shepon, Eshel, Noor, & Milo, 2018). Meanwhile, the food industry is increasingly using plant protein components, particularly from legume seeds as an alternative to animal-based sources due to their diversity, nitrogen-fixing ability, higher availability, low price, and consumer perception of health and sustainability (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015; Boye, Zare, & Pletch, 2010). The mixtures of plant and animal proteins have also been considered to address food transition concerns and to explore synergistic effects in terms of consumer acceptance, nutrition, digestibility, and techno-functional properties of such systems (Alves & Tavares, 2019; Guyomarc'h et al., 2021). Among the new sources of proteins, pea proteins are increasingly attractive, and several studies have targeted their behaviour when mixed with dairy proteins, to form gels, emulsions, or foams (Chihi, Mession, Sok, & Saurel, 2016; Hinderink et al., 2021a). On the other hand, no study has explored mixtures of pea and egg proteins, which may be an interesting perspective for the development of ovo-vegetarian products.

Pea proteins represent ≈ 23 % (w/w on a dry basis (d.b.)) of the dry seeds (Gueguen, 1983; Tzitzikas, Vincken, de Groot, Gruppern, & Visser, 2006) and are mainly composed of globulins (≈ 70 %), i.e., legumin 11S and vicilin/convicilin 7S (Sharif, et al., 2018) and the rest corresponds mainly to the 2S albumin fraction (≈20%) and other insoluble proteins. Legumin is a hexameric protein of 360-400 kDa, comprising six subunits of ~60 kDa associated with non-covalent interactions. Each monomer consists of an acidic (~40 kDa) and an alkaline (~20 kDa) subunit linked by a disulfide bond (Dziuba, Szerszunowicz, Natecz, & Dsiuba, 2014; Shand, Ya, Pietrasik, & Wanasundara, 2007). Vicilin (7S) is a trimeric protein of around 150 kDa. Each monomer ~50 kDa has two cleavage sites possibly generating small fragments during pea seed development: α (~20 kDa), β (~13 kDa), γ (~12-16 kDa), αβ and βγ

polypeptides (Gatehouse, Croy, Morton, Tyler, & Boutler, 1981; Tzitzikas, et al., 2006; Shand et al., 2007; Liang & Tang, 2013). A third minor globulin, convicilin, is a multimeric protein of 210-290 kDa whose subunit (~70 kDa) has a highly homologous core amino acid sequence with vicilin monomer, yet possesses an extended hydrophilic N terminus (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004a).

Among animal-rich protein products, egg white is a desirable ingredient used in many foods such as bakery products, meringues, and meat products, because of its excellent foaming and gelling properties (Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007). It usually contains about 11 % proteins which consist of more than 40 different kinds of proteins. Ovalbumin (54 %), ovotransferrin (12 %), ovomucoid (11 %), lysozyme (3.5 %), and ovomucin (1.5-3.5 %) are among the major proteins of egg white (Belitz, Grosch & Schieberle, 2009; Guha, Majumder, & Mine, 2019). Ovalbumin (44.5 kDa) consists of a peptide chain of 385 amino acid residues and contains four thiols and one disulfide group. Its isoelectric point is estimated at 4.5. With high pH and temperature-dependent denaturation, ovalbumin converts into a thermally stable form known as S-ovalbumin (Smith & Back, 1965). Ovotransferrin consists of one peptide chain of 686 amino acids and contains one oligosaccharide unit made of four mannose and eight N-acetylglucosamine residues. Its molecular weight and isoelectric point are around 77.7 kDa and 6.1 respectively. Lysozyme (14.4 kDa) is a relatively small secretory glycoprotein, consisting of 129 amino acids linked by four disulfide bonds with an isoelectric point of 10.7. Recently, Iwashita, Handa, & Shiraki (2019) highlighted that ovotransferrin can co-aggregate with lysozyme, and Wei, Cheng & Huang (2019) confirmed heteroprotein complex formation between ovotransferrin and lysozyme. Ovomucoid is a heat-stable glycoprotein containing 186 residues with a molecular weight of 28 kDa and pI of 4.1 (Julià et al., 2007; Winiarska-Mieczan & Kwiecień, 2007). It contains 9 disulfide bonds and has three different domains which are crosslinked only by the intra-domain disulfide bonds (Huopalahti, Anton, López-Fandiño, & Schade, 2007). Ovomucin is a sulfated glycoprotein that is responsible for

the jellylike structure of egg white (Abeyrathne, Lee, Jo, Suh, & Ahn, 2016; Strixner & Kulozik, 2011). The molecular weight of ovomucin is 1.8–8.3×10^3 kDa (Abeyrathne, Lee, & Ahn, 2014; Baumgartner & Schubert-Ullrich, 2010). Based on their composition and characteristics, ovomucin subunits may be classified into two types: α- and β-ovomucin (Huopalahti et al., 2007).

At an industrial level, egg white is available in pasteurized liquid or frozen form, or powder after spray drying. During processing, the egg white is pasteurized at moderate temperatures between 54 and 57 °C for a few minutes to prevent the coagulation of the heat-sensitive egg white proteins. The egg white can also be subjected to higher temperatures during spray drying operating up to 180 °C. To develop a mixed ingredient with pea proteins undergoing treatments analogous to egg white alone, it appears necessary to evaluate the thermal behaviour of the mixture from native proteins. To our knowledge, egg proteins have been rarely studied in association with plant proteins except for soybean (Su et al., 2015; Zhang et al., 2019), and the physicochemical properties of egg white and pea protein mixture have not yet been considered in previous works.

In this paper, we mainly evaluate the thermal properties by differential scanning calorimetry (DSC) of isolated pea globulins (PPI) and egg white (EW) mixtures at different PPI-EW mass ratios (100/0, 75/25, 50/50, 25/75 and 0/100) at pH 7.5 and 9. The nitrogen solubility profile of the pure proteins and 50/50 mixture systems, and their polypeptide composition using electrophoresis were also characterized. Additionally, the gelling temperature (point) of the 10 % protein PPI-EW mixtures was investigated by small amplitude rheology to anticipate possible pasteurization temperature adjustment in future manufacturing.

5.3 Results and discussion

5.3.1 Solubility profile of protein systems

Solubility is a prerequisite for proteins in a variety of high-moisture food applications. It is also a key determinant parameter for many important functional properties including emulsifying, foaming, and gel-forming capabilities (Malik & Saini, 2017). Shand et al. (2007) reported that the solubility of pea protein is highly dependent on ionic strength and pH. The solubility of PPI, EW, and the PPI-EW mixture at the weight ratio of 50/50 are shown in Figure 5-1. Solubility regarding PPI and EW only was discussed in Chapter 3.1.4. In general, PPI showed a U-shaped nitrogen solubility profile, with a minimum around pH 5, and increased and reached values of around 85 % and 89 % for pH values below 3 and above 7. The nitrogen solubility of egg white was always over 88 %, with a little lower solubility of EW at around pH 4, close to the isoelectric point of ovalbumin (pH 4.5).

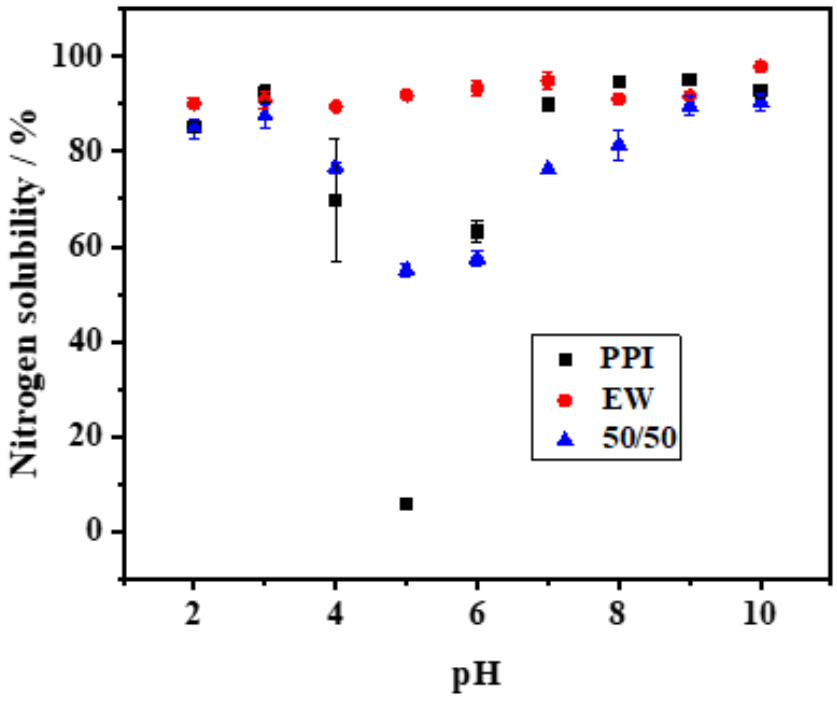


Figure 5-1: Nitrogen solubility of PPI, EW, and PPI-EW mixture at the weight ratio of 50/50 in 0.1 M NaCl.

Regarding the PPI-EW mixture, the solubility profile showed the same shape compared to PPI with a minimum of solubility at around pH 5. Meanwhile, recalculated NS values data gave a reference (Table 5-1). When pH was smaller than 4, the measured NS values of the 50/50 mixture showed no significant difference from the recalculated ones. However, at pH 5 the measured NS value was significantly higher than the recalculated one while above this pH the measured NS profile showed significantly lower values. The mixture of both proteins could either favor protein solubility (around pH 5) or enhance the formation of insoluble (co-)aggregates that decrease protein solubility above pH 5. This could be explained by hydrophobic or electrostatic interactions between protein molecules of different natures. Close to pH 5, ovalbumin, the main egg white protein, is indeed less charged and may interact with PPI, thus increasing their solubility while at higher pH, PPI could form aggregated complexes with lysozyme via electrostatic interaction that could influence their solubility (Chapter 4).

Table 5-1: Nitrogen solubility (NS) values of the PPI-EW mixture at the ratio of 50/50 at different pH. Recalculated NS values were obtained from experimental NS values of individual PPI and EW suspensions.

pH	NS (%)	Recalculated NS (%)
2	84.8±1.2a	87.6±0.5a
3	87.5±1.4a	91.5±1.3a
4	76.6±0.7a	78.3±1.6a
5	55.0±0.8a	48.9±0.2b
6	57.5±0.8a	78.6±1.3b
7	76.2±0.1a	92.5±0.7b
8	81.3±1.8a	92.9±0.4b
9	89.7±1.2a	93.4±0.3b
10	90.5±1.0a	95.3±0.5b

Means followed by a different lowercase letter for the same row are significantly different.

5.3.2 Polypeptide composition

Figure 5-2 shows the electrophoresis profile of the 50/50 weight ratio PPI-EW mixture before and after centrifugation under reducing and non-reducing conditions at pH 7.5 and 9. The electrophoretic profile of the PPI and EW prepared at pH 7.5 and 9 was already discussed in chapter 3.1.3, and showed great similarities, respectively. In general, three main polypeptide components were shown in egg white samples ovotransferrin (76 kDa), ovalbumin (44 kDa), and lysozyme (14.6 kDa). PPI contained a molecular weight of lipoxygenase around 88 kDa, convicilin around 71 kDa, and legumin 11S 60 kDa concluding acidic subunits Lα (~38-40 kDa) and basic subunits Lβ (~20-22 kDa) under reducing conditions, main vicilin fractions around i) 15 kDa, ii) 20-37 kDa responding to fragments α (≈20 kDa), α:β (~30-36 kDa), and β:γ (~25-30 kDa), iii) 19 kDa, iiii): vicilin monomer around 50 kDa.

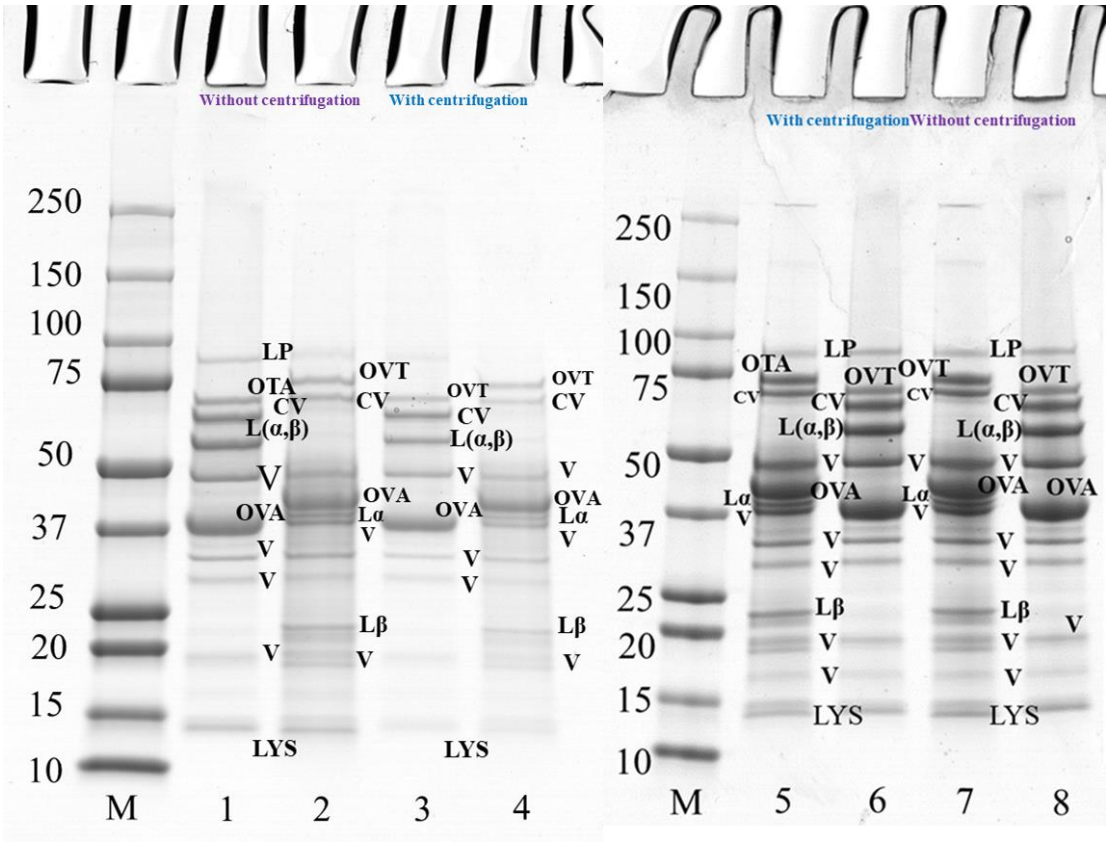


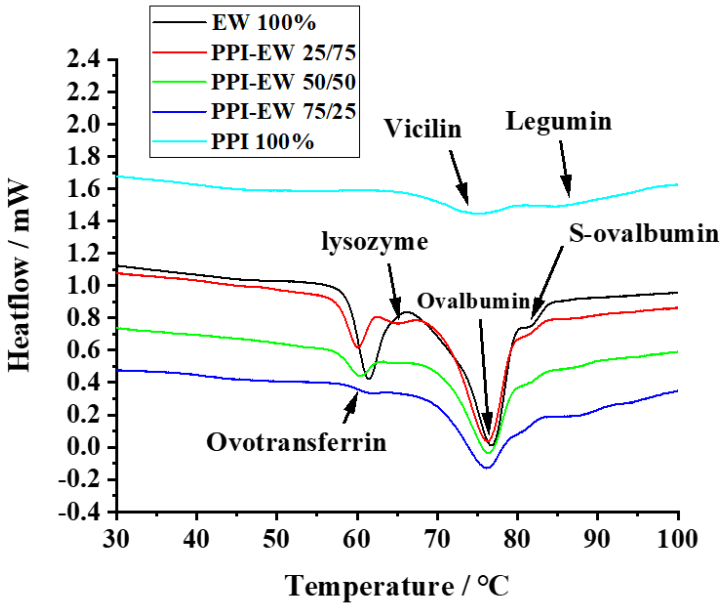
Figure 5-2: SDS-PAGE profile of PPI-EW suspensions prepared at pH 7.5 (Lanes 1-4) and 9 (Lanes 5-8), at the weight ratio 50/50 with and without centrifugation. The

samples on lanes 2, 4, 5, and 7 were treated under reducing conditions with SDS+DTT reagents. Lane M: molecular weight (Mw) markers; Lanes 1-2, 7-8: PPI-EW at the weight ratio 50/50 without centrifugation; Lanes 3-4, 5-6: PPI-EW at the weight ratio 50/50 with centrifugation; LP, lipoxygenase; L (α, β), legumin; CV, convicilin; Lα, legumin acid polypeptide; Lβ, legumin basic polypeptide; V, vicilin; OVA, ovalbumin; OVT, ovotransferrin; LYS, lysozyme.

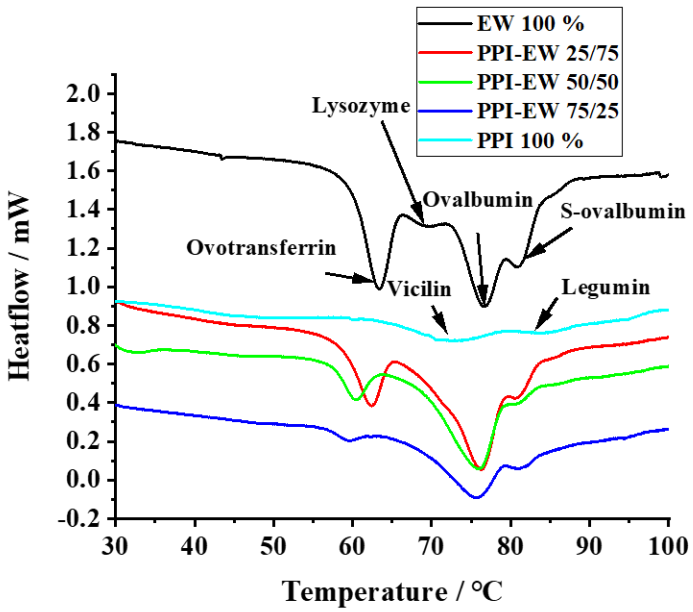
The polypeptide profile of PPI-EW mixtures at a weight ratio of 50/50 at pH 7.5 (lanes 1-4) and 9 (lanes 5-8) under reducing and non-reducing conditions with and without centrifugation was shown in Figure 5-2. Regarding the line 1 (at pH 7.5) and 8 (at pH 9) for PPI-EW mixtures under non-reducing conditions, the profile showed the presence of L (α, β) polypeptide around 60 kDa, which is consistent with the result of Figure 3-1 (Chapter 3, Section 3.1.3). In reducing conditions lane 2 (at pH 7.5) and 7 (at pH 9), L (α, β) was separated into Lα and Lβ because of the rupture of disulfide bonds. Major components of the pea protein isolate and egg white have been found under non-reducing conditions, such as vicilin (around 50 kDa, 20-37 kDa, 19 kDa), convicilin, Legumin (L (α, β)), ovalbumin, ovotransferrin and lysozyme (Figure 5-2, lanes 1, 8, 6). When compared to the polypeptides under reducing conditions between Figure 5-2 (lanes 2, 7) and Figure 3-1 (lanes 2,4, and 6,8 for PPI and EW, respectively), no bands disappeared. However, with centrifugation and regarding supernatant, the bands of vicilin (around 19 kDa) decreased at pH 7.5 (Figure 5-2, lane 3) but not at pH 9 (Figure 5-2, lane 5). Moreover, the bands at pH 9 were more intense than those at pH 7.5, which could be explained by the solubility that was higher at pH 9.0. At pH 7.5, the bands attributed to lipoxygenase and lysozyme (Figure 5-2, lanes 3 & 4) strongly decreased after centrifugation compared to those before centrifugation (Figure 5-2, lanes 1 & 2). This would suggest the contribution of these two proteins in the aggregates lost after centrifugation. Interestingly, this did not occur at pH 9.0 (Figure 5-2, lane 5-8) when lysozyme was less positively charged.

