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Effect of Radiofrequency Assisted Thermal Processing on the Structural, Functional and Biological Properties of Egg White Powder

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EFFECT OF RADIOFREQUENCY ASSISTED THERMAL PROCESSING ON THE STRUCTURAL, FUNCTIONAL AND BIOLOGICAL PROPERTIES OF EGG WHITE POWDER

by

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EFFECT OF RADIOFREQUENCY ASSISTED THERMAL PROCESSING ON THE STRUCTURAL, FUNCTIONAL AND BIOLOGICAL PROPERTIES OF EGG WHITE POWDER

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Radiofrequency (RF) assisted thermal processing is a novel and energy-efficient method used for pasteurization, which heats the product volumetrically as opposed to traditional hot room (HR) pasteurization. RF-assisted thermal processing accelerates the heating rate of egg white powder (EWP), which has a low thermal conductivity and further affect its gelling properties. The effect of processing methods (RF and HR) due to pH and treatment temperature combination was monitored by subjecting neutral EWP (6.4 pH) to 70 and 80 ℃ and alkaline EWP (pH 9.9) samples to 70 ℃ over 0, 5, 10, 15 and 20 d. At 80 ℃ and 0 d, the gel firmness was 48.6% higher than HR, indicating RF could potentially improve the functionality as well as reduce the come-up time to less than a day as opposed to the traditional hot room processing used in the industry which takes 5-6 days. With increased treatment severity, the gel firmness substantially increased without changing the soluble protein contents, and insoluble contents.

The secondary protein conformations evaluated using surface hydrophobicity, CD spectroscopy and Raman spectroscopy for EWP processed at 80 ℃ and 0 d, exhibited no significant effect of RF assisted thermal processing over HR processing. An in-vitro digestion model on egg white gels demonstrated that the accessibility of pepsin and trypsin to egg white peptides was not compromised despite a stronger gel network in RF

assisted thermal processing at 80 ℃ and 0d. An *in-silico* analysis of the peptides in the egg white gel hydrolysates demonstrated that Ovalbumin and Ovotransferrin were the parent protein of most of the unique peptides generated, and minor structural differences were accounted for these peptides. The transport of soluble egg white gel hydrolysates, the cell viability, and tight junction proteins in the Caco-2 cells were not affected by RF assisted thermal processing.

To my family and friends

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PREFACE

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Chapter I: Introduction

1.1. Background

Egg white has been used for decades in the food industry for its multifarious functional properties. More recently, the transition in the sector has inclined towards food products with a longer shelf life and shelf stability. This led to the development of egg white powder (EWP), which further alleviated the hurdles in the applications (Asghar & Abbas, 2012; Ayadi et al., 2008; Handa et al., 2001; Wei et al., 2019). With a low thermal conductivity, EWP requires 2 weeks of storage at 56-67 ℃, for pasteurization, by a process known as hot room processing (Baron et al., 2003; Bhandari et al., 2013; Handa et al., 1998; Mine, 1995, 1997; Wei et al., 2019). It takes roughly 5 days for a 9 Kg batch to reach the pasteurization temperature, making the whole process energy intensive (Boreddy et al., 2014; Chen et al., 2019). Therefore, radiofrequency (RF) assisted thermal processing has been suggested as an alternative to the traditional hot room processing. The dielectric heating mechanism with RF processing has a larger penetration depth that leads to heat generation from within the food product (Awuah et al., 2015; Birla et al., 2004; Jiao et al., 2018). Because of volumetric heating in radiofrequency processing, it is faster and has a lower come-up time than traditional hot room (HR) processing. RF processing coupled with traditional HR processing, also called RF assisted thermal processing, has shown considerable improvement in functional properties such as gelling, foaming and emulsification of egg white powder over traditional hot room processing (Boreddy et al., 2014, 2016).