5.3.3 Thermal properties of the mixtures

The thermal properties of proteins directly reflect their native status and can be evaluated by calorimetry.



(A)



(B)

Figure 5-3: Typical DSC thermograms for PPI, EW, and PPI-EW at different weight ratios at pH 7.5 (A) and 9 (B), respectively.

Figures 5-3 A and B show typical DSC thermograms for pea proteins, egg white proteins, and PPI-EW mixture at different mass ratios at pH 7.5 and 9, respectively.

The thermal denaturation temperature (Td) and enthalpy of PPI and EW were discussed in chapter 3.1.5. In general, PPI thermal curves showed the two endothermic peaks for 7S (~76, 71 °C) and 11S globulins (87, ~85 °C) with an enthalpy of 10.8 and 3.6 J/g at pH 7.5 (former) and 9 (latter), respectively. EW had 4 main peaks, and in order were ovotransferrin, lysozyme, ovalbumin, and S-ovalbumin, while lysozyme was overlayed by ovotransferrin at pH 7.5.

T_d values of PPI, EW, and PPI-EW mixtures at different weight ratios were compared at pH 7.5 and 9 in Tables 5-2 and 5-3, respectively. In these tables, the peaks were assigned to the different proteins present in PPI, EW, and their mixtures. For PPI, T_d3 and T_d5 corresponded to vicilin and legumin, respectively. For EW, T_d1, T_d2, T_d3, and T_d4, corresponded to ovotransferrin, lysozyme, ovalbumin, and S-ovalbumin, respectively. For the PPI-EW mixtures, T_d3 resulted from the superimposition of ovalbumin and vicilin peaks which could also overlay the peak of lysozyme at pH 9. T_d4 and T_d5 corresponded to S-ovalbumin and legumin denaturation, respectively.

Table 5-2: Thermal denaturation temperatures of EW, PPI, and PPI-EW mixtures at different weight ratios at pH 7.5.

samples	T_d1(°C)	T_d 2(°C)	T_d 3(°C)	T_d 4(°C)	T_d 5(°C)
EW 100%	61.1±0.1a	-	76.7±0.1a	83.5±0.8a	-
PPI-EW 25/75	60.1±0.1a	64.4±0.6a	76.0±0.1ab	81.6±0.1a	85.4±0.3a
PPI-EW 50/50	60.5±0.4a	64.5±0.6a	76.2±0.1ab	81.5±0.1a	85.6±0.1a
PPI-EW 75/25	61.4±0.1a	-	76.0±0.1ab	81.3±0.2a	85.0±0.7a
PPI 100%	-	-	75.8±0.4b	-	87.4±0.5b

All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different. T_d1: ovotransferrin or ovotransferrin and lysozyme; T_d2: lysozyme; T_d3: ovalbumin (EW), vicilin (PPI) or ovalbumin and vicilin (mixture case), T_d4: s-ovalbumin; T_d5: legumin.

Table 5-3: Thermal denaturation temperatures of EW, PPI and their mixtures (PPI-EW) at different weight ratios at pH 9.

samples	T_d1(°C)	T_d 2(°C)	T_d 3(°C)	T_d 4(°C)	T_d 5(°C)
EW 100%	63.2±0.1a	69.5±0.1	76.4±0.1a	83.1±0.7a	-
PPI-EW 25/75	62.1±0.1b	-	76.1±0.1ab	81.3±0.1a	86.3±0.7a
PPI-EW 50/50	60.3±0.1c	-	75.3±0.2b	81.4±0.1a	86.1±0.1a
PPI-EW 75/25	59.5±0.1d	-	75.5±0.1b	81.4±0.1a	86.9±0.2a
PPI 100%	-	-	71.3±0.4c	-	84.5±0.2b

All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different. T_d1: ovotransferrin or ovotransferrin and lysozyme; T_d2: lysozyme; T_d3: ovalbumin (EW), vicilin (PPI) or ovalbumin, vicilin and lysozyme (mixture case), T_d4: s-ovalbumin; T_d5: legumin.

At pH 7.5 (Table 5-2), no significant difference in T_d of ovotransferrin between pure EW and PPI-EW mixtures occurred. However, with the addition of PPI, the peak corresponding to lysozyme appeared. This may be due to either a slight shift of ovotransferrin signal toward lower temperatures thus resulting in a better separation of ovotransferrin and lysozyme signals or to an increase of lysozyme denaturation temperature due to its stabilization through interactions with PPI proteins as evidenced previously (Chapter 4, Section 4.3.1). When PPI was mixed with EW, the pea proteins could indeed form electrostatic interactions with lysozyme since they are strongly oppositely charged at pH 7.5. This result agreed with SDS-PAGE results that suggested the implication of lysozyme in aggregates that precipitated with centrifugation. Moreover, the T_d value of legumin (T_d5) decreased by about 2 °C in PPI-EW mixtures compared to pure PPI suspension. This means that legumin proteins were more sensitive to temperature in mixtures. Conformational changes towards a more unfolded state of legumin molecules could thus be hypothesized in the presence of egg white proteins. Modification of the hydration environment of molecules influenced by the new composition in the mixture, and/or interactions with egg white proteins could explain the partial unfolding of legumin to a less stable form.

As vicilin and ovalbumin have close denaturation temperatures, only one mean Td value ($T_d3 \approx 76$ °C) was recorded for the PPI-EW mixtures. This value was not significantly affected by the PPI-EW weight ratio and corresponded to the mean of ovalbumin and vicilin values in pure EW and PPI systems, respectively. T_d of S-ovalbumin decreased slightly but not significantly for the mixtures compared to the pure EW sample.

At pH 9,0 (Table 5-3), the T_d value of ovotransferrin (T_d1) decreased significantly from ~63 °C to ~59 °C with the increase in PPI content in admixture. Ovotransferrin is thus more sensitive to heat denaturation in presence of PPI at pH 9 than at pH 7.5, assuming the unfolding of ovotransferrin in presence of PPI or a decrease in electrostatic interactions with lysozyme due to the competition with PPI proteins as suggested by the increase in T_d value of legumin in the mixture at this pH (T_d5). No peak corresponding to lysozyme (T_d2) was detected for PPI-EW mixtures at pH 9 although it was observed for pure EW samples at this pH. We assumed that the peak of lysozyme may be overlapped by the larger peak of ovalbumin and vicilin. In presence of PPI, the total peak shifted slightly to a lower T_d value. As in pure systems, T_d values for 7S proteins and lysozyme are lower compared to ovalbumin one, the superimposition of the three denaturation temperatures could explain the resultant average lower T_d. It could not be excluded also that the 7S peak and/or lysozyme peak increased to T_d values > 71 °C closer to T_d of ovalbumin in admixture due to a cooperative denaturation effect or thermo-protective effect of respective proteins. For instance, Mession, Roustel, & Saurel (2017a) reported that when mixing casein with pea legumin and vicilin enriched fractions, the Td of the latter proteins increased by about 4 °C. Otherwise, Zheng et al. (2021) found that the T_d temperature of the mixture of lysozyme and β-Conglycinin was higher than single lysozyme, which indicates the thermal stability of lysozyme was improved via partial unfolding of β-Conglycinin during complexation. T_d of S-ovalbumin showed no significant difference for all the samples at both pHs, which confirmed the high thermostability of this protein.

Table 5-4: Denaturation enthalpy of (ΔH) of EW, PPI, and their mixtures (PPI-EW) at different weight ratios at pH 7.5 and 9.

	pH 7.5		pH 9	
samples	ΔH（J/g）	Recalculated ΔH（J/g）	ΔH（J/g）	Recalculated ΔH（J/g）
EW 100%	22.3±0.5*	-	23.8±0.2**	-
PPI-EW 25/75	18.6±0.2a	19.4±0.4b	18.3±0.3a	18.7±0.1a
PPI-EW 50/50	14.1±0.2a	16.6±0.2b	12.4±0.1a	13.7±0.1b
PPI-EW 75/25	12.6±0.1a	13.7±0.1b	8.6±0.1a	8.6±0.2a
PPI 100%	10.8±0.1*	-	3.6±0.2**	-

All data were given as mean ± SD of triplicate measurements. Means in a row bearing the same letter are not significantly different at the same pH. Means followed by different numbers of * for the same row are significantly different.

Recalculated ΔH: $\Delta H_{recalculated} = \Delta H_{ew} \times ratio + \Delta H_{ppi} \times ratio$.

Table 5-4 shows the specific enthalpy (ΔH) values for suspensions of PPI and EW and their mixtures at different weight ratios at pH 7.5 and 9. Concerning the PPI-EW mixtures at different weight ratios, the ΔH value was recalculated by equation (4) to know if the measured values of ΔH resulted from the additive denaturation of PPI and EW proteins considering their relative content in the mixtures. Mixtures at pH 7.5 presented measured ΔH values significantly lower than recalculated ones with differences comprised between 0.8 to 2.5 J/g. These differences may reflect the loss of solubility of some proteins as revealed for the 50/50 mixture in Sections 5.3.1 and 5.3.2 at this pH as the precipitated protein part is less prone to contribute to the total enthalpy.

PPI-EW mixture at 25/75 and 75/25 ratios at pH 9, showed recalculated ΔH values similar to measured ones, which could probably indicate that the interactions that structure the different proteins were not significantly modified in the mixture in these conditions. However, the 50/50 ratio at pH 9 showed lower measured values than recalculated ones. This could not be attributed to a decrease in solubility since it was not the case for the mixture at this pH but maybe to a specific ratio effect in a complex

manner, as the decrease in enthalpy was also significantly higher for this ratio at pH 7.5.

5.3.4 Gelation temperatures

Temperature sweeps were performed by small amplitude rheology to understand the sol-gel transition behaviour of the different protein suspensions upon thermal treatment. The gelling temperatures were reported in Table 5-5. For EW and the PPI-EW mixtures containing at least 50 % EW, two transition temperatures were measured whatever the pH. For pure EW at pH 7.5 and 9, these gelling temperatures could be attributed preferentially to the denaturation of ovotransferrin and ovalbumin at ~60 and ~75 °C, at pH 7, respectively, as reported by Barhut & Findlay (1990) and Ferreira et al. (1997). It can be noticed that these temperatures are close to the thermo-denaturation temperature identified for these proteins in DSC thermograms (Tables 5-2 & 5-3).

Table 5-5: Gelling point temperature of PPI, EW suspensions and their mixtures at 10% protein at pH 7.5 and 9.

Samples	pH 7.5		pH 9	
	1st gelling point / °C	2nd gelling point / °C	1st gelling point / °C	2nd gelling point / °C
EW 100%	59.3±0.2a	75.4±0.3a	58.8±0.3a	75.2±0.4ab
PPI-EW 25/75	59.3±0.2a	75.1±0.3a	60.7±0.3c	75.5±0.4a
PPI-EW 50/50	59.9±0.2a	75.1±0.3a	61.6±0.2c	74.0±0.3b
PPI-EW 75/25	none	73.2±0.3b	none	75.6±0.3a
PPI 100%	none	75.6±0.3a	No gelation	

All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different. 1st gelling point was linked to ovotransferrin, 2nd gelling point linked to ovalbumin and vicilin compared to the results of thermal properties.

For the mixtures containing at least 50 % of EW, considering that pure PPI suspensions didn't show any transition temperature around 60 °C whatever the pH, the presence of a first gelling point can also be associated mainly with ovotransferrin. This

first transition temperature was not affected by the EW/PPI ratio at pH 7.5 whereas it increased by nearly 3 °C with the increase in PPI in the mixture at pH 9.0. At this latter pH, the gelation point assigned to ovotransferrin was therefore delayed even though the DSC results reported in Section 5.3.3 showed a slight decrease in the denaturation temperature of this protein in admixture with PPI. The presence of the pea globulins carrying highly negative charges at this pH, far from their pI, could be prone to hamper ovotransferrin molecules/particles association until more advanced denaturation (or aggregation) was achieved at slightly higher temperatures. Similar results were observed by Watanabe et al. (2002) with dry-heated ovalbumin inhibiting ovotransferrin heat aggregation and coagulation.

Besides, the PPI-EW mixtures at the 75/25 weight ratio at pH 7.5 and 9 didn't show any sharp G' rise in this temperature range meaning that no early sol-gel transition can be associated with ovotransferrin in this mixture. It could be hypothesized that even if thermal denaturation of ovotransferrin occurred around 60 °C, the resulting unfolded/aggregated proteins were not numerous enough to interact and form a three-dimensional network, and/or their association was sterically hindered by the presence of the pea globulins in the mixture.

The second transition temperature was observed in all cases except for the pure PPI sample at pH 9 that didn't reach gelation during the temperature sweep. In this last case, the formation of a three-dimensional network of unfolded/aggregated pea globulins was hindered by high repulsive forces within protein particles because the negative charges dominated at this pH far from the pI of globulins (pI = 4.5-4.8). For all other mixtures, the second sol-gel transition around 75°C was associated with ovalbumin and 7S globulins which denatured in the same range of temperature, as previously evidenced by DSC analysis in Section 5.3.3. No sol-gel transition was specifically associated with legumin which presented a maximum denaturation temperature of around 85 °C as observed in Section 5.3.3. This range of temperature rather corresponded to the slowing threshold of G' towards maximum stable values.

Table 5-6: Final G' and G'' values at 95 °C of PPI, EW, and PPI-EW mixtures at different weight ratios at pH 7.5 and 9.

Samples	Heating final G' / Pa		Heating final G'' / Pa	
	pH 7.5	pH 9	pH 7.5	pH 9
EW 100%	4865±156a	5496±131a	425±21a	369±6a
PPI-EW 25/75	2552±149b	2077±117b	210±17b	167±8b
PPI-EW 50/50	1189±100c	953±65c	137±26bc	83±1c
PPI-EW 75/25	742±181c	106±6d	94±29c	17±1d
PPI 100%	30±26d	2.2±0.4d no gel	2±1d	1±1d no gel

All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different.

In order to consider, the overall contribution of all proteins in the systems upon the thermal treatment, we measured the final values of G' and G'' at the end of heating (Table 5-6). In a first approach, these values could be representative of the level of association of the proteins undergoing denaturation up to 95 °C. Moreover, it could indicate that only the contribution of hydrophobic interactions and covalent SS bond formation can be considered in thermal protein aggregation upon temperature sweep, as electrostatic interactions such as hydrogen bonds were considerably weakened in such a temperature range. The work of Wang, Luo, Zhong, Cai, Jiang, & Zheng (2017) on wheat gluten gel formation and Chronakis (2001) on the formation of Spirulina protein thermal gels indeed highlighted that disulfide bonds and hydrophobic interactions were dominated during the heating progress while hydrogen bonds and electrostatic interactions did not significantly contribute to gel formation but may reinforce the network rigidity of the protein on cooling.

Except for the pure PPI sample at pH 9 that didn't gel, all the protein systems presented a final G' >> G'' by a factor of about 10. The G' and G'' values decreased significantly and gradually when the proportion of EW decreased in the protein suspensions. Considering that for the pure PPI sample at pH 7.5, the viscoelastic parameters were very low, and no gel was formed at pH 9, it could be deduced that the

sol-gel transition upon heating in the EW-containing systems mostly reflects the contribution of EW proteins. Native EW proteins were able to gel at elevated pH as reported in other studies (Handa, Takahashi, Kuroda, & Froning, 1998; Croguennec, Nau, & Brule, 2002). The decrease in G' values when adding PPI was explained by a lower concentration of EW in the system and a possible steric hindering caused by pea globulin unfolded/aggregated molecules formed all along the temperature sweep. As already indicated, the repulsive force between pea proteins at the pHs used was not favorable for the self-association of globulin molecules or aggregation through hydrophobic interactions, neither in the pure PPI sample nor in PPI-EW mixtures. Regarding the pH effect, the G' and G'' values were significantly higher and lower at pH 9 (vs pH 7.5) for pure EW samples and PPI-EW mixtures, respectively (Tables 5-6). It has been already reported that EW gels present higher gel strength when prepared at a pH of around 9 compared to lower pH (Handa et al., 1998; Croguennec et al., 2002). The inverted behaviour between pH 9 and pH 7,5 observed in the case of PPI-EW mixtures confirmed the hindering effect of highly repulsive pea protein particles on the EW protein association. This effect was found favorable to delay the sol-gel transition of ovotransferrin at pH 9 as already supposed when considering the first gel point in the system. This result could be considered positive in order to apply higher pasteurization temperature at elevated pH during ingredient processing.

5.4 Conclusion

The thermal behaviour of PPI-EW mixtures at pH 7.5 and 9 was evaluated by determining the thermal denaturation parameters by DSC and the sol-gel transitions by dynamic rheology upon heating. The thermal parameters (T_d values of each protein and total ΔH) of the protein systems were found slightly or not different compared to those measured for the pure proteins solution of EW and PPI. The slight differences observed for ΔH could be explained by a limited loss (<10 %) of protein solubility in the mixtures. However, the slight shifts of T_d value observed for some proteins in the mixed system

could be explained by interactions between some proteins (main lysozyme with ovotransferrin and/or legumin) acting positively or negatively on the thermal stability of the proteins depending on the pH. The pH played indeed a significant role as the result of protein unfolding at pH far from the pI of pea proteins (pH 7.5 and pH 9) could affect structural modification upon heating. The repulsive forces between proteins created at elevated pH could also hinder the self-association of proteins in the system as observed from the thermal sol-gel transition data. The heat-induced gelation behaviour of the PPI-EW mixtures seemed governed by the EW proteins in the systems that were partially hindered by the presence of pea proteins that underwent denaturation but insufficient self-association to contribute to the gel network during heating. In this case, the early sol-gel transition of ovotransferrin around 60 °C was slightly delayed by ~3 °C but only at pH 9, which is the natural pH of egg white. So, the maintenance of pH 9 should be considered to optimize the heat treatment of the PPI-EW mixtures in the production of mixed ingredients. Further investigations into the thermal coagulation properties of these protein mixtures are also expected for adequate applications in food.