However, there are two major drawbacks with using RF processing. Firstly, even though the dielectric heating results in the product to reach higher treatment temperature

rapidly, to hold the product further at such high temperatures in a larger scale commercially is not feasible. Secondly, the biological implications associated with RF processing have not been delved upon. Therefore, it necessitates the requirement of an optimal time-temperature combination which would substantially improve the functional properties of EWP, while not affecting the feasibility of the process. Processing steeps into deeper levels when the processed EWP is further used into applications based on their functional properties. Among the various functional properties associated with EWP, gelation is the most heat intensive property because heating causes formation of the gel matrix through protein-protein and water-protein interaction (Alleoni, 2006; Chang et al., 1999; Handa et al., 2001). RF processing being a non-uniform heating technique (Cao et al., 2019; Wei et al., 2020; Zhu et al., 2017) could have possible implications on the egg white protein structure in the gel matrix. The associated protein structural conformations could not just affect the gel firmness but also have possible implications on the protein bioaccessibility and bioavailability, thereby affecting protein assimilation. However, processing technologies using electromagnetic waves have received skepticism with regards to possible toxicity, allergenicity and microbial safety aspects in proteins (Akharume et al., 2021; Pojić et al., 2018; Ponne & Bartels, 1995). A microbial challenge study using continuous RF heating for 55 minutes at 80 ℃ followed by 2 h holding time demonstrated > 6 log reduction of Salmonella in egg white powder (Wei et al., 2019, 2020). The implications on toxicity and allergenicity are yet to be explored with respect to electromagnetic waves on egg white proteins.

RF assisted thermal processing is expected to improve the gelling properties of EWP with increased treatment temperature, time and pH. Furthermore, it is expected that

secondary protein conformations, and protein-protein and protein-water interactions, would cause the increase in the gel firmness of EWP processed by RF assisted thermal processing. The changes in the interactions between egg white proteins and water is also expected to affect the bio-functional properties such as bioaccessibility and bioavailability.

1.2. Goals and objectives

The goal of this dissertation is to optimize RF assisted thermal processing of EWP to maximize its gelling properties and evaluate the subsequent effect on the egg protein conformation and its biological functionality. There are two main objectives to achieve the overall goal:

Objective 1

Investigating the effect of temperature, holding time and pH in improving the gel firmness of EWP, and comparative analysis of RF assisted thermal processing and HR processing.

Objective 2

Investigating the impact of RF processing on the egg white protein structure and bio-functionality.

1.3. Dissertation organization

Chapter II includes the literature review about the relevance of egg white powder in the food industry, processing steps involved in its production and pasteurization, functional and biological properties of egg white powder, and introducing an alternative

approach of processing through radiofrequency assisted thermal processing of egg white powder.

Chapter III evaluates the effect of processing temperature, time, and pH on the gel firmness and solubility of egg white powder. It also demonstrates the comparison between using radiofrequency assisted thermal processing over traditional hot room processing on improving the efficacy of the process as well as the functionality.

Chapter IV evaluates the effect of RF processing on the secondary protein conformations of egg white powder. It further evaluates and compares the bioaccessibility and bioavailability of the egg white protein in egg white gels prepared from RF assisted thermally processed and traditional hot room processed egg white powder.

Chapter V summarizes the results from Chapter III and Chapter IV and presents recommendations for future work.

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Chapter II: Literature Review

2.1. Introduction

Egg white has remained an essential food ingredient for decades across the globe because of its nutritional and functional properties. Even though egg white comprises of 10-11% protein and 84-89% water, egg white protein provides all the essential amino acids (Wang et al., 2018; Lassé et al., 2015). Egg white protein is composed of 6 major proteins namely ovalbumin (54%), ovotransferrin (12-13%), ovomucoid (11%), ovoglobulins (2%), ovomucin (1.5-3%) and lysozymes (3.5%), and some minor proteins such as ovastatin, ovoflavoproteins, and avidin (Abeyrathne et al., 2013; Bragg & Hough, 1961; Mine & Zhang, 2013; Sharif et al., 2018). Egg white is also rich in various enzymes (such as lysozyme, phosphatase, catalase, and glycosidase), vitamins (such as biotin, niacin, and riboflavin), and minerals (majorly sulfur, potassium, sodium, and chlorine, and in traces comprising of calcium, phosphorous and magnesium) (Sharif et al., 2018). Abundance of such diverse components in egg white further translates into its wider functionality such as gelling, foaming, binding, and emulsification (Arntfield & Murray, 1981; Ayadi et al., 2008; Campbell et al., 2003a).