Chapter 6 Nature of protein-protein interactions during gelation of mixtures between pea protein isolates and egg white proteins

6.1 Introduction

To match the increasing protein demand, it is now a need to expand the plant-rich protein products. Meanwhile, associations between plant and animal proteins have become increasingly attractive in the formulation of high-protein food products in terms of nutritional, economic advantages, functional and organoleptic properties (Chili, Sok, & Saurel, 2018; McCann, Guyon, Fischer & Day, 2018). Egg proteins have been widely used in the food industry owing to their capacity to form gels with favorable nutritional and texture properties (Mine, 1995; Valverde et al., 2016; Li, Zhang et al., 2018). It is rich in ovalbumin (about 54%), ovotransferrin (about 12%), ovomucoid (about 11%), and lysozyme (about 3.4%) (Mine, 2007; Guha, Majumder, & Mine, 2019). Its gelation is a complex process involving protein denaturation, aggregation, and formation of a gel network (Mine, 1995). The gel characteristics of egg white mainly depend on the medium conditions such as pH, ionic strength, and type of salts (Croguennec, Nau, & Brulé, 2002; Nasabi, Labbafi, Mousavi, & Madadlou, 2017). Gelation of egg proteins is a two-step process: the first includes changes in protein structure or partial denaturation; the second involves additional aggregations of denatured proteins, which results in an exponential increase in viscosity and the creation of a continuous network (Alleoni, 2006). During gel formation, non-covalent bonds (i.e., hydrophobic interaction during heating and hydrogen/ionic bonds during cooling) and covalent bonds (disulfide bonds) develop the ordinates aggregation of unfolded chains of polypeptides (Campbell et al., 2003; Razi et al., 2022). In previous studies, Raikos, Campbell, and Euston (2007) reported that increasing pH and the addition of NaCl will result in elevated gelation temperatures of egg white proteins.

As an alternative to animal proteins, pulse proteins such as yellow pea (Pisum sativum L.) proteins are gaining attention due to their cheaper price, allergen-free, gluten-free chain (Aluko, Mofolasayo, & Watts, 2009; Havemeier, Erickson, & Slavin, 2017; Alves & Tavares, 2019; Burger & Zhang, 2019). Pea seeds contain four main protein fractions. The major fractions are globulins (55-65 % of total proteins), which are soluble in saline solutions; albumins (18-25 %) which are soluble in water;

prolamins (4-5 %) which are soluble in hydroalcoholic solutions, and glutelins (3–4 %) which are soluble in highly alkaline solutions (Lu et al., 2019). Pea globulins are oligomeric storage proteins, which are composed of legumin (11S) with a hexameric structure of 360 - 400 kDa. It contains 6 monomers (~60 kDa), linked by non-covalent interactions. Each monomer consists of an acidic polypeptide subunit of ~40 kDa and a basic subunit of ~20 kDa, connected by a disulfide bond (Barać et al., 2010; Shand, Ya, Pietrasik, & Wanasundara, 2007). Vicilin (7S) is a trimeric glycosylated protein of molecular weight of 150 - 200 kDa, distinguished by the absence of cysteine, which prevents it from participating in intramolecular or intermolecular disulfide bond formation (Shewry, Napier, & Tatham, 1995). Each monomer ~50 kDa has two cleavage sites possibly generating small fragments during pea seed development: α (~20 kDa), β (~13 kDa), γ (~12-16 kDa), αβ and βγ polypeptides (Liang & Tang, 2013; Shand et al., 2007; Tzitzikas et al., 2006). A third minor 7S globulin, convicilin, is a multimeric protein of 210-290 kDa which is formed by weak interactions association of monomers (~71 kDa). This non-glycosylated protein has a nearly identical amino acid profile (80 %) to vicilin. During the heating process, proteins undergo unfolding and aggregation until self-supporting networks are formed. Multiple types of molecular interactions, such as hydrogen bonds, dipole−dipole interactions, and hydrophobic and electrostatic interactions are involved during thermal aggregation and gelation (Sun & Arntfield, 2012a; Shand, Ya, Pietrasik, & Wanasundara, 2007). The contribution of disulfide bonds in these heat-induced phenomena seems limited (O'kane et al, 2004 a & c; O'kane et al., 2005; Sun & Arntfield,2012a; Mession et al., 2015).

Gelation property is one of the important functional properties of proteins, which provide unique texture, senses, and flavor for food products (Zhang et al., 2019; Harfmann, 2016). The cross-linking of polypeptide chains to form a three-dimensional network is known as protein gelation (Sun & Arntfield, 2012a). Protein cross-linking is induced by several molecular forces, which may include hydrogen bonds, ionic attractions, disulfide bonds, hydrophobic associations, or a mix of the aforementioned (Otte, Schumacher, Ipsen, Ju, & Qvist, 1999; Sun & Arntfield, 2012a). Some reagents

have been used to investigate the potential methods and molecular forces responsible for the binding reactions (Table 6-1).

To eliminate the primary molecular forces in protein gels, bond-disrupting chemicals can be utilized. They destabilized the protein's original structure and caused irreversible molecular rearrangements, resulting in variations in protein gel characteristics (Yu, Xu, Jiang & Xia, 2017; Wang & Arntfield, 2016). The molecular forces involved in the gel network are determined by the protein nature and the protein structure which can be altered by the protein extraction methods used (Sun & Arntfield, 2012a; Shimada & Matsushita,1980; Utsumi & Kinsella, 1985).

Urea and guanidine hydrochloride (GuHCl) may disrupt protein hydrogen bonds and hydrophobic interactions. Urea denatures a protein molecule by preferential adsorption with charged protein solutes, dehydrating the molecules and inducing protein repulsion, therefore stabilizing the unfolded state (Wallqvist, Covell, & Thirumalai, 1998). Shan et al. (2014) showed that urea inhibited network formation in soy protein gel and significantly reduced gel stiffness by breaking down hydrogen bonds and hydrophobic interactions.

Dithiothreitol (DTT) reacts with sulfhydryl groups to form a stable alkyl derivative, preventing the formation of disulfide bonds between protein molecules. Several studies have illustrated that sulfhydryl/disulfide interchange is involved in soy protein gelation based on the reaction of the gel (a loss of the gel integrity) (McKlem, 2002; Utsumi & Kinsella, 1985; Wolf, 1993). In terms of electrostatic forces, several studies have shown that it can be involved in gel formation by the effect of pH and salts (Sun & Arntfield, 2012a; O'Riordan, Kinsella, Mulvihill, & Morrissey, 1988; O'Riordan, Mulvihill, Kinsella, & Morrissey, 1988).

Table 6-1 Effect of various reagents on molecular forces existing in protein.

	Non-covalent bonds			Covalent bond	References
	Ionic effect/ Electrostatic interaction	Hydrophobic interaction	Hydrogen bond	Disulfide bond	
Dithiothreitol (DTT)				Disrupt	**Rüegg & Rudinger (1977), Léger & Arntfield (1993), Sun & Arntfield (2012a),**
Guanidine-HCl (GuHCl)	disrupt	weaken	Disrupt		**Tanford (1968), Léger & Arntfield (1993), Sun & Arntfield (2012a)**
Propylene glycol (PG)	Promote	Disrupt	Promote		**Tanford (1962), Ustunol et al. (1992), Utsumi & Kinsella (1985)**
Urea		Disrupt	Disrupt		**Gordon & Jencks (1963), Uruakpa & Arntfield (2006b), Ustunol et al. (1992)**

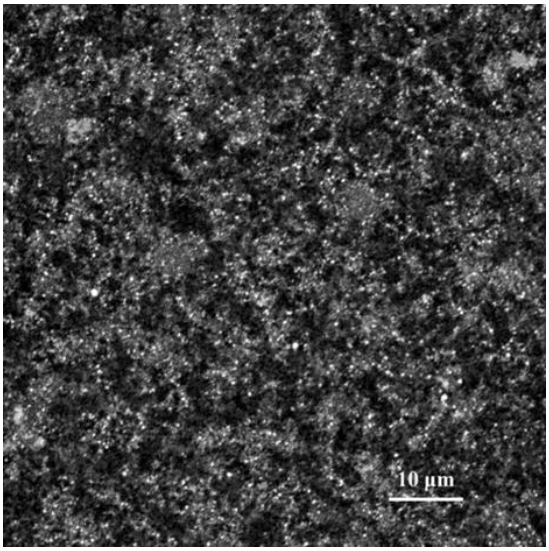
However, fewer studies have been reported about the heat-induced gels formed by egg proteins and plant proteins, such as egg white with soy proteins (Su et al., 2015); whole egg or egg yolk proteins with soy proteins (Zhang et al., 2019); egg white with hempseed proteins (Alves, Emam-Djomeh, & Chen, 2020). For pea protein isolate, gelation of PPI and animal proteins concerned mainly milk proteins, and most of them were about acid gels (Mession, Roustel, & Saurel, 2017b; Ben-harb et al., 2018; Chihi, Sok, & Saurel 2018; Oliveira et al., 2022) and rarely heat-induced gels (Wong, Vasanthan, & Ozimek, 2013; Silva, Balakrishnan, Schmitt, Chassenieux, & Nicolai, 2018). To our knowledge, there is no study regarding the heat-induced gelation of egg white and pea protein mixtures. In previous work, the storage modulus (G') of the mixed protein systems during heating was found to decrease with the EW content, and

interactions between proteins changed the thermal denaturation temperature of ovotransferrin, lysozyme, and pea legumin (Chapter 5, Section 5.3.3). Meanwhile, more experimental data are needed to better understand the importance of various forces in network formation. Thus, the work aimed to prepare and investigate the gelling properties, texture properties, and microstructure of composite gels based on the liquid egg white (EW) and pea protein isolate (PPI) by considering various weight ratios (PPI/EW 100/0, 25/75, 50/50, 75/25, 0/100) at pH 7.5 and 9, respectively. Meanwhile, the characterization of intermolecular interactions during gel formation and rheological properties was also investigated to identify gelling behavior of composite protein systems.

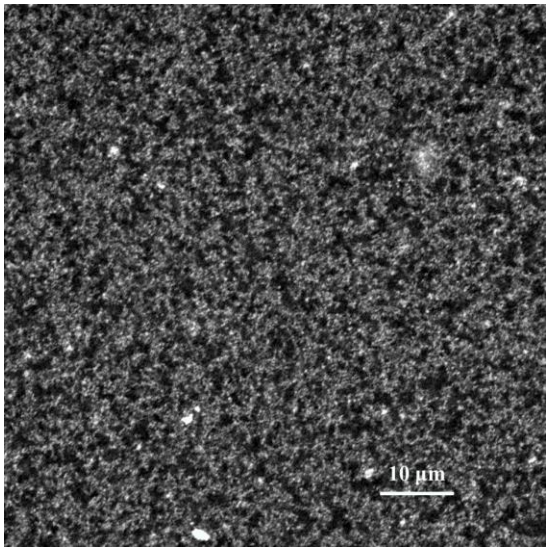
6.2 Results and discussion

6.2.1 Confocal imaging

Figure 6-1 shows the network of 10% (w/w) mixed proteins gel systems at the various PPI-EW weight ratios (0/100, 25/75, 50/50, 75/25, 100/0) and different pH. Proteins show gray and white on confocal micrographs due to Fast green labeling, whereas related pores containing aqueous phase look black. It is important to mention that both types of proteins (EW and PPI) were labeled and that one cannot distinguish between them in the images.



A



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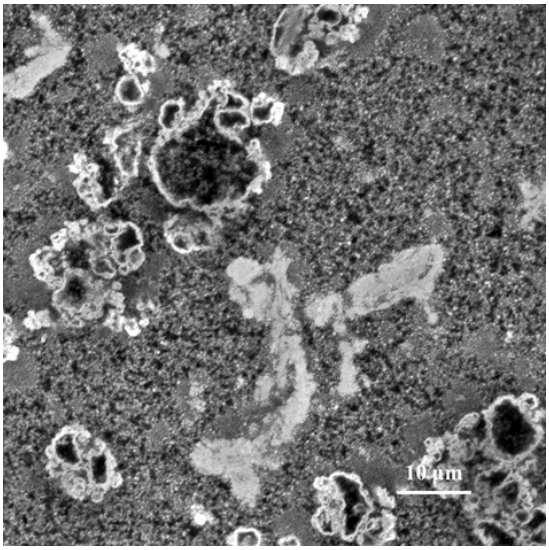
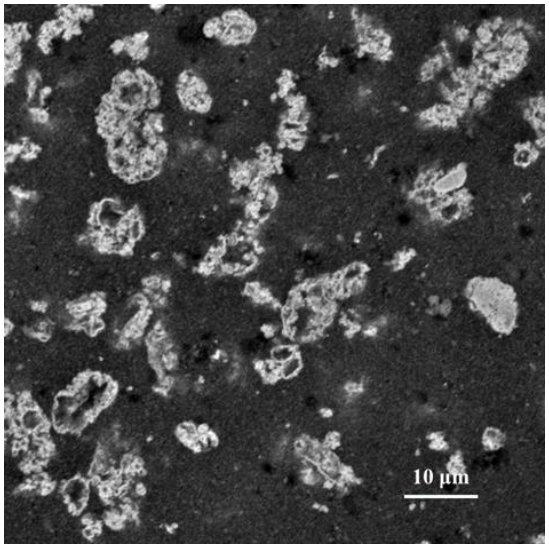


B



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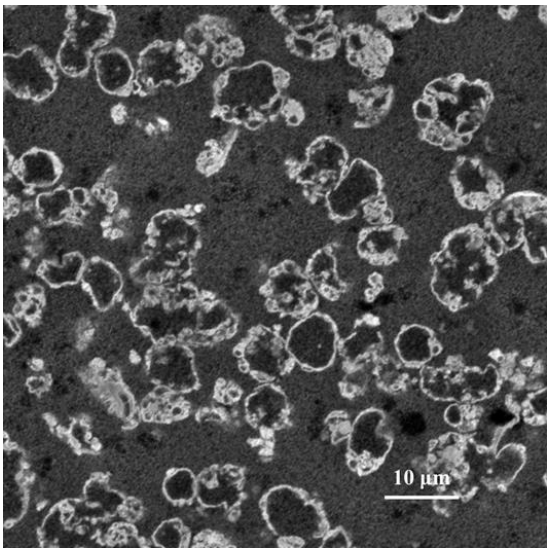
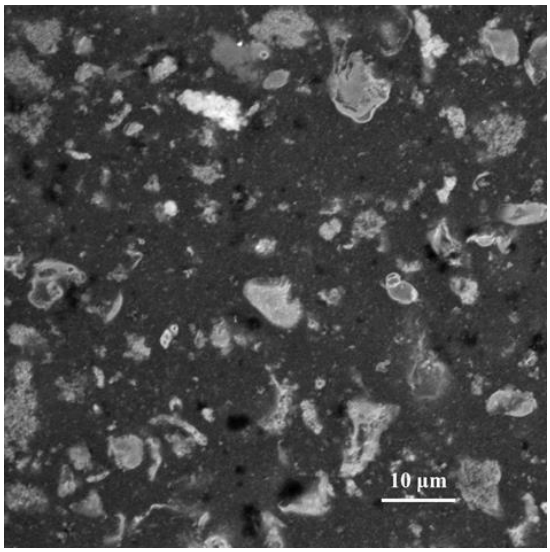
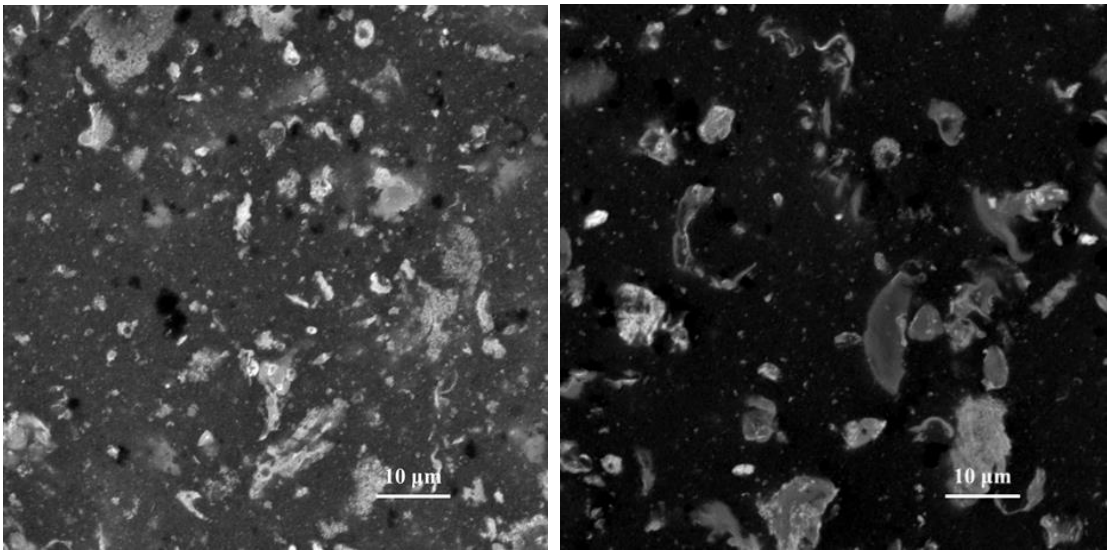


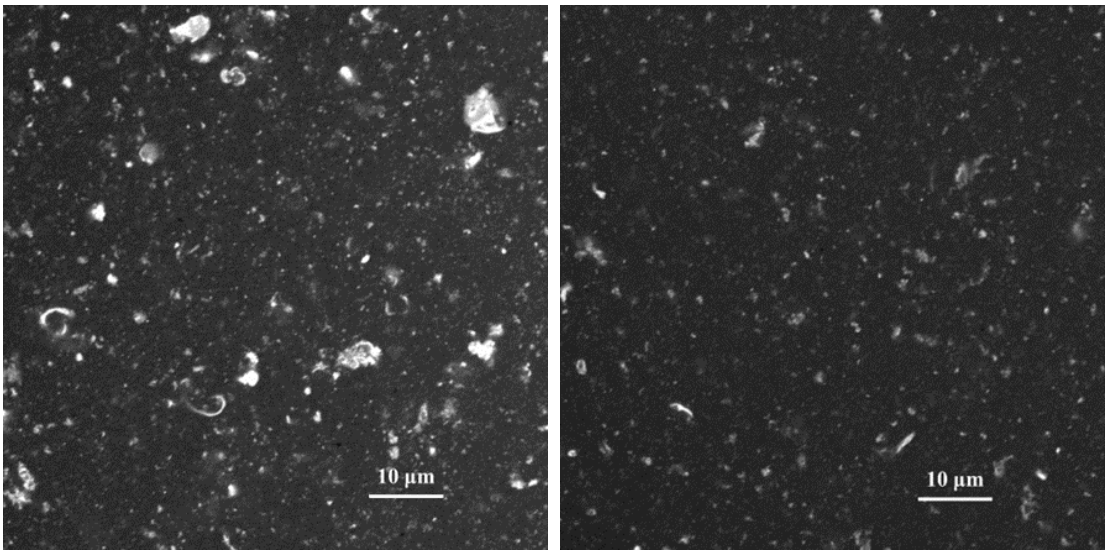
C



c



D d



E e

Figure 6-1: CLSM images visualizing the microstructure of PPI-EW protein mixtures at 63x magnification. A: EW gel at pH 7.5, a: EW gel at pH 9; B: PPI-EW 25/75 at pH 7.5, b: PPI-EW 25/75 at pH 9; C: PPI-EW 50/50 at pH 7.5, c: PPI-EW 50/50 at pH 9; D: PPI-EW 75/25 at pH 7.5, d: PPI-EW 75/25 at pH 9; E: PPI-gel at pH 7.5, e: PPI-gel at pH 9.