Gelation in egg white is a result of multiple chemical modifications in the glycoproteins (ovalbumin, lysozyme, and ovomucin) through acetylation, phosphorylation, and methylation. Furthermore, protein-protein interactions such as lysozyme-ovomucin complex formation, and protein interaction with other components, such as water-protein interactions, also play a vital role in gelation (Mine & Zhang, 2013; Robinson & Monsey, 1972). Foaming, on the other hand has been associated with surface

active properties of egg white proteins, mostly globulins, ovalbumin, ovotransferrin, lysozyme, ovomucoid, and ovomucin (Strixner & Kulozik, 2011). Different charge characteristics and conformational changes of these proteins enables the formation of a cohesive viscoelastic film, which contributes to foaming.

However, several adjoining concerns associated with transportation, storage and handling of liquid egg white has resulted in the growing propensity towards dehydrated egg white or egg white powder (EWP). The ease of application in food formulation, better uniformity during application, and lower microbial risk have also been critical in driving the shift towards EWP.

Processing EWP relies upon preserving the functional and nutritional properties of liquid egg white. Raw egg white undergoes a combination of mechanical and thermal energy through filtration, pH adjustments, concentration and desugarization before being spray dried as demonstrated by Lechevalier et al. (2007) in an 11-step process. Since egg white proteins are heat sensitive, therefore it is pasteurized after drying to prevent heat coagulation in raw egg white. EWP is stored in hot rooms, which are almost similar to a forced convection oven maintained between 56-67 ℃ for 15 days for pasteurization (Baron et al., 2003; Wei et al., 2019).

Like most powders or low moisture food products, EWP also has a low thermal conductivity of less than 0.1 $Wm^{-1}K^{-1}$, which further accounts for a longer come-up time. Come-up time is the time required for heat to penetrate the coldest location of the sample, so as for it to reach the pasteurization temperature. With a low thermal conductivity, it takes about 5 days for a ~9 Kg batch of egg white powder to reach the pasteurization temperature (60 ℃) in hot room (Boreddy et al., 2014; Chen et al., 2019). It is further

held for 10 days to render the product microbially safe. However, some studies by Baron et al. (1999a, 1999b); Liu et al. (2018) have stressed on the ability of powders to facilitate microbial infestation in a food matrix with other ingredients of higher water activity. Therefore, pasteurizing EWP effectively is pertinent, and more so important is to make the process of pasteurization efficient. Therefore, a novel approach for reducing the come-up time and improving the microbial safety was introduced with radiofrequency (RF) assisted thermal processing.

RF assisted thermal processing uses electromagnetic waves which penetrates the food surface and volumetrically heats the food. Ionic depolarization and dipole rotation due to the alternating electric field in dielectric heating results in friction and generation of heat from within the food matrix (Piyasena et al., 2003; Jiao, Luan, and Tang, 2014). Since RF waves have a larger penetration depth over microwaves, therefore they can rapidly heat low moisture products (Boreddy et al., 2014; Chen et al., 2019).

Earlier works have demonstrated that thermal processing and pH specifically aids in improving the gelling and foaming properties of EWP (Kato et al., 1989; Lechevalier et al., 2007; Mine, 1996). Therefore, EWPs are processed in an application specific manner such as: for non-whipping binding and baking applications, high gelation and binding applications, sports or nutritional beverages, and antioxidant for salad dressings (Ayadi et al., 2008; Boreddy et al., 2014; Chang et al., 2020; Lu & Chen, 1999; Moriano & Alamprese, 2020; Nolasco et al., 2020; Song et al., 2009). Thermal processing further causes a significant effect on the protein conformations as well as biological functionality of egg white while enhancing the functionality. However, the specific impact of temperature, holding time, pH, and dielectric heating on the functionality, and the

structure-function relationship, as well as the adjoining impact on biological properties of EWP has not been widely explored yet. Since RF assisted thermal processing has been proposed as an energy efficient alternative, this chapter summarizes the literature on EWP, the effects of processing on EWP, use of RF assisted thermal processing as an alternative to traditional thermal processing, effect of processing on the functional properties, egg white protein structure and biological functionality of EWP.