As shown in Figure 6-1 A and a, for the pure EW system, the microstructural organization of the gel appeared quite different at pH 7.5 and 9. At pH 7.5, the EW gel

presented a more porous, loosely packed, and heterogeneous protein network. At pH 9, the protein network became denser and more homogenous. This finding was consistent with previously published SEM and cryo-TEM data showing granular (pH 7) and smooth (pH 9) EWGs (egg white gels) microstructure (Nyemb, et al., 2016, Clark, Kavanagh, & Ross-Murphy, 2001), and other authors observed by CLSM a more homogenous structure of EW gels at pH 9 than at lower pH 5 (Somaratne et al., 2020a). The explanation for the different gel structures according to the pH was that the various egg white proteins (EWPs) present (particularly ovalbumin (OVA) and ovotransferrin (OVT)) were predicted to react differently when forming EW gels. These two proteins account for over 70% of total EWP and consequently have a large influence on the EW gelation process (Nyemb et al., 2016). At pH 7.5, OVT was more disposed to form random and spherical aggregates and dominated because it was close to its pI (6.5), whereas OVA began to form linear branching aggregates because it was far from its pI (4.5) (Nyemb et al., 2016). As a result, in this case, egg white gel was made up of a variety of aggregated structures: dispersion of OVT spherical aggregates in the protein network of OVA linear branching aggregates. Van der Plancken et al. (2006) highlighted that the net protein charge and the electrostatic repulsions were greatly enhanced at pH 9, and the activation energy barrier required to unfold the protein was lowered. In this case, the proteins tended to unfold to form a homogeneous protein network rather than spherical aggregates (Clark, Kavanagh, & Ross-Murphy, 2001).

Figures 6-1 E and e showed the microstructure of heated PPI at pH 7.5 and 9, respectively. It was found that, at pH 9, the particles and small aggregates kept moving (no gel was formed), while a more solid-like gel was found at pH 7.5, which agreed with previous results that protein-rich fraction prepared by alkaline extraction-isoelectric precipitation, shown a more heterogenous network for PPI at pH 7 compared to other pea protein fractions prepared by other methods and conditions of extraction (the former one extracted at natural pH, the latter one by only with alkaline extraction) (Kornet et al., 2021a). Meanwhile, larger particles were seen at pH 7.5 (Figure 6-1 E) and a loose network of small particles was apparent compared to the pH 9 condition

(Figure 6-1 e), where predominantly smaller individual aggregates were observed. The higher repulsive force between protein particles at high pH (far from the pI of pea globulins (~4.5) could explain the formation of smaller aggregates with insufficient interconnections to form a solid network.

Focused on the pictures from Figure 6-1 B to D and b to d at pH 7.5 and pH 9, respectively, different structures were shown. For the composite gel at pH 7.5 (Figure 6-1 B), it was found that aggregates of irregular shapes (> 10 μm) were formed, surrounded by a white homogeneous protein network when PPI took the proportion of 25 % in PPI-EW mixtures. We hypothesized that this homogenous network could be attributed to egg protein as egg white is predominant in the mixture, surrounding PPI aggregates. When PPI occupied half of the mixtures (Figure 6-1 C), more aggregates with black holes were formed and the area of surrounding egg-white colloids decreased. With more PPI concentration in PPI-EW mixtures, the gel structure seemed to be more heterogeneous and formed more random clusters of smaller sizes. In contrast, PPI-EW gel at pH 9 (Figure 6-1 b-d) showed some differences. When egg white was the dominant part, the gel looked brain-sharp surrounded by continuous egg white gel. When PPI took 50 % of the mixtures, the gel contained more clusters with smaller sizes. When PPI was the dominant part, the random clusters formed spread farther and farther in the gel structure. Similar observations regarding mixed gels were found in previous literature. Kornet et al. (2021a) found that whey protein-PPIc gel formed large clusters at high pea protein concentrations. Silva, Cochereau, Schmitt, Chassenieux, & Nicolai, (2019) showed that mixtures of micellar caseins and PPI at pH 5.8 formed gels with protein clusters, whereas more homogeneous gels were obtained for individual proteins. McCann et al. (2018) and Roesch & Corredig (2005) observed a discontinuous network in soy protein-whey protein gel at a total protein concentration of around 6 %, indicating phase separation, while Gómez-Mascaraque, & Pinho (2021) found microgel structure between soy and whey protein gel.

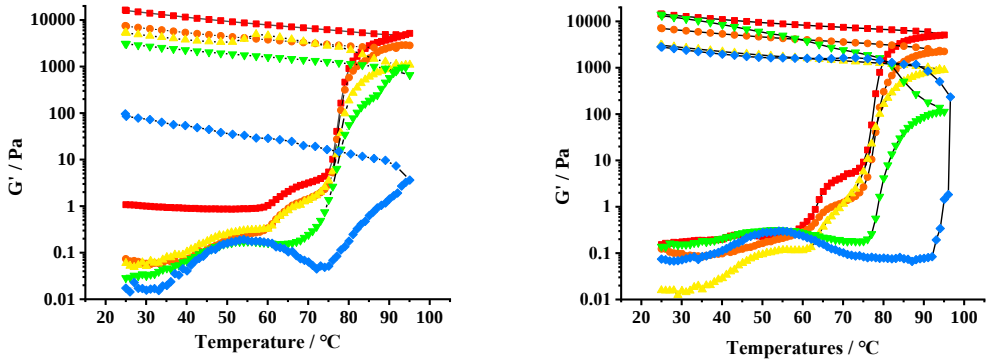
It was worth mentioning that, as can be seen from the structure of the abovementioned mixed gels, the network structure of these gels was not as tight as that

of egg white with the formation of large clustered aggregates which also did not exist for the pure PPI systems. In the mixtures, it is suggested that the egg white proteins could constitute the basic architecture of the protein network, and that gelation is accompanied by the formation of protein aggregates which may be pure PPI aggregates or mixed aggregates consisting of pea globulins and some egg white proteins. Particularly, LYS can form complexes with pea proteins as shown in chapter 4, section 4.3.1. The total or partial phase separation between EW and pea proteins could be caused by depletion or thermodynamic incompatibility phenomena (Tolstoguzov, 1995 & 2003; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Even if thermodynamic incompatibility is widely described between food proteins and polysaccharides, these phase separation phenomena could exist between proteins of different natures with a favorable effect of denaturation (Polyakov, Grinberg, & Tolstoguzov, 1997). In our systems, these phenomena would be undoubtedly amplified by the lower temperature coagulation of the OVT. Indeed, we have shown previously (Chapter 5, Section 5.3.3) that the gel point appeared at a temperature < 59 °C in egg-based systems and this early coagulation was assigned to OVT. The primary gel network that is thus formed would be susceptible to exclude the other protein particles in formation during heating, constituted mainly of the nascent pea protein aggregates that would reassemble into large clusters. This was confirmed by the following section 6.2.2, G' decreased with the decreasing proportion of EW in the mixtures, these aggregates would rather have a passive role in the structure of the gel and the properties of the gel would be dominated by the network created by the egg white proteins, OVT firstly and OVA later, even at high proportions of PPI in the mixture. The differences in gel structure noted at pH 9 would be due to greater difficulty for pea proteins to associate due to the repulsive forces between protein particles at this pH. Indeed, smaller aggregates would be formed in this case, and fewer interconnections in the protein network would be possible.

6.2.2 Viscoelastic properties during thermal gelation

Typical storage modulus (G') vs. temperature curves of PPI, EW, and PPI-EW

mixtures at pH 7.5 and 9 are shown in Figure 6-2. The loss modulus (G'') vs. temperature curves of PPI, EW, and PPI-EW mixtures at pH 7.5 and 9 are shown in Figure Annex-3.



(A) (B)

Figure 6-2: Temperature sweep G' of egg white protein (red), PPI (blue), and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at different pH 7.5 (a) and 9 (b).

The values of G' of EW, PPI, and PPI-EW mixtures at pH 7.5 and 9 during temperature sweep at the end of each ramp (heating and cooling) were reported in Table 6-2. The gelling temperatures for the different protein systems were already commented in the previous paper (Chapter 5, Section 5.3.4), where two thermal sol-gel transitions were observed at around 60 °C and 75 °C in EW-containing systems coinciding with T_d of ovotransferrin and ovalbumin/S-ovalbumin/7S globulins, respectively. Especially, the first transition associated with ovotransferrin shifted by about + 2-3 °C at pH 9.

According to our previous study on the thermal properties of EW/PPI mixtures (Chapter 5, Section 5.3.3), the maximum denaturation temperatures of PPI-EW were around 83-87°C, which assumed that complete denaturation of proteins by thermal treatment was achieved over 87 °C. Although there was no constant temperature stage in this test, compared to Zhang et al. (2019) where the time sweep for hen egg proteins-soybean mixtures was maintained at 85 °C for 10 min, in this test, the temperature sweep was increased until 95 °C, where we assumed that resulting molecular

interactions between proteins were significantly promoted. The formation of a three-dimensional network is indeed caused by various molecular forces, including hydrogen bonding, ionic attractions, disulfide bonding, hydrophobic interactions, or a combination of these forces (Sun & Arntfield, 2012a; Zhao, Sun, Li, Liu, & Kong, 2017).

All systems formed gels except PPI at pH 9. In this case, heat treatment led to the aggregation of pea proteins giving just a heterogeneous coagulum that was not forming a solid gel. This can be explained by the repulsive force existing between pea proteins at this pH far from their pI impeding protein particles to form a regular three-dimensional network. At pH 7.5, a very weak PPI gel was formed, especially during the cooling phase (G' < 100 Pa). There is indeed less repulsive force at this pH; however, given the low storage modulus, the number of connections between protein aggregates and the inter-particular forces was limited. Sun & Arntfield (2010) studied the minimum gelling concentration (MGC) of heat-induced gel formed by salt-extracted pea protein isolate at pH 5.65 was around 5.5 % (w/v), while commercial pea protein isolate (PPIc) was around 14.5 % (w/v). O'Kane et al. (2005) reported the MGC of the heat-induced gel formed by pea protein isolate near neutral pH (pH 7.1) extracted by isoelectric-precipitation (IEP) was around 16 % (w/v), due to high denaturation of pea proteins by the IEP methods. Adebiyi & Aluko (2011) illustrated that the pea proteins prepared by alkaline extraction and isoelectric precipitation (AE-IEP) could form a thermal gel at an MGC of 20% (w/v), while alcohol-soluble (AS) fraction at around 10% (w/v), but no gel was formed from water-soluble (WS) and salt-soluble (SS) fractions. It can be concluded that extraction methods, pH, and ionic strength have an impact on the gelation properties of pea protein isolate.

Finally, G' of all gels further increased during the subsequent cooling process. The appearance of growth was called gel reinforcement, a typical characteristic of protein gels, which was generally attributed to the formation of many non-covalent interactions such as hydrogen bonds (O'Kane et al., 2004a; Renkema & van Vliet, 2002) and ionic interactions between proteins (Clark & Lee-tuffnell, 1986). It is noteworthy that the G′

of the mixture and EW samples increased around 3-5 times during cooling than during the heating.

The EW samples presented the higher G' values after the heating and cooling stages (Table 6-2). These values decreased by increasing the proportion of PPI in the mixture confirming that PPI is less susceptible or unable to participate in the gel network in these conditions. According to the previous gelling point data (Chapter 5, Section 5.3.4), the first gelling point around 59 °C corresponded to ovotransferrin present in EW. This early gelation stage could strengthen the system, then the gel was reinforced by later ovalbumin coagulation; probably in mixed systems, the PPI did not contribute significantly to the gelled network.

It is noteworthy that up to a 50/50 ratio the EW-containing systems presented the same final G' values at both pHs (values not significantly different) at the end of treatment indicating probably that EW proteins governed the gelation of these systems. The PPI-EW at a ratio of 75/25 wasn't taken into this conclusion, as the standard deviation was too high for this ratio at pH 9.

However, for pure pea protein isolate at pH 7.5, the storage modulus is lower than 100 Pa after temperature sweep as already mentioned in Kornet et al. (2021b) who worked at 10 % of protein content and pH 7. So, it could be concluded that pea protein isolate formed a very weak gel. Table 6-2 showed the same tendency of G' after heating and cooling at pH 9 compared with that at pH 7.5 except for the EWP-PPI mixture at the 25/75 weight ratio. Surprisingly, the G' of this mixture after cooling is higher (not significantly) than that of other mixtures containing less pea protein at pH 9. We noticed that the standard error of the pure PPI and this mixture was large, which could indicate the gelation phenomena of the samples were not repeatable. This effect could be explained by the formation of large protein aggregates that increased the viscosity of the system but without forming a solid gel as was presented before by studying the gel microstructure of the systems at the same weight ratio (Figure 6-1F).

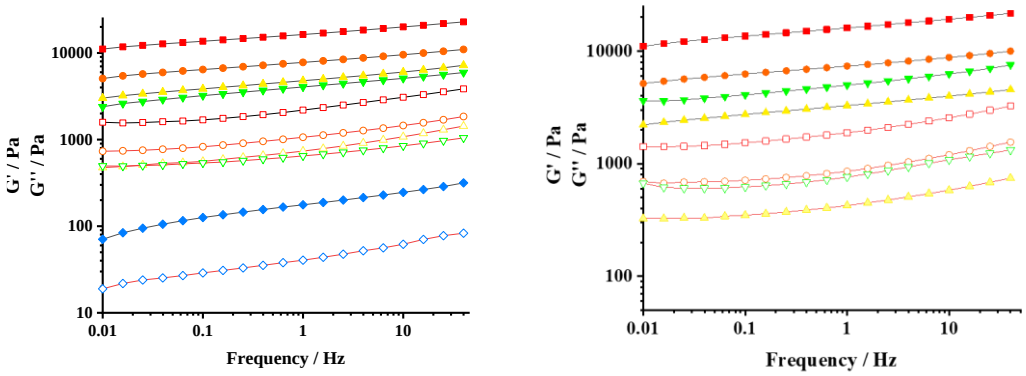
Table 6-2: Rheological analysis comparing the storage modulus (G') values for pea protein isolate (PPI), egg white protein (EW), and their composite gels at 95 °C (end of heating cycle), and 25 °C (end of cooling cycle).

samples	pH 7.5		pH 9	
	G' (Pa) at 95 °C after heating	G' (Pa) at 25 °C after cooling	G' (Pa) at 95 °C after heating	G' (Pa) at 25 °C after cooling
EW 100%	4865 ± 156aA	15115 ± 632a*	5496 ± 131aB	14446 ± 413a*
PPI-EW 25/75	2552 ± 149bA	7284 ± 192b*	2077 ± 117bA	7204 ± 281ab*
PPI-EW 50/50	1189 ± 100cA	4725 ± 324c*	953 ± 65cA	4182 ± 440b*
PPI-EW 75/25	742 ± 181cA	3446 ± 331c*	106 ± 6dB	9237 ± 3249ab*
PPI 100%	30 ± 26 d	97 ± 4 d	no gel	no gel

All data were given as mean ± SD of at least triplicate measurements. Means in a column bearing the same letter are not significantly different. Means at different numbers * and different uppercase letters in a row show a significant difference.

6.2.3 Frequency sweep test

The frequency sweep tests for PPI, EW, and PPI-EW mixtures at different weight ratios at pH 7.5 and 9 are shown in Figures 6-3 A and B, respectively. According to these data, G' and G'' were frequency-dependent and both increased with increasing frequency. In addition, the amount of G' was higher than G'' for all the applied frequencies, revealing a gel-like network structure of the EW-PPI mixtures. Similar findings have previously been reported for soy protein-egg white protein mixtures (Su et al., 2015), egg white-oat globulins (Ma, Yiu, & Harwalkar, 1990), and whey protein-pectin (Raei et al., 2018) mixtures.



(A) (B)

Figure 6-3: Storage modulus (G') (solid one) and loss modulus (G'') (hollow one) from frequency sweep test of the egg white protein (red), PPI (blue), and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at different pH 7.5 (A) and 9 (B).

Meanwhile, the loss factor of the PPI-EW mixture (calculated at 1Hz, 1 % strain) under different weight ratios and pH, and the respective frequency slopes of G' of the PPI-EW mixture are shown in Table 6-3.

Tan (δ) < 0.1 revealed the formation of a strong gel (Clark & Ross-Murphy, 1987). The loss factor of pure egg white is close to 0.1, which means pure egg white tended to form a strong gel. With the addition of pea protein isolate, the loss factor tended to increase significantly when the proportion of PPI exceeded 50 % which means the mixtures formed weaker gels. According to Tunick (2010), the larger the slope the weaker the gel. Hence, with the addition of PPI, the slope tended to increase but with no significant difference (except for the PPI-EW mixture at 75/25 ratio at pH 9).

Table 6-3 Loss factor of PPI-EW mixtures at different weight ratios, at 1% strain, 1Hz frequency, and frequency slope of G' of PPI-EW mixtures at different weight ratios at pH 7.5 and 9.

samples	pH 7.5		pH 9	
	Tan (δ) or Loss factor	Frequency slope of G'	Tan (δ) or Loss factor	Frequency slope of G'
EW 100%	0.135 ± 0.002aA	0.080 ± 0.001a*	0.115±0.001aB	0.076±0.001a**
PPI-EW 25/75	0.138 ± 0.001abA	0.087 ± 0.001a*	0.118±0.003abB	0.077±0.002a**
PPI-EW 50/50	0.151 ± 0.002bcA	0.092 ± 0.001a*	0.134±0.004abB	0.086±0.002a*
PPI-EW 75/25	0.157 ± 0.003cA	0.097 ± 0.003a*	0.158±0.008abA	0.124±0.011b*
PPI 100%	0.227 ± 0.005dA	0.161 ± 0.015b	0.229±0.060bA	(no gel)

All data were given as mean ± SD of at least triplicate measurements. Means in a column bearing the same letter are not significantly different. Means at different numbers of * and different uppercase letters in row show a significant difference.

6.2.4 Strain sweep

The typical strain sweep curves of G' and G'' presented in Figure 6-4, show a distinct linear and non-linear viscoelastic region (LVR) which is also the case for all the other gel samples given in Figure Annex-4 sections.

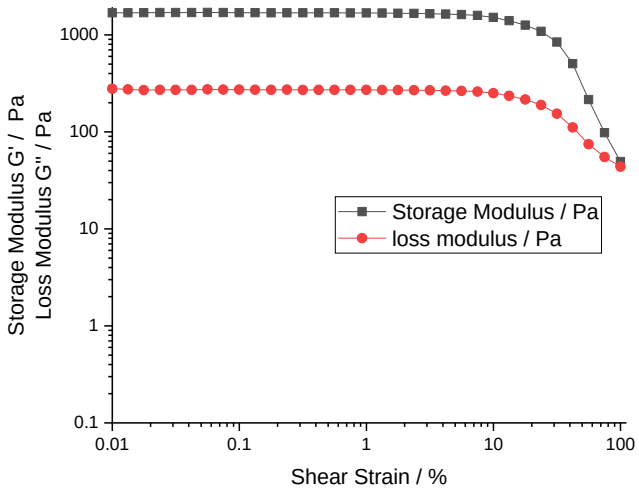


Figure 6-4: Strain sweep curves of G' and G'' on PPI-EW at a weight ratio of 50/50 at pH 7.5.

In LVR, the gels deformed elastically, with storage modulus (G') larger than loss modulus (G''), indicating the gel-like nature of samples. Beyond that region, G′ decreased due to the breakdown of the network structure. Yield points are given in table 6-4. With the addition of PPI, the LVR first increased, until pea protein isolate took the majority in the mixture (the 50/50 weight ratio presented a maximum at both pHs). Then, LVR decreased and there was no significant difference with pure PPI. It could be suggested that with the addition of PPI, pea globulins aggregates were formed and disturbed the network of egg white protein giving more weaker gel. On the opposite, at lower concentrations of PPI, egg white proteins were concentrated giving a gel with more elastic properties and higher LVR. Furthermore, the decrease of LVR when PPI exceeded 50 % in the mixture would indicate lower connections in the protein network due to decreased EW content leading to earlier network ruptures.

The region of the linear response also increased with pH values, suggesting that the protein gel network was stronger and more deformable at pH 9 (Wang & Chen, 2011). According to the previous research of Handa, Takahashi, Kuroda, & Froning (1998), it was found that the gel hardness and elasticity were stronger at pH 9 than at pH 7. The same results were found in the study of Alleoni & Antunes (2005), when the

pH ranged from 9.0 to 9.45, the hardness in egg white gels was greater than when the pH was between 7.7 and 8.1. These authors thought that it was due to the s-ovalbumin along with other proteins that can improve the hardness of albumen gels, as the increase in the proportion of s-ovalbumin in egg white during storage at pH 9 is higher than at pH 7.0. In our case, it could be likely because egg white gels formed a more compact and microstructurally homogenous gel at pH 9, which was confirmed by the CLSM results (Figure 6-1 A & a). Somaratne et al. (2020b) found that the hardness of egg white gel at pH 9 was higher than that at pH 5, due to a more homogenous network at pH 9 compared to heterogeneous proteins made of larger aggregates particles at pH 5.

Table 6-4 Yield point (%) of PPI, EW, and PPI-EW gels at different weight ratios in strain sweep at pH 7.5 and 9.

samples	yield point / %	
	pH 7.5	pH 9
EW 100%	5.5 ± 0.1a	16.6 ± 0.6a
PPI-EW 25/75	9.7 ± 0.6b	41.6 ± 5.4b
PPI-EW 50/50	11.4 ± 0.6b	52.3 ± 2.0b
PPI-EW 75/25	3.9 ± 0.2a	9.7 ± 3.8a
PPI 100%	5.6 ± 0.3a	no gel

All data were given as mean ± SD of triplicate measurements. Means in a column bearing the same letter are not significantly different.

6.2.5 Intermolecular interactions

Typical protein gel can be stabilized by both non-covalent and covalent forces. Chang & Chen (2000) illustrated that hydrophobic interactions, disulfide bonds and hydrogen bonds stabilized the heated egg protein gels. Yang, Wang, Vasanthan & Chen (2014) confirmed that disulfide bonds and hydrogen bonding contributed to the gel network formation of canola protein gels. To evaluate the role of the types of interactions in PPI-100 % gel, EW-100 % gel, and PPI-EW mixture-based gels at pH 7.5 and 9, respectively, a dissociation approach was investigated and compared with the predicted effects. The utilization of urea, propylene glycol, DTT, and Guanidine-HCl

as dissociating agents allowed us to assume interactions between proteins in various gels. Urea, DTT, propylene glycol, and Guanidine-HCl were used to disrupt hydrogen bonds, disulfide bonds, and hydrophobic interactions, respectively. The effect of different dissociating agents on the dissociation of proteins from PPI-100 %, EW-100 % gels, and PPI-EW mixtures gels, at pH 7.5 and pH 9, are reported in Figures 6-5, 6-6, 6-7, and 6-8, respectively. In general, DTT weakens the existing disulfide bonds between cysteine residues and prevents the formation of inter- and intramolecular disulfides (Léger & Arntfield, 1993; Sun & Arntfield, 2012a). According to Zou, Habermann-Rottinghaus & Murphy (1998), urea binds to amide groups via hydrogen bonds, decreasing the hydrophobic effect through dehydration of the protein molecule and indicating that both hydrophobic and hydrophilic groups are involved in urea-induced denaturation. Walstra (2003) pointed out that the denaturing effect of urea was caused by the dehydration of urea-bound peptide bonds, which also weakens hydrophobic interactions. Meanwhile, Tanford (1968) indicated that urea is a strong denaturing agent which can induce an extensively unfolded state, in which the protein molecule behaves like a random coil. GuHCl is a strong ionic denaturing agent, which weakens hydrophobic interactions and inhibits hydrogen and ionic bonds (Tanford, 1968; Sun & Arntfield, 2012a). Tanford (1968) concluded that GuHCl produced the most extensively unfolded state, in which protein molecules lack their native conformation and behave as random coils. GuHCl is a more effective denaturant than urea, unfolding proteins at two to three times lower concentrations (Greene & Pace, 1974), and it is chemically stable. Propylene glycol (PG) disrupts hydrophobic interactions but enhances hydrogen bonds and electrostatic interactions by lowering the dielectric constant of solvent and reducing the energy barrier to protein-protein interaction enough to enable structure formation (Ustunol et al., 1992; Utsumi & Kinsella, 1985).

6.2.5.1 Effect of dissociating agent on PPI-100% and EW-100% gel, at pH 7.5 or pH 9

Figures 6-5 and 6-6 show that the percentage of proteins that were solubilized by the dissociating agent varied with both the kind of gels and the pH. In PPI-100 % gel as compared with the control (21.5 ±0.6 %), both urea and guanidine-HCl led to 3 times higher solubilization of protein (Figure 6-5 A). On the contrary, in EW100 %-gel, only DTT and urea have a significant effect on total protein solubilization (2 times increase) (Figure 6-5 B) as compared with the control (3.7 ±0.1 %) or the other agents when used alone. The mixing of the 4 agents (urea + DTT + PG + Gu-HCl) showed an overwhelming increase in protein solubility for all samples, showing the synergistic effect of the four dissociation agents whatever the pH and the type of gel.

Whatever the type of dissociating agent (control including) the amount of total protein dissociated from the PPI-100 % gel is always much higher than that of the EW-100 % gel. The remaining protein in the gel (in particular for the EW sample) represented those that were still interacting despite the presence of dissociating agents. This means that, in addition to the phenomena described above, there may be other interactions (covalent bonds, ionic interactions) not affected by the dissociating agents or that the intrinsic solubility of the particles released was not complete. What should be kept in mind was that there are some limits to using solubility to identify the interaction during gelation. In the sense of the method, solubility corresponds to the dissolution of the gel under the effect of dissociating agents. While certain chemical reagents were added to the protein gel, the constitutive protein of the gel would dissolve (Jiang & Xiong, 2013). Thus, a high solubility indicates that polypeptides or small-sized protein particles have detached from the gel thanks to the breaking of certain interactions and that a part of these is non-sedimentable. The remaining protein in the gel represented those that were still interacting despite dissociating agents suggesting very strong interactions between proteins. Nevertheless, the results must be taken with caution because the chemical agents can break interactions, but the protein particles can

remain large and therefore remain insoluble due to their unfavorable surface properties (i.e., lack of hydrophilicity); moreover, new interactions created between released particles could lead to their precipitation. The efficiency of the agent in dissolving the gel is therefore relative. Subsequently, we will consider that the more the gel is dissolved in the presence of a chemical agent, the more the agent can destroy interactions releasing soluble protein particles. This represents the ability of the agent to solubilize the gel considering the interactions it can affect. Dissolution of gels in 100 mM Tris buffer (used as control) allows knowing which fraction of the protein system is dissociated in the absence of any dissociating agent. It could be hypothesized that this solubility corresponds to protein particles not bound to the gel network or that certain interactions were weakened by the buffer-releasing part of the protein material. Tris ($C_4H_{11}NO_3$) is a very polar molecule having 1 amine and 3 hydroxyl groups (a weak base); it is therefore positively charged with a pKa of 8.3 close to the 2 pHs studied. At a concentration of 100 mM, the properties of the molecule can affect hydrogen and ionic bonds, which would explain the partial protein dissociation from the gels in this buffer. The EW-100% gel was poorly dissociated in this buffer (~4 % at both pHs) and the solubility increased up to ~21 % for PPI-100 % at pH 7.5. The significant difference between the network structure of PPI-100 % and EW-100% gel at pH 7.5 resulted in the majority of the protein interactions. So, based on our results obtained at pH 7.5, we can suggest that disulfide bonds between EW-proteins are significantly involved in the EW-100 % gel (Figure 6-5B). Huang et al. (2019) investigated the intermolecular force of egg white gelation by using 30 Mm dithiothreitol, 2 M Gu-HCl, and 2 M urea. It was found that disulfide bonds involved in the production of the egg gel outnumbered the hydrophobic interaction. Jin, Chen, Zhang, & Sheng (2021) also reported that disulfide bonds play the primary role in gel formation followed by hydrophobic interactions, hydrogen bonds, and ionic bonds no matter how long the heat-induced time. Wang et al. (2020) found the same results, that the primary intermolecular forces in the development of heat-induced native egg white protein gel were disulfide bonds, with hydrophobic interactions playing a minor role. On the contrary, both hydrogen, and

hydrophobic interactions would be strongly involved in the PPI-100 % gel formation (Figure 6-5A). These results were consistent with the results of Sun & Arntfield (2012a), that mentioned hydrophobic interactions and hydrogen bonds were involved in heat-induced pea protein gelation with 0.3 M NaCl and pH 5.65, while disulfide bonds were not involved in gel formation. Tanger, Müller, Andlinger, & Kulozik (2022) found that the main protein interactions in pea protein gels were non-covalent bonds regardless of pH and ionic strength, and disulfide bonds only at a lower extent.

At pH 9, the quantity of protein dissociated from both PPI-100 % (rather a coagulum in this case) or EW-100 % gel is generally higher than at pH 7.5 (Figure 6-6 A & 6-6 B), suggesting the greatest part of i) low-energy interactions and/or ii) proteins not associated to the protein network. This hypothesis is consistent with previous microscopy results showing that aggregates of PPI-100% after the heat-induced process kept moving at pH 9. This agreed with the finding of Tanger et al. (2022), who reported that pea protein isolates with 15 % protein content formed rather an entangled solution than a continuous gel network at pH 9 and 0.9 M NaCl.

At pH 9, the solubility of protein from EW-100 % gels remained low in all cases (<= 11.5 % in the case of urea at pH 9). This means that even if some interactions are affected by the chemical agents, the gel particles released remain insufficiently soluble, which reveals a combination of strong interactions. The EW gels, therefore, remain particularly insoluble even if the agents are used simultaneously, as already noted since only 35.8 % of proteins are solubilized. These gels are especially sensitive to urea and DTT, which would indicate an important role of hydrogen bonds and disulfide bonds in the structure of the gel. The effect of DTT is present for both types of gel (PPI-100 % and EW 100 % at both pH), but even if disulfide bonds are reduced, few soluble particles are released, because other interactions structure the remaining gel particles. Finally, we observed that adding all the chemical agents at the same time does not allow to solubilize the whole gel except in the case of PPI at pH 9 (but no gel was formed in this case); the total solubility values varied from 65 % minimum to 97.5 % maximum.

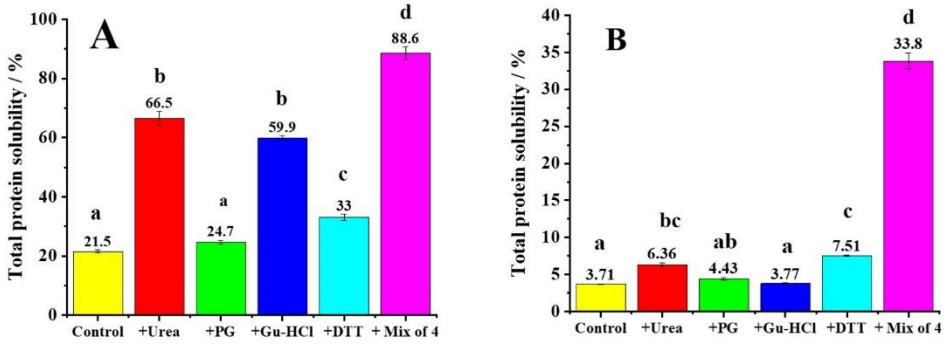


Figure 6-5: Effect of different dissociating agents on total protein solubilization from PPI-100% gel (A) or EW-100% gel (B) at pH 7.5. Control: 100 mM Tris-HCl.

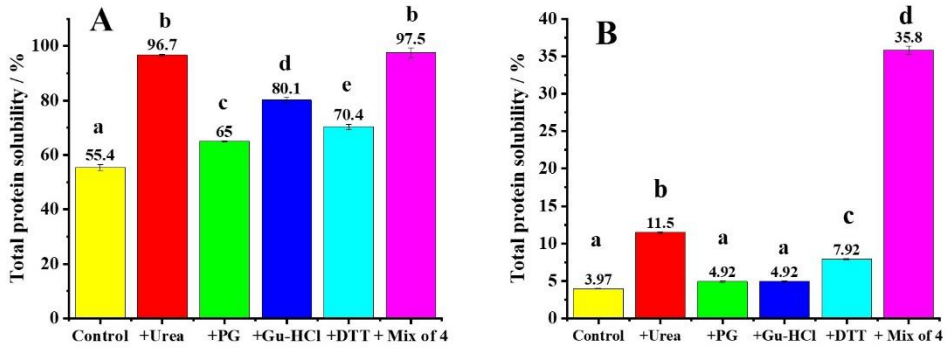


Figure 6-6: Effect of different dissociating agents on total protein solubilization from PPI-100% gel (A) or EW-100% gel (B) at pH 9. Control: 100 mM Tris-HCl.

6.2.5.2 Effect of dissociating agent on PPI-EW mixed gel, at pH 7.5 or pH 9

Figures 6-7 and 6-8 show that the protein solubility of PPI-EW mixed gel at different weight ratios at pH 7.5 and pH 9, respectively, increased in presence of dissociating agents as the proportion of PPI protein in mixed gels increased. The protein solubility in mixed gels was generally higher at pH 9 than at pH 7.5. It could be hypothesized that this solubility corresponded to protein particles not bound to the gel

network or that certain interactions were weakened by the buffer-releasing part of the protein material. Mixed gels rich in EW proteins (such as PPI-EW 50/50 and PPI-EW 25/75) remain particularly insoluble even if the agents were used simultaneously. Nevertheless, these gels were especially sensitive to urea and DTT, which would indicate an important role of hydrogen bonds, hydrophobic interactions, and disulfide bonds in the structure of the mixed gel. On the contrary, when the mixed gel was enriched in PPI proteins (PPI-EW 75/25), the agents that allow the most protein particles to be solubilized were urea (47.8 % ±0.9) and guanidine-HCl (29 % ±0.5) at pH 7.5 (Figure 6-7). This result indicates a combination of non-specific and lower energy interactions as in the case of PPI-100% gel with a dominance of hydrogen and hydrophobic bonds. The gels were not very sensitive to propylene glycol; this agent would be ineffective because its effect on hydrogen bonds could be masked by the TRIS-HCl buffer used.

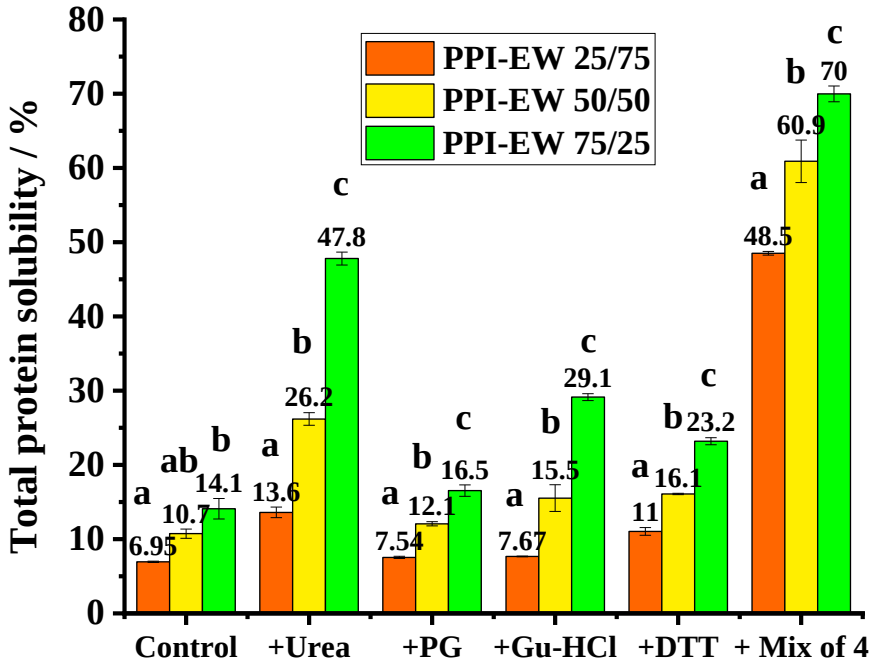


Figure 6-7: Effect of different dissociating agents on total protein solubilization from PPI-EW gel at pH 7.5. Control: 100 mM Tris-HCl.

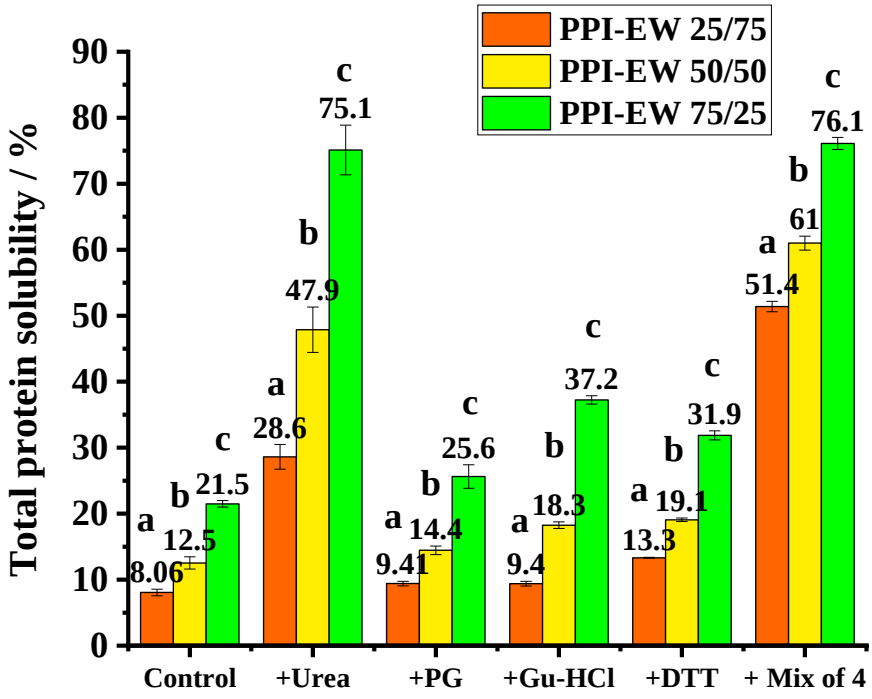


Figure 6-8: Effect of different dissociating agents on total protein solubilization from PPI-EW gel at pH 9. Control: 100 mM Tris-HCl.