2.2. Relevance of EWP in the food industry

EWP has been used over liquid egg whites due to the multifarious advantages associated with dehydration such as easier transportation, lower storage costs, less susceptibility to microbial infestation etc. They can be stored under anaerobic conditions up to 10 years with refrigeration, and up to 12 months without refrigeration (Sharif et al., 2018). However, the most valuable effect due to the dehydration of egg white for the food processing industry was in obtaining better uniformity of product, which further resulted in higher control during formulation and processing of food products (Bergquist, 1995). Therefore, EWP have effectively replaced liquid egg white in bakery and confectionary (Asghar & Abbas, 2012; Penfield & Campbell, 1990), mayonnaise, salad dressings (Drakos & Kiosseoglou, 2008), ice creams and various other convenience food items.

2.3. Production of EWP

Raw egg white is initially adjusted for pH, followed by filtration using a high-pressure pump. At this stage, it is concentrated and undergoes enzymatic glucose hydrolysis for further desugarization (Lechevalier et al., 2007). Desugarization facilitates glucose removal and prevents caramelization and browning, while also improving the

shelf stability (Muschiolik, 1974; Quan & Benjakul, 2019; Sharma et al., 2012). The next stage of processing is drying. Drying method used for manufacturing EWP from liquid egg white is often dependent on the end use associated.

A non-commercial egg white drying method called pan drying produces yellowish and course particles, and the product could be rehydrated to be used in omelets, fried eggs and scrambled eggs (Sue, 2016; Erich, 2012). Foam drying of egg white is associated with premium quality products. This is because foaming or whipping of the egg whites precede the drying step, resulting in a higher total surface area between particles due to air incorporation between them. This, in turn results in the requirement of lower drying temperature and drying time. The egg white foam could also be subjected to freeze drying at -40 ℃ to obtain egg white powder (A et al., 2015; Muthukumaran et al., 2008). Using oven drying for manufacturing EWP has shown to minimally affect the nutritional and functional properties of egg white (Penfield & Campbell, 1990). Spray drying is by far the most widely used commercial method for manufacturing EWP, and it significantly affects the functionality of EWP (Ayadi et al., 2008; Franke & Kießling, 2002; Galyean & Cotterill, 1979; Hill et al., 1965; Ma et al., 2013).

2.4. Thermal processing of EWP

2.4.1. Effects of spray-drying on EWP

Spray drying of egg white considerably increases the alkalinity by exposing some basic amino acids present such as lysine and arginine, and the loss of dissolved carbon dioxide in the liquid egg white by a process called decarbonatation (Ayadi et al., 2008; Hammershoj et al., 2006; Mine, 1997). Slight protein denaturation and some chemical

changes due to deamination of amino acid residues also results in a decreased protein content (Ayadi et al., 2008).

Ayadi et al. (2008) concluded that at the highest spray drying temperature, with the longest residence time, the pH of the resulting EWP increased to pH 10, and the gel strain of reconstituted egg white was highest. The argument was supported with findings from earlier works of Chang et al. (1999); Handa et al. (1998); Kato et al. (1989); Lee & Chen (1999), where gelation in EWP increased with an increase in the alkalinity.

The inlet air temperature during spray drying influenced the solubility of EWP. With increased temperature, and residence time, the protein solubility decreased (Katekhong & Charoenrein, 2018; Van der Plancken et al., 2007).

On the other hand, Lechevalier et al. (2007) demonstrated that spray drying had rather detrimental effects on the functionality of egg white due to heat treatment and wider air-product interface. However, it was also observed that desugarization prior to drying enhanced the foaming properties of egg white (Lechevalier et al., 2007). The removal of sugar led to a fall in the pH to pH 6, which facilitated enzymatic reactions favoring protein interfacial unfolding (Damodaran & Paraf, 2017). Because of increased interfacial unfolding of the egg white proteins, they had a higher flexibility to adsorb at the interface, which eventually improved the foaming properties (Gekko & Yamagami, 1991; Kato et al., 1989).

Some earlier works by Galobart et al. (2001) and Wahle et al. (1993) have reported the influence of spray drying temperature on the biological functionality of whole egg proteins. A higher temperature and larger air to product surface exposure

could increase the rate of fat oxidation and subsequently deteriorate the natural antioxidants and tocopherols present in egg white.