Overall, an increase in protein solubility is observed when the proportion of PPI increases in the system regardless of the chemical agent. The mixed gels have intermediate behaviors with increasing solubility values between EW-100 % and PPI-100 % systems. Thus EW-based gels are not very dissociable up to the 50/50 ratio, which shows that EW proteins remained dominant in the structure of the gels; indeed, the solubility values become significantly different from the EW-100 % system from or beyond the 50/50 ratio. In all cases, the gels at pH 9 are more dissociable than at pH 7.5 because, at this higher pH, the charge of the protein particles is greater generating more repulsive forces within proteins during the gel formation.

In conclusion, we can say that EW-based gels are less dissociated by the different chemical agents, indicating that egg white proteins form gels with more numerous and stronger molecular interactions regardless of pH. This conclusion agrees with the data of dynamic rheology where the gels showed increased elastic behaviour with the

proportion of EW.

6.2.6 Textural properties

The textural properties of the gels are quite important in determining the quality of food. It can imitate the actions applied to the gels by the tongue and teeth, therefore this test was performed to compare the eating performance of the gels. Hardness and springiness were typically regarded as more relevant measures of gel performance (Li et al., 2018; Alavi, Emam-Djomeh & Chen, 2020). The changes in TPA parameters (hardness, springiness, and cohesiveness) of gel samples are presented in Table 6-5, and gel appearance of EW and PPI-EW combination gels at different weight ratios are shown in Figure 6-9. The color of the gels obtained from different PPI-EW mass ratios changed from pale yellow to light brown at pH 7.5 and dark brown at pH 9 with the increasing proportion of PPI. Obviously, the color of PPI-EW mixtures gels at pH 9 was darker than the one at pH 7.5. However, the color appearance of egg white gel between pH 7.5 and 9 was hard to distinguish. Since PPI hardly or even did not gelify at pH 9, it was hard to experiment with 100% PPI gels. Therefore, only TPA parameters of EW 100 % and PPI-EW at different weight ratios were reported in Table 6-5. At both pHs, 100 % EW-gel showed the highest gel hardness, and the hardness of gel decreased significantly with the increasing proportion of PPI content (from 0 to 75 %). This is consistent with the rheological data where elastic modulus and loss factor decreased with increased PPI content (see Section 6.2.3 & 6.2.4). A similar tendency was found in the literature on egg white-hempseed protein mixtures (Alavi, Emam-Djomeh & Chen, 2020) and egg white-soy protein composite gels at higher protein concentrations (Su et al., 2015).

Springiness is a textural quality that is connected to sample elasticity. Springiness in TPA is related to the height that the food recovers during the time that elapses during the end of the first bite and the start of the second bite (Chandra & Shamasundar, 2015). High springiness is caused by the gel structure splitting into a few big pieces during the initial TPA compression, whereas low springiness is caused by the gel breaking into

numerous tiny pieces (Lau, Tang, & Paulson, 2000). As shown in Table 6-5, springiness decreased with higher PPI concentration in composite gels meaning that compression led to more irreversible deformation.

Cohesiveness is often used as an indicator of the ability of the gels to maintain an intact network structure. Higher values of cohesiveness indicate the strength of more intact network structures (Handa, Takahashi, Kuroda & Froning, 1998; Fernandez-Lopez, et al., 2006). In the present study, cohesiveness data showed that cohesiveness had the same tendency as springiness and hardness. 100% EW gel had higher values and it decreased with increasing PPI proportion in composite gels. This result was consistent with microstructure observations in Figure 6-1 (section 6.2.1) which has been discussed in the previous section.

Table 6-5: Parameters of texture profile analysis (TPA) of EW 100 and PPI-EW mixture gels at different weight ratios at pH 7.5 and 9.

Samples	pH 7.5			pH 9		
	Hardness /N	Cohesiveness	Springiness	Hardness /N	Cohesiveness	Springiness
EW 100%	3.10±0.21a	0.73±0.02a	0.95±0.04a	3.90±0.08a	0.75±0.01a	0.93±0.02a
PPI-EW 25/75	2.78±0.17a	0.67±0.02a	0.92±0.04ab	3.45±0.16a	0.74±0.01ab	0.91±0.03ab
PPI-EW 50/50	1.69±0.03b	0.58±0.02b	0.84±0.00ab	1.84±0.08b	0.71±0.00bc	0.89±0.01ab
PPI-EW 75/25	0.79±0.00c	0.59±0.02b	0.80±0.01b	0.51±0.01c	0.67±0.01c	0.81±0.01b

Different superscripts in each column chart represent a significant difference ($p < 0.05$).

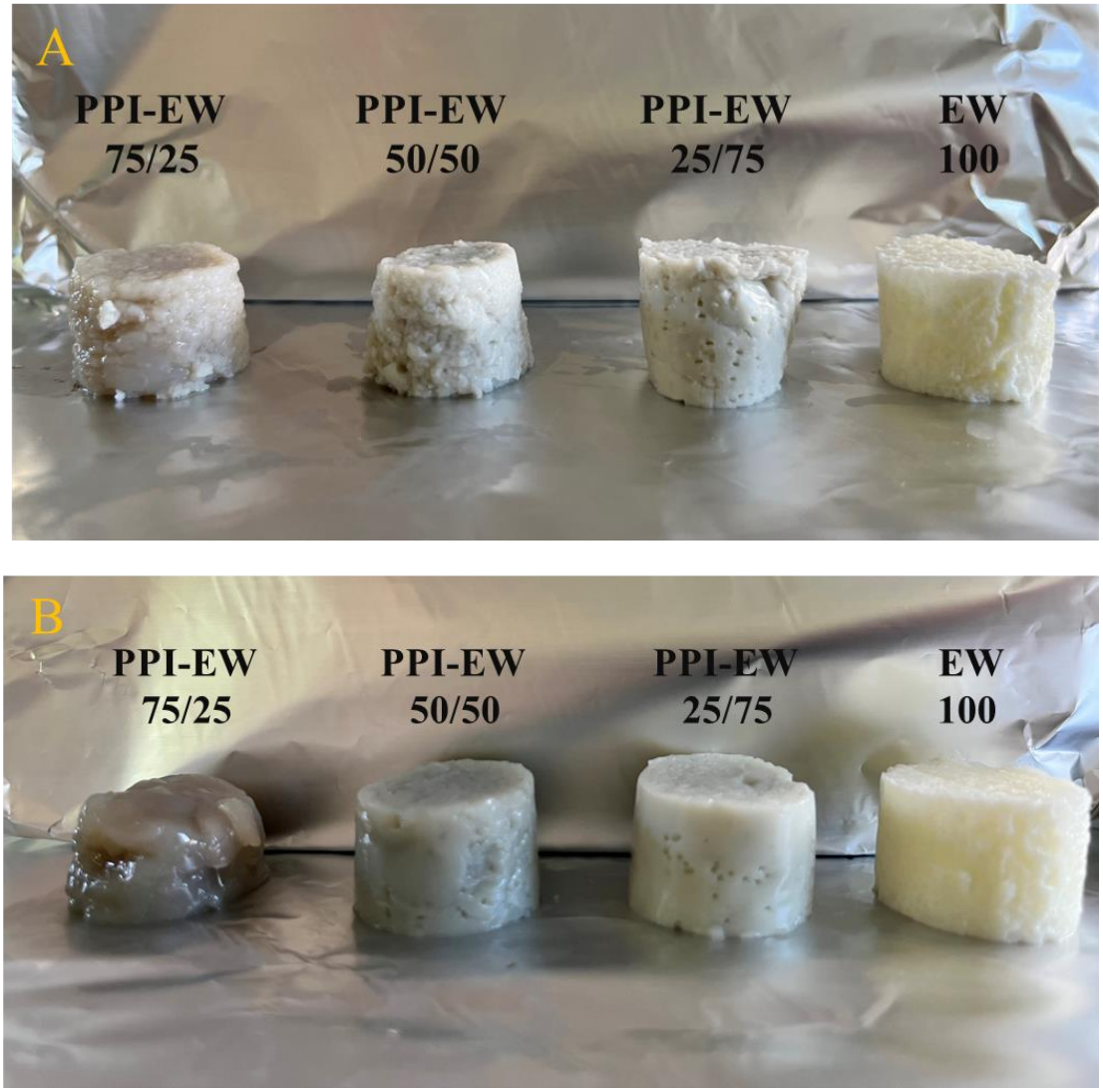


Figure 6-9: Gel appearance of EW, and PPI-EW gels at different weight ratios. A: samples prepared at pH 7.5, B: samples prepared at pH 9.

6.2.7 Mechanism of gelation

Combining the results of dissociating agent tests, texture, microscopy, and dynamic rheology data, a possible mechanism of PPI-EW gel formation can be proposed. With the temperature increasing, egg white proteins started to be partially denatured resulting in the unfolding of the proteins. Ovotransferrin, a protein sensitive to temperature, was the first to be denatured as its thermal denaturation temperature was around 61 and 63 ℃ at pH 7.5 and 9, respectively (Chapter 5), followed by lysozyme and ovalbumin. According to the research of Wang et al. (2022), ovotransferrin,

lysozyme, and ovomucin were involved in the early formation of egg white gels, moreover, in binary mixtures lysozyme co-aggregated with ovalbumin or ovotransferrin through disulfide bonds and physical interactions (Matsudomi, Takasaki, & Kobayashi, 1991; Matsudomi, Oka, & Sonoda, 2002; Iwashita, Handa, & Shiraki, 2017; Iwashita, Handa, & Shiraki, 2019). Association or molecular aggregation of denatured proteins increased with involved protein-protein interactions, such as hydrophobic interactions, hydrogen bonds, and disulfide bonds as suggested by the results from the dissociation tests. During cooling, gel strength increased due to the creation of hydrogen bonds (as G' increased in the dynamic rheology test), resulting in a continuous network. However, a denser and more homogenous network was formed at pH 9 due to higher net protein charges and the electrostatic repulsions at this pH, rather than a more porous, loosely packed, and heterogeneous protein network as obtained at pH 7.5. For heat-induced PPI gelation, as globular proteins, the proteins were firstly partially denatured leading to their unfolding; aggregates would then be formed between pea globulins, hydrogen bonds, and hydrophobic interactions taking the major role during gel formation. While at pH 7.5, a more solid-like gel was formed, at pH 9, no gel formed, possibly due to high repulsive force at higher pH leading to insufficient interconnections. For the combined PPI-EW heat-induced gel systems, during the heating process, the protein was denatured leading to their unfolding and exposed protein subunits. Protein-protein interactions were involved and led to protein aggregates, such as the aggregate between pea protein and lysozyme or the possible aggregate with ovotransferrin or ovalbumin. During the cooling step, with the increasing proportion of PPI, hydrogen bonds, and hydrophobic interactions took a major role in the gel network formation, while disulfide bonds and electrostatic interactions took a minor role. When EW prevailed in the mixtures, the network had less irregular shapes leading to higher hardness and springiness compared to lower EW proportions in mixtures. Increasing the PPI proportions, larger clustered aggregates were formed making the structure not as tight as pure egg white gel, leading to the decrease in the texture properties. The different results between the mixtures at pH 7.5 and pH 9 may be due to high repulsive forces

and negative charges at higher pH.

6.3 Conclusion

In this study, the gelling properties of egg white proteins and pea protein isolate were analyzed by dynamic rheology, texture analysis, dissociation test after gelation, and CLSM. The gels obtained from the mixtures were probably constituted of a primary network of egg white proteins, mainly associated with hydrogen and disulfide bonds, including pea protein aggregates driven by electrostatic and hydrophobic interactions. However, there was good evidence that hydrophobic interactions and hydrogen bonds took a major influence on the composite gel formation with the increasing proportion of pea protein isolate, by contrast, disulfide bonds had less effect on gel formation. This can be confirmed by the rheological data which indicated that weaker gels were formed with the increase in PPI proportion. Consistently, adding more proportion of PPI to egg white led to a decrease in the hardness, springiness, and cohesiveness of the gels. A more homogeneous network was obtained for pure egg white at pH 9, while PPI-only didn’t form a gel at this pH. For the combined gels, it showed a difference in the gel network that was more heterogeneous and formed more random clusters of smaller size at pH 7.5 when the dominant part was PPI. At pH 9, when increasing the proportion of PPI, random clusters were formed and spread farther and farther in the gel structure. This kind of gel was lack of tight connections in the gel network, resulting in a lower hardness. This study gives us the first time to understand what interaction could happen in the PPI-EW composite gels and give more understanding for further application in food design.

General conclusion and perspective

General conclusion and perspective

General conclusion

The purpose of the thesis was to partially replace egg white proteins (EW) with pea protein isolate (PPI) under different physico-chemical conditions (total protein concentration at 10% w/w, different weight ratios of PPI/EW, as well as different pHs) in order to further understand the possible interactions between both types of proteins after mixing. The impact on the functional properties of the mixture such as thermal and gelling properties was also studied.

The study was developed in two main steps. The first part was to know which kind of interactions could happen in the aqueous mixture, to give us an idea for further experiments. The second part was dedicated to the study of the thermal and gelation properties of the samples prepared by mixed systems and single proteins. The mechanical and structural properties of the different composite systems as well as the pure PPI and EW ones were studied by different biochemical and physicochemical analytical tools.

In order to carry out this project, it was essential, first of all, to obtain qualitative raw materials including pea globulins, and ovalbumin in sufficient quantity to cover our needs during this study. Two protocols were used for the extraction and purification of these proteins. Pea globulins were obtained by alkaline extraction (pH 8) followed by isoelectric precipitation (pH 4.8). The purification procedure by ultrafiltration/ diafiltration was carried out using a cassette with a cutoff of 10 kDa. Subsequently, the diafiltered protein solution was then freeze-dried to obtain a “pure” globulin powder containing 89 % protein on a dry basis (N= 5.44). Ovalbumin was extracted by adjusting egg white pH to 6 to precipitate ovomucin and adjusting pH to 8.4 with covered supernatant. After centrifugation, the supernatant was filtered and injected into anion exchange chromatography Q-Sepharose to separate the OVA from the other egg white proteins. Finally, the solutions were lyophilized, and the ovalbumin was obtained. Several physicochemical analyses on the raw materials were carried out, especially

purity, solubility, thermal parameters, and polypeptide composition. It was found that pea globulins were rich in legumin (~40 %) and vicilins and convicilins (~60 %) subunits. Egg whites were made up of ovalbumin, ovotransferrin, and lysozyme from SDS-PAGE. Extracted pea protein isolate showed higher solubility at alkaline pH over 7 (> 89 %), and acidic pH (< 3) (> 85 %) which decreased significantly around the isoelectric point region (pH 4.5-5). Meanwhile, egg white showed high solubility (>88 %) whatever the pH; just a small decrease in solubility was observed around pH 4 close to the major ovalbumin fraction's isoelectric point (~pH 4.5). The pH chosen at pH 7.5 and 9 for further experiments provided a good solubility of proteins and the solubility of the mixtures of both proteins at these pHs seemed to be slightly affected. The thermograms obtained by MicroDSC confirmed that pea globulins were low-denatured proteins by the chemical and physico-chemical treatments during the extraction phases and therefore usable for the rest of our study. Two thermal peaks at around 76, 87 °C (pH 7.5) and 71, 85 °C (pH 9) regarding vicilin and legumin were shown respectively. The thermal enthalpy of PPI was 10.8 J/g at pH 7.5 and 3.6 J/g at pH 9. Both thermal peak and enthalpy decreased at higher pH (pH 9, in our case), it could be due to the partial unfolding of proteins molecules at pH far from pI, leading to increased intramolecular net charges and repulsive forces. For egg white, it showed four thermal peaks regarding ovotransferrin (~63 °C), ovalbumin (~76 °C), lysozyme (~ 70 °C), and s-ovalbumin (~83 °C) at pH 9, while at pH 7.5, the peak of lysozyme was overlapped, maybe due to co-aggregation and heteroprotein formation between ovotransferrin and lysozyme. The enthalpy of egg white was ~ 22 and ~24 J/g at pH 7.5 and 9, respectively.

To have a better understanding of which fractions of egg white protein interact with pea protein isolate in a mixed system, the first main part of the work focused on the study of the interactions between pea protein isolate and purified egg white proteins (ovalbumin (OVA), ovotransferrin (OVT) and lysozyme (LYS)) in solution. Isothermal titration calorimetry (ITC) was first used to identify which binary solution (PPI-LYS, PPI-OVA, PPI-OVT) could have interactions. The results showed strong exothermic

interactions between PPI and LYS at both pHs, while no or very weak interactions were detected between OVT or OVA and PPI whatever the pH. Then, zeta potential was used to determine the nature of interactions in PPI-LYS systems. Electrostatic interaction was involved between lysozyme and pea protein isolate at both pHs. During ITC, we found that the titration thermograms underwent two distinct events, as the height of the exothermic peaks continuously increased with the addition of LYS in the first phase until a critical value of LYS/PPI molar ratio beyond which the trend was reversed; further addition of LYS decreased the exothermic intensity of the signal (phase 2) until saturation. As a result, we performed dynamic light scattering, laser granulometry, confocal laser scanning microscopy, and optical microscopy to identify the influence of the proportion of lysozyme in PPI-LYS systems on the particle size and structure. Particle size increased firstly with the increasing LYS concentration until the critical molar ratio of LYS/PPI around 5 (pH 7.5) and 13 (pH 9), leading to a formation of heterogeneous aggregates with irregular shapes due to strong attractive interactions between the two oppositely charged proteins. When more LYS was added, the size decreased. It could be hypothesized that mixed aggregates became more and more compact and more and more individualized from this threshold. This phenomenon was confirmed by the microstructure results. According to the above results of this part, we proposed a possible mechanism for the interaction–aggregation that occurs when LYS is mixed with PPI, which contained two major structuring step processes: (i) the first step leads to the spontaneous formation of soluble complexes, and (ii) the second step involves the aggregation of these structures to form large separated aggregates with higher size centered around 20-25 μm. The transition from step 1 to step 2 is governed by pH-dependent protein stoichiometry needed to achieve opposite charge compensation. This transition occurs at a lower LYS/PPI molar ratio at pH 7.5 (~5) thanks to the higher surface positive charge of LYS as compared to it at pH 9 with a higher LYS/PPI molar ratio of around 13.

After having highlighted the electrostatic interactions and possible repulsive forces in PPI-LYS systems, we turn to the second main part of our study, which was the

influence of different weight ratios and pHs on the functional properties of the PPI-EW composite system regarding thermal and gelation properties. As the system was based on egg white, the total protein concentration was settled at around 10% w/w protein of egg white and quantified by the Kjeldahl method (N = 6.25). As mentioned before, to have a better quantity of soluble proteins in the mixtures, high pH at 7.5 and 9 were selected. The other reason for choosing these pHs is because the pH of egg white is just after laying (7.5) or a few days after laying (9). Different weight ratios were chosen as PPI/EW 0/100, 25/75, 50/50, 75/25, and 100/0. The solubility of PPI-EW at a weight ratio of 50/50 showed a lower nitrogen solubility profile than recalculated ones, which may be attributed to the formation of aggregates between lysozyme and pea proteins through electrostatic interactions, especially at pH over the isoelectric point of pea protein isolate. From SDS-PAGE data, we could not evidence any new bond was formed for the 50/50 PPI-EW mixture.

For the design of egg products, thermal properties are an important value regarding pasteurization treatment. We performed MicroDSC to obtain the thermal denaturation temperature (T_d) and the changes of enthalpy (ΔH) of the proteins. Thermal properties of egg white only and PPI only were discussed in the previous graph. When taking the different protein mixtures into account regarding the T_d of PPI-EW mixtures, it was found in general that there were 5 peaks corresponding to T_d1 of ovotransferrin, T_d2 of lysozyme, T_d3 of superimposition of ovalbumin and vicilin peaks which could also overlay the peak of lysozyme at pH 9, T_d4 of S-ovalbumin and T_d5 of legumin. The T_d was slightly or not different compared to those measured for the pure protein solution of EW and PPI. Especially, the T_d value of ovotransferrin (T_d1) increased significantly at pH 9 from ~59 °C to ~63 °C with the increase in PPI content in admixture, possibly due to OVT being more sensitive to heat at this pH leading to its unfolding in presence of PPI or a decrease in electrostatic interactions with lysozyme due to the competition with PPI proteins. The T_d2 of lysozyme performed differently in the PPI-EW mixture at both pH; in detail, it appeared at pH 7.5 but not at pH 9. The former one would be due to either a slight shift of ovotransferrin signal toward lower temperatures at pH 7.5

thus resulting in a better separation of ovotransferrin and lysozyme signals or to an increase of lysozyme denaturation temperature due to its stabilization through interactions with PPI proteins as mentioned before. The latter one was assumed to be overlapped by the larger peak of ovalbumin and vicilin. The slight differences observed for ΔH could be explained by a limited loss (<10 %) of protein solubility in the mixtures. We conclude that pH played indeed a significant role as the result of protein unfolding at pH far from the pI of pea proteins (pH 7.5 and pH 9) could affect structural modification upon heating.

Gelation properties are one of the most important properties of globular proteins and are one of the principal means to give desirable texture to food products. Meanwhile, a heat-induced gel is the most common gelation process, especially for pea protein isolate and egg white proteins. As a result, we prepared the gel at a total protein concentration of 10%, from the mixtures at different mass ratios, at pH 7.5 and 9. The work presented aimed to study the interactions during gelation, as well as the gel characteristics in terms of viscoelastic properties, microstructure, and textures depending on the conditions, to propose a gel formation mechanism.

First of all, storage modulus (G') as a function of temperature during temperature sweep was measured to understand the sol-gel transition behaviour of the mixtures. Two transition temperatures were obtained for pure egg white and PPI-EW composite mixtures containing at least 50% of EW whatever the pH (1st gelling point assigned to ovotransferrin, the 2nd gelling point assigned to ovalbumin and vicilin), and the temperatures were close to the thermal denaturation temperature obtained by DSC experiments. In particular, the first gelling temperature increased by nearly 3 °C with the increase in PPI in the mixture at pH 9.0 rather than no changes occurring at pH 7.5, due to highly negative charges of pea proteins at pH 9.0. This resulted in hampering ovotransferrin molecules/particles association until more advanced denaturation (or aggregation) was achieved at slightly higher temperatures. So, the maintenance of pH 9 should be considered to optimize the heat treatment of the PPI-EW mixtures in the production of mixed ingredients.

The PPI-EW mixtures at the 75/25 weight ratio at pH 7.5 and 9 didn't show any early sol-gel transition previously associated with ovotransferrin in this mixture. It could be hypothesized that even if thermal denaturation of ovotransferrin occurred around 60 °C, the resulting unfolded/aggregated proteins were not numerous enough to interact and form a three-dimensional network, and/or their association was sterically hindered by the presence of the pea globulins in the mixture. G' and G'' at the end of the heating step both decreased with the increase of PPI content. Only the contribution of hydrophobic interactions and covalent SS bond formation can be considered in thermal protein aggregation upon temperature sweep, as electrostatic interactions such as hydrogen bonds were considerably weakened in such a temperature range. This means that both former interactions could have a structuring effect during gelation all the more strongly as the proportion of egg white is high.

At pH 9, PPI only formed a heterogeneous coagulum rather than a gel, due to the high repulsive forces at this pH preventing the protein particles from forming a regular three-dimensional network. This was confirmed by the microstructure study of the systems where the formation of small aggregates was observed at this pH. At pH 7.5, there was a solid-like gel structure formed with larger particles and a loose network of small particles. The application of several dissociating agents indicated that hydrogen bonds and hydrophobic interactions played an important role in PPI heat-induced gel formation while disulfide bonds only have a minor role.

Egg white gels, in general, had the highest hardness, springiness, and cohesiveness, confirmed by the lowest loss factor, with a porous, loosely packed, heterogeneous network at pH 7.5 and a denser, more homogenous network at pH 9. Hydrogen and disulfide bonds played a major role in egg white heat-induced gel formation.

With the increased proportion of PPI in admixture with EW, the loss factor increased, as well as the frequency slope of G', meaning weaker gels were formed. Also, hardness, springiness, and cohesiveness decreased with the increasing proportion of PPI, with a more heterogenous network including more random clusters of smaller size.

As a result, we proposed a possible mechanism of gelation for PPI, EW, and PPI-

EW mixtures. Combining the results of dissociating agent test, texture, microscopy, and dynamic rheology, a possible mechanism of PPI-EW gel formation can be proposed. For heat-induced EW gelation, egg white proteins were partially denatured resulting in the unfolding of the proteins, with involved protein-protein interactions, such as hydrophobic interactions, hydrogen bonds, and disulfide bonds. During cooling, gel strength increased due to the creation of hydrogen bonds (as G' increased in the dynamic rheology test), resulting in a continuous network. However, a more homogenous network was formed at pH 9 due to the higher net protein charges and the electrostatic repulsions at this pH, rather than a more porous and heterogeneous protein network obtained at pH 7.5. For heat-induced PPI gelation, in our case, it is difficult to form a gel at pH 9, compared to a solid-like gel formed at pH 7.5, it could be due to higher repulsive force at higher pH (9, in this case) leading to an insufficient interconnection. For the combined PPI-EW heat-induced gel systems, proteins were denatured with increased temperature, leading to their unfolding and exposed protein subunits. Protein-protein interactions were involved and led to protein aggregates, possibly containing self-aggregates of PPI, aggregates between lysozyme and ovotransferrin, lysozyme and ovalbumin, or combined aggregates between LYS, OVT, and OVA. In addition, PPI could form aggregates with LYS in a PPI/LYS binary system (Chapter 4), therefore, it could have PPI/LYS aggregates in PPI/EW mixtures. During the cooling step, with the increasing proportion of PPI, hydrogen bonds, and hydrophobic interactions took a major role in the gel network formation, while disulfide bonds took a minor role. When EW prevailed in the mixtures (75% of the mixture), it showed higher hardness and springiness compared to other proportions of mixtures and the network appeared irregular shapes surrounded with homogenous egg white colloids. Increasing the PPI proportions, larger clustered aggregates were formed making the structure not as tight as pure egg white gel, leading to a decrease in the texture properties. The different results between the mixtures at pH 7.5 and pH 9 may be due to high repulsive forces and negative charges at higher pH.

Perspectives

The study contributed to the understanding of the mechanisms of interactions in the formation of pea protein isolate and egg white protein systems. The thermal properties of the mixtures depended on pea protein isolate content, and the characteristics of the heat-induced gel formed were markedly improved compared to the pure thermal gel of pea proteins. In solution, except for the evidenced complexation between pea protein and lysozyme, the properties of the mixture seemed relatively driven by the additive effects of each type of protein. In the structured mixed systems as gels, we could not observe any synergy even if a specific structure of the mixed gel was highlighted (i.e., continuous protein network including other protein aggregates in which it was supposed that egg white proteins and pea proteins dominated in each domain respectively). Regarding this specific microstructure, two different dying agents could be added to stain separately PPI and EW, in order to give more detailed information on the gel structure. Nevertheless, there were still many other questions to consider experimentally. As already known, the type and concentration of salts influence the gelation properties and thermal properties of proteins, therefore, adding salts inside the mixture could help us to have a better understanding of the techno-functional properties of composite systems between plant and animal proteins. As fat is another constituent of food products in general, the impact of a lipid-dispersed phase on gelling properties could be also investigated to design new food products.

In addition, regarding the co-precipitate of the proteins between lysozyme and ovalbumin or ovotransferrin, the mechanism of thermal results was not well understood when PPI was added to egg white, it could be a viable solution to have a hybrid ternary protein system, such as PPI/OVT/LYS, PPI/OVT/OVA, PPI/OVA/LYS, to know more information of the aggregate or precipitate between PPI and egg white fractions. Furthermore, regarding two fractions of legumin and vicilin in pea globulins, it could be useful to obtain purified 11S and 7S globulins first and then mixed with egg white, leading to a more specific understanding of the co-aggregates, or functional properties.

To complete the study on the functional properties of the system, it could be

interesting to investigate emulsifying and especially foaming properties of the mixed system, as the formation of foam is one of the sought-after qualities of egg white ingredients.

Moreover, regarding the technological point in the design of new mixed ingredients, the pasteurization treatment could be performed to evaluate the impact of this treatment on the physicochemical and functional properties of egg white and pea protein mixtures. Other treatments such as drying are expected to cause further denaturation/aggregation among proteins likely to change the final functional properties of the powdered ingredients. Moreover, dry heating of egg white powder (40 to 80 °C for a few days) is a classical treatment applied in industry to enhance the functionality of this ovoproduct. Improved foaming and gelling properties are obtained in this way (Lechevalier et al., 2017; Yuno-Ohta et al., 2021). So, we could take advantage of this treatment to functionalize the present pea and egg white protein mixtures. Indeed, encouraging preliminary results (unpublished) on the application of the method to pea proteins and egg white system have been obtained by the UMR STLO/UMR PAM consortium in the framework of the VeggIn project (2018-2021) supported by the Carnot Qualiment in France.

In addition, one of the limitations of using egg white proteins is their proven allergenicity. Pea proteins are less allergenic than soy proteins for example and are thus interesting plant proteins to attenuate the allergenicity in mixtures. The possible mechanism leading to allergy reduction could be then explored in future studies. Furthermore, as mentioned in section 1.4, it could be very interesting in using different treatments to modify egg white and pea protein isolate to favor protein interactions and increase the functional properties, such as gelation properties.

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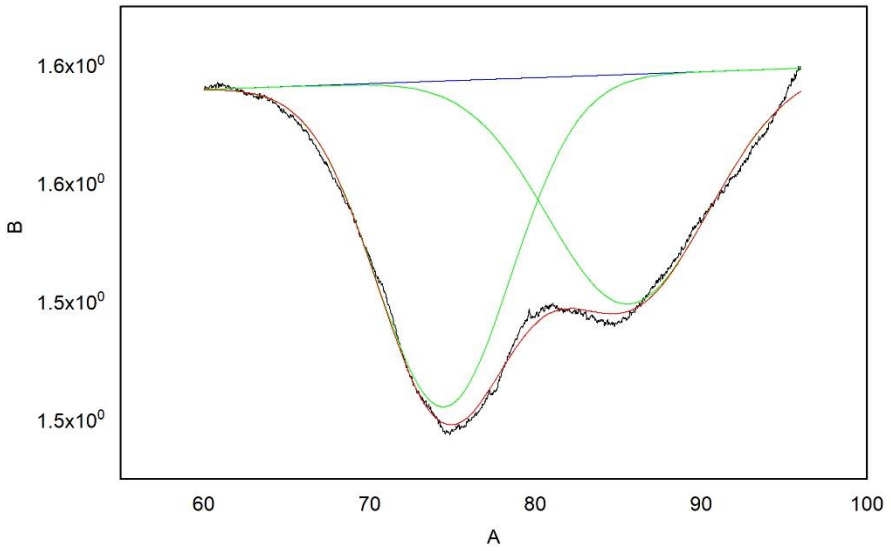
Annex

Annex-1

Peak Analysis

Data Set:[Book1]"05-23 2"!B — Date:2022/10/21
BaseLine:Straight
Chi^2=9.24121E-006 — Adj. R-Square=9.95805E-001 — # of Data Points=2208
SS=2.03491E-002 — Degrees of Freedom=2202



Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1	Gaussian	-1.39786	9.52443	-0.1379	74.47282	-54.59793
2	Gaussian	-1.16242	11.42034	-0.09718	85.59854	-45.40207

A

Peak Analysis

Data Set:[Book1]04-07-3!B Date:2022/10/21

BaseLine:Straight

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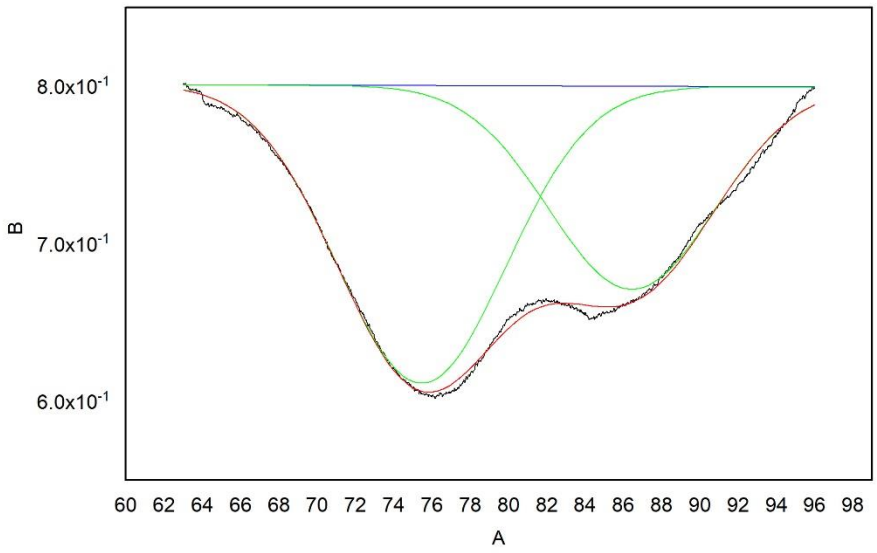
SS=2.67945E-002 Degrees of Freedom=1917



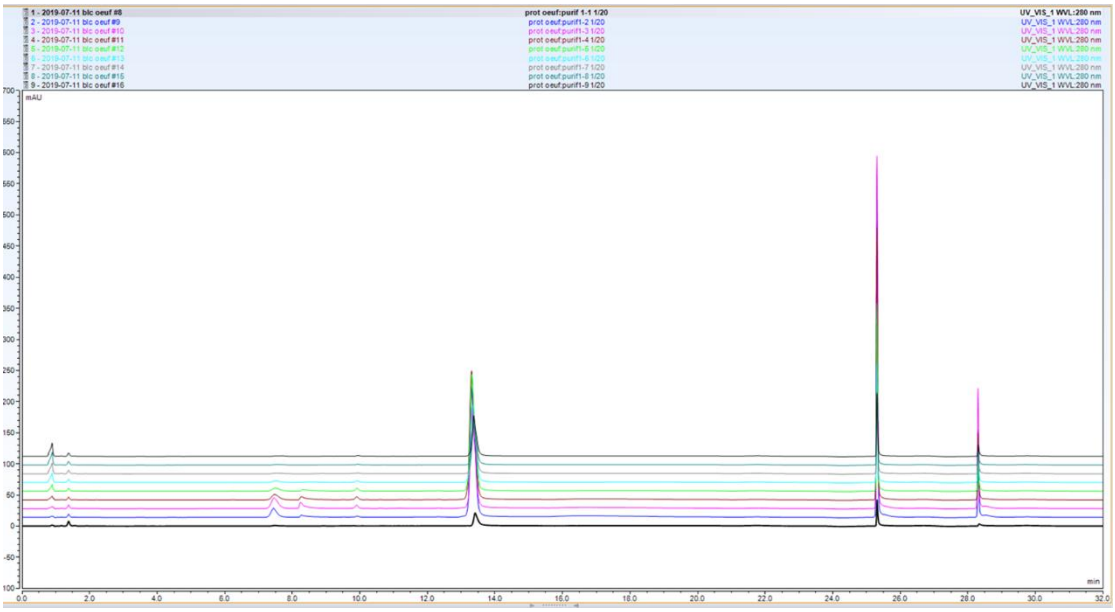
Fitting Results

Peak Index	Peak Type	Area Intg	FWHM	Max Height	Center Grvty	Area IntgP
1	Gaussian	-2.08143	10.37533	-0.18891	75.46291	-60.12663
2	Gaussian	-1.38031	10.20391	-0.12885	86.43952	-39.87337

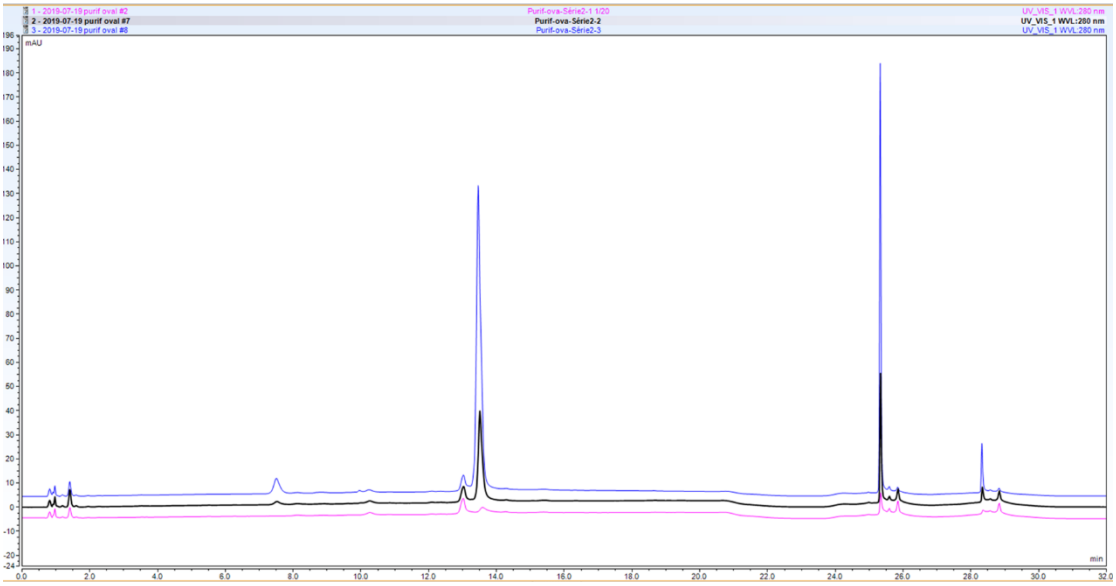
B

Figure A-1 The rest deconvolution results of pea protein isolate 10% (A-B).