2.4.2. Pasteurization of EWP

Interestingly, egg white does not undergo pasteurization before being dehydrated because it coagulates at temperatures higher than 57 ℃. Spray drying of egg white does not render it safe from pathogenic infestation. Even though EWP is a low moisture product, it has a water activity of ~ 0.3, at which *Salmonella* cannot grow, but can survive (Blessington et al., 2013; Keller et al., 2013). As a result, when EWP is used in a food matrix containing ingredients with a higher water activity, the pathogens could potentially grow. Therefore, it is essential to pasteurize it after spray-drying to render it microbially safe for consumption (Baron et al., 1997; LiCari & Potter, 1970; Wei et al., 2019).

Traditionally, EWP is pasteurized in hot rooms maintained at 56 to 67 ℃ for 15 days (Handa et al., 2001; Talansier et al., 2009). EWP has a low thermal conductivity ($<0.1\ Wm^{-1}K^{-1}$), which results in its longer come-up time during pasteurization (time required for the coldest spot in the batch to reach the treatment temperature).

For a batch of 9.07 kg of EWP, it takes 5 days for the coldest spot to reach 60 ℃ (Boreddy et al., 2014; Handa et al., 2001). Thus, the process is both energy and time intensive, and could potentially alter the functional properties of EWP. However, the traditional hot room (HR) pasteurization method has been prominently used at the commercial level.

2.4.3. Effect of thermal processing on the functional properties of EWP

Thermal processing in EWP has been frequently associated with affecting its functional properties because of heat lability of egg white protein. However, past studies have explored the potential of effectively using thermal processing to enhance the functional properties of EWP, specific to the application. Controlled heating temperature, time and moisture content were prime factors that impacted the egg white functionality (Kato et al., 1989; Rao & Labuza, 2012; Van der Plancken et al., 2007). The improvement in the functional properties were a result of conformational changes in the egg white protein (Hammershoj et al., 2006; Matsudomi et al., 2002; Nicorescu et al., 2011; Van der Plancken et al., 2007).

Kato et al., (1989, 1990) reported the increase in the gelling, foaming, and emulsifying properties of EWP concordantly with increased temperature and heating time.

Such an increase was explained by exposure of the buried hydrophobic amino acid residues on the egg white protein surface, which increased the surface hydrophobicity (Kato & Nakai, 1980). As a result of dry heating, there is formation of low molecular weight aggregates, which also contribute to improved gelation of egg white proteins (Mine, 1996).

2.4.3.1. Gelation

Heat induced egg white gelation is a result of egg white protein partial-unfolding, which results in protein aggregation and coagulation to bind and retain water (Handa et al., 1998). The gelation process starts with partial protein unfolding, followed by aggregate formation due to sulfhydryl-disulfide interchange reactions, and further

followed by multiple hydrogen bonds formation increasing the gel hardness and elasticity. The gel network formation is influenced by the balance of attractive and repulsive forces between the protein molecules (Campbell et al., 2003a; Mine, 1996). The resulting gel morphology depends on the relative speed of protein unfolding and aggregation, which further determines the gel homogeneity (Hermansson, 1994). Gelation begins at around 65 ℃ and builds up the rigidity to shift from a viscous to visco-elastic structure between 71-74 ℃. Kato et al. (1990) revealed that enhanced gel firmness was a result of heterogenous protein interaction instead of the action of a single protein. With EWP, ovalbumin and ovotransferrin were the two major egg white proteins, which coagulated at ~80 ℃ and 61-65 ℃ respectively and influenced the gel network formation (Kato et al., 1990).

Ovotransferrin is the most thermolabile protein. It has 686 amino acids folded into 2 lobes, the N-lobe (1-332) and the C-lobe (342-686), with two iron binding sites (Wu & Acero-Lopez, 2012; Yamashita et al., 1998). An increased saturation of the iron binding sites has shown to increase the molecular stability of ovotransferrin by increasing the denaturation temperature to about 80 ℃. However, dry heated EWP shows a denaturation peak between 60-65 ℃, suggesting the absence of saturated iron binding sites (Yamashita et al., 1998). The gel formation in egg white proteins is also influenced by glycoproteins, in which covalent bonds such as N-acylglycosylamine linkages links the polypeptide chain to the carbohydrate moiety. The interaction between different glycoproteins like ovalbumin, ovomucin and lysozyme, improves the gel rigidity. For instance, due to dry heating, cysteine residues in ovomucin and lysozyme results in the formation of ovomucin-lysozyme complex, linked through disulfide bonds.