Annex-2



A



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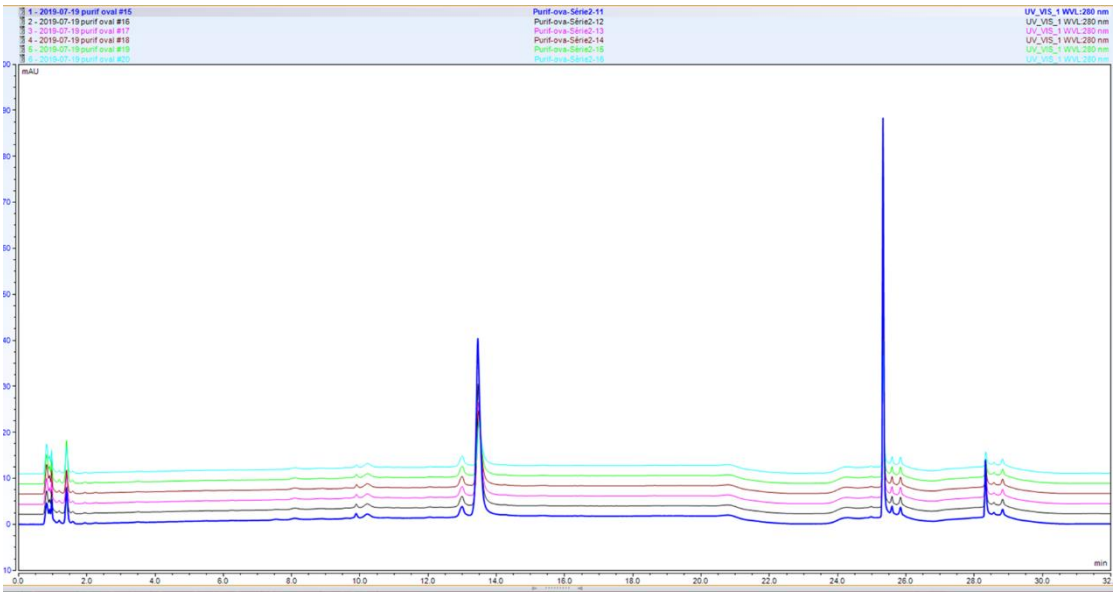
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5 - 2019-07-19 purif oval #19
Purif-ova-Série2-11
Purif-ova-Série2-12
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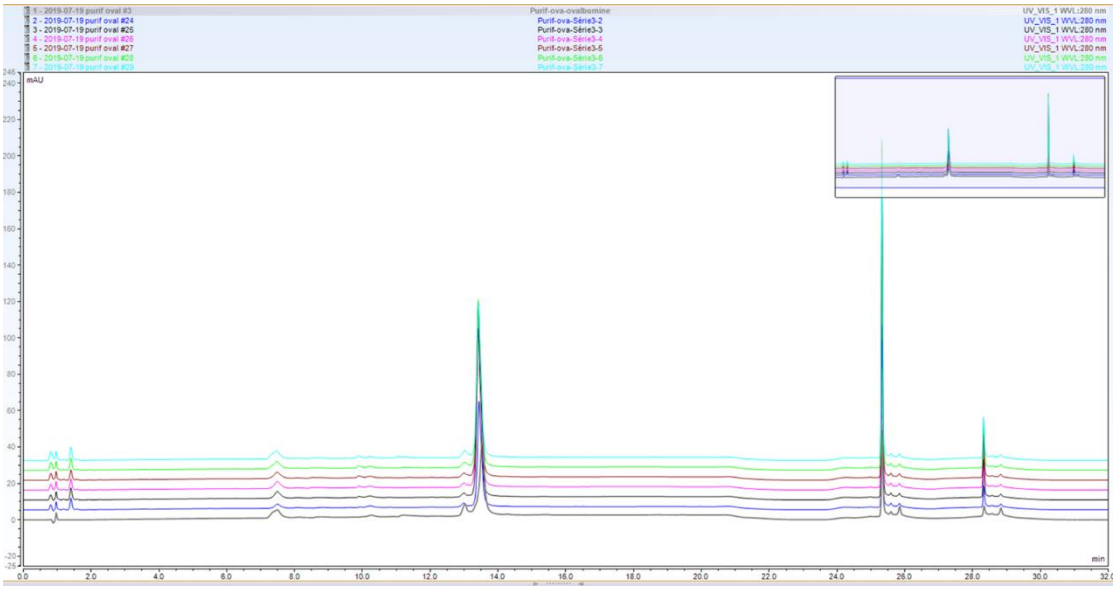
mAU
min

C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Purif-ova-Série3-2
Purif-ova-Série3-3
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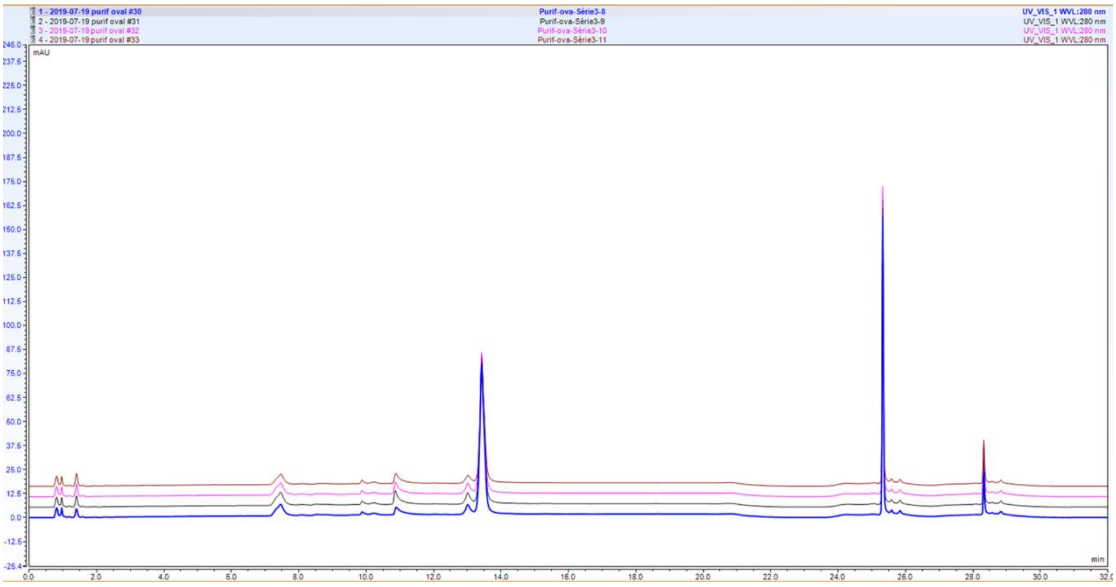
D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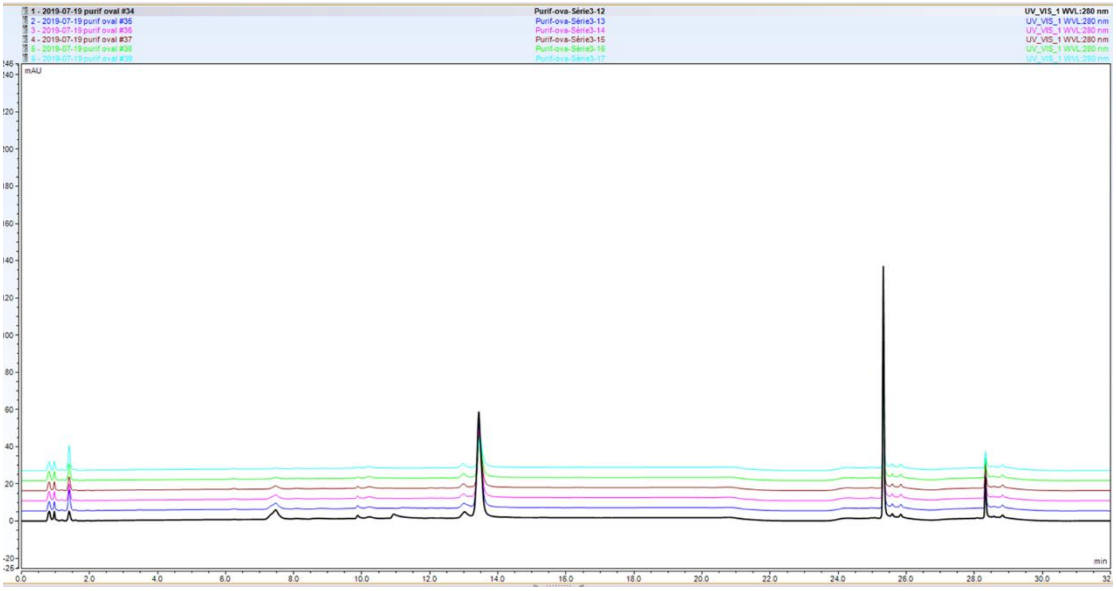
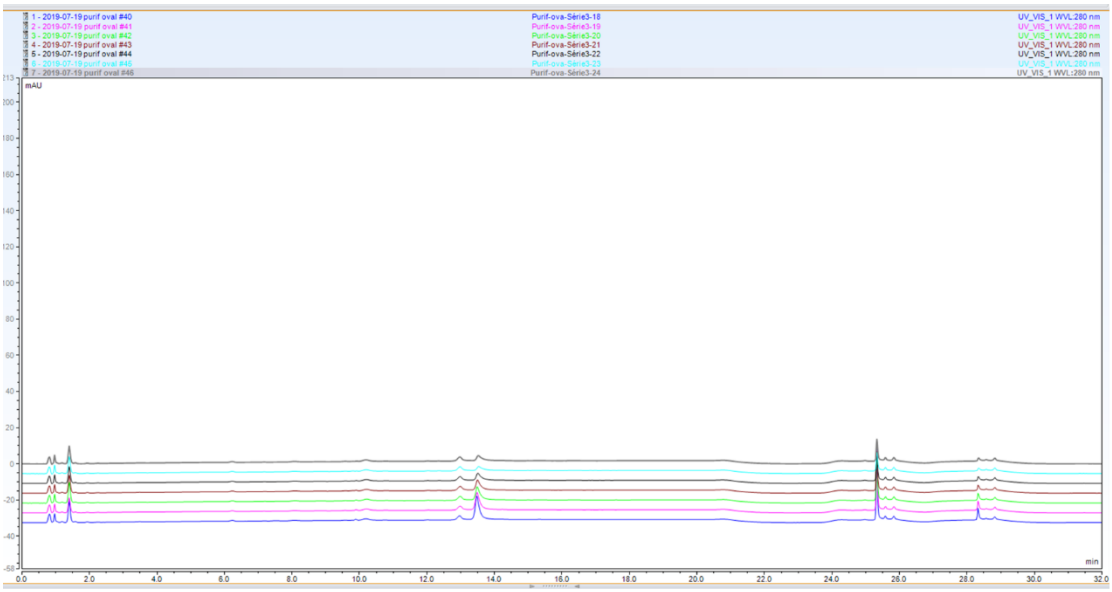
min

E


1 - 2019-07-19 purif oval #34
2 - 2019-07-19 purif oval #35
3 - 2019-07-19 purif oval #36
4 - 2019-07-19 purif oval #37
5 - 2019-07-19 purif oval #38
Purif-ova-Série3-12
Purif-ova-Série3-13
Purif-ova-Série3-14
Purif-ova-Série3-15
Purif-ova-Série3-16
UV_VIS_1 WVL:280 nm
mAU
min

F



G

Figure A-2 Different batches of extracted ovalbumin analyzed by RP-HPLC (A-G).

Annex-3

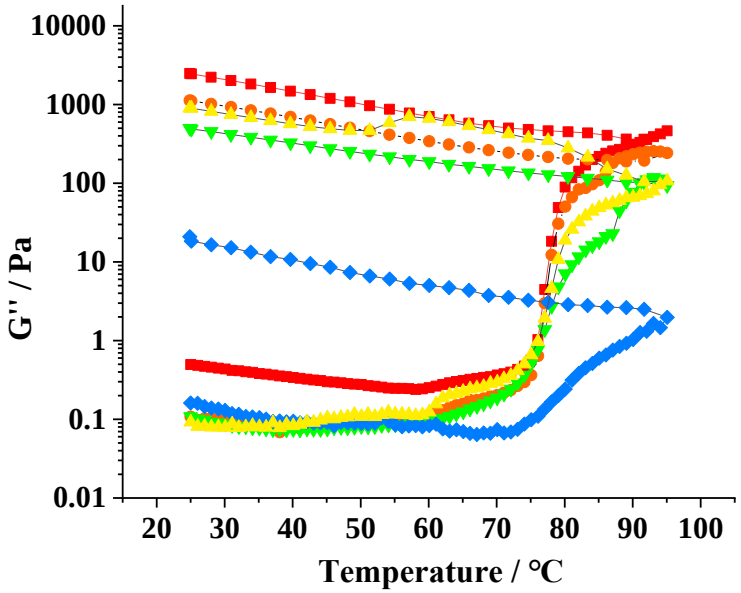


Figure Annex-3-1 Temperature sweep G'' of egg white protein (red), PPI (blue) and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at different pH 7.5.

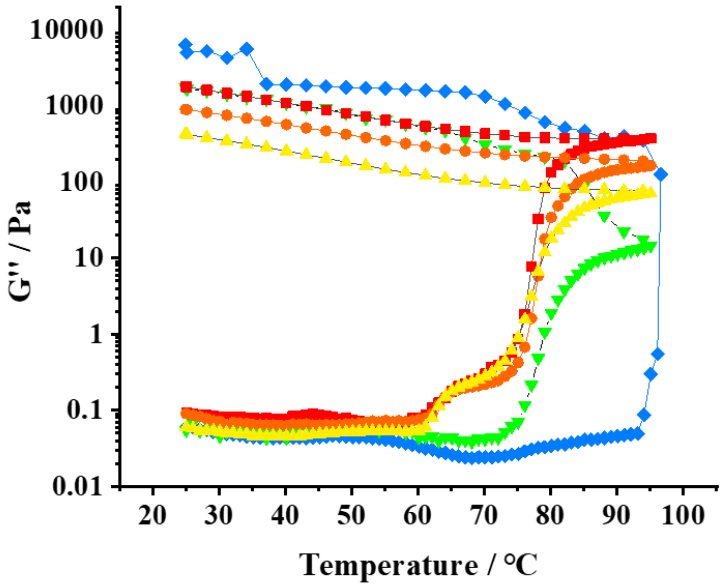


Figure Annex-3-2 Temperature sweep G'' of egg white protein (red), PPI (blue) and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at different pH 9.

Annex-4

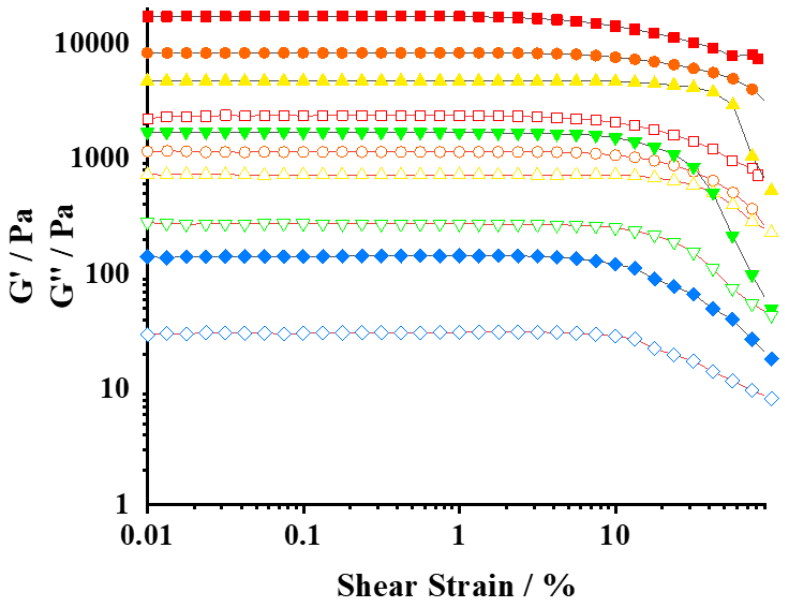


Figure Annex-4-1 Storage modulus (G') (solid one) and loss modulus (G'') (hollow one) from strain sweep test of the egg white protein (red), PPI (blue) and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at pH 7.5.

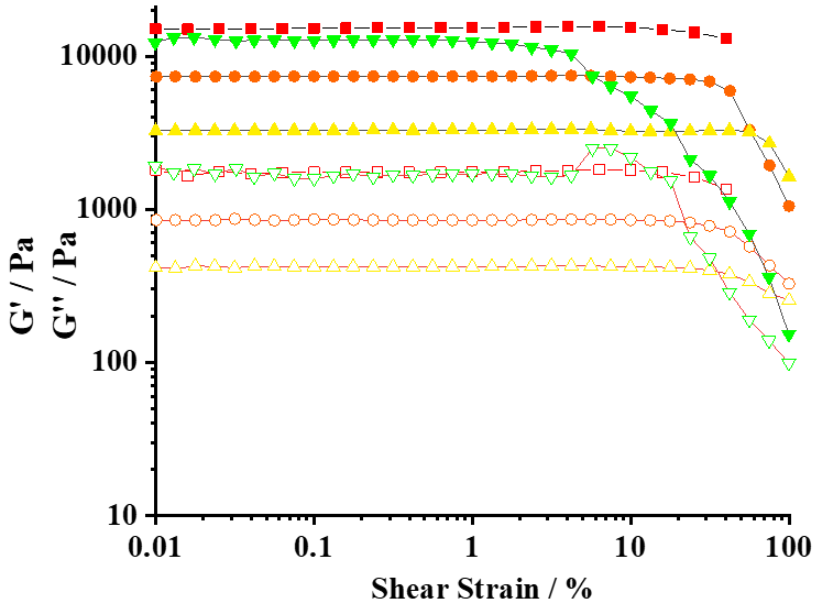


Figure Annex-4-2 Storage modulus (G') (solid one) and loss modulus (G'') (hollow one) from strain sweep test of the egg white protein (red), and PPI-EW mixtures at different weight ratios (75/25 in orange, 50/50 in yellow, 25/75 in green) at pH 9.