Egg white gel formation is driven by multiple factors such as protein concentration, ionic strength of protein-water solution, heating time and temperature used in gel formation, pH of protein, and interaction of protein with other components. The gel hardness and shear stress increase with an increase in protein concentration, pH, temperature and time (Ziegler & Foegeding, 1990).

The protein secondary structural conformation is also significantly affected by these factors that determine the gel hardness. There is an increase in the intermolecular β-sheeted structure from α-helix structure in ovalbumin, because of protein unfolding with exposure to dry heating. These aggregates formed as a result are water soluble, which also enhances the water-protein interaction (Mine et al., 1990; Nicorescu et al., 2011). As the protein unfolds because of heat application and other external factors, the denaturation enthalpy of the protein decreases in egg white (Ahmed et al., 2007; Arntfield & Murray, 1981; Donovan et al., 1975; Fitzsimons et al., 2007). The denaturation enthalpy in egg white gels is a representation of the balance between endothermic unfolding and exothermic aggregation. Furthermore, an increase in the gel firmness has been correlated with a decrease in the denaturation enthalpy, suggesting increased protein unfolding due to dry heating of EWP (Kato et al., 1990). An increase in thermal denaturation in egg white protein due to dry heating also explains for a tandem rise in peak broadening (endotherm from differential scanning calorimetry). Talansier et al. (2009) reported an increase in the peak broadening with an increase in temperature from 60 ℃ for a holding time of 3 days to 80 ℃ for a holding time of 5 days.

Increasing the intensity of dry heating, combined with increasing the pH, increased the extent of egg white protein denaturation. Dry heating impacts the coagulum

formed during gelation, which further is affected by hydrophobic interchange reactions. The free sulfhydryl groups only present in core of ovalbumin undergoes sulfhydryl-disulfide interchange reactions with the disulfide bridges present in other egg white proteins, to increase the surface hydrophobicity (Matsudomi et al., 2002; Mine, 1995, 2002, p. 200). The gelling property of egg white in particular, has often been associated with thermal processing.

Mine (1996), found that not only did an increased pH and intensity of dry heat treatment increase the gel firmness but also, it subsequently increased the rate of deamidation. Even a small increase in the deamidation of proteins has had a significant improvement in the functional properties (Hamada & Swanson, 1994; Shih, 1987; Shih & Kalmar, 1987; Wong et al., 2009; Wu et al., 1976). However, the solubility of egg white powder did not change with improved functionality, until the pH exceeded pH 10.

2.4.3.2. Foaming

Egg white proteins offer high foaming capacity and foam stability because of the interaction of its constituent proteins to form a cohesive viscoelastic film through a surface tension gradient (Mine, 1995; Hammershøj et al., 1999; Ayadi et al., 2008). The unique foaming capacity of egg white could be attributed to the excellent surface-active properties of its constituent proteins. As a result, egg white readily absorbs in the air-water when whipped, undergoing a rapid conformational change and rearrangement in the interface (Mine, 1995). Subsequently it forms a cohesive viscoelastic film through intermolecular interaction. The charge characteristics of constituent proteins: globulins, ovalbumin, lysozyme largely affect the surface-active properties affecting the foam formation (Strixner & Kulozik, 2011). Foaming capacity and foam stability have shown

to increase with increased treatment time and temperature of EWP. Thermal processing increases the flexibility of egg white proteins, specifically ovalbumin which partially unfolds into a stable intermediate stage, also called the 'molten globule' (Kato et al., 1989). The molten globule stage decreases the surface tension and aids the protein to adsorb more easily to the air-water interface. This phenomenon eventually helps retain water and enhances the foam stability (Campbell et al., 2003b; Kato et al., 1989).

Van der Plancken et al. (2007) demonstrated that the moisture content of EWP also had a significant role in affecting the degree of unfolding of proteins and exposing their sulfhydryl groups and hydrophobic residues. Higher solubility and foamability were associated with lower moisture content (<6.8%) and a greater extent of unfolding was observed with higher moisture content (6.8-10%) of EWP.

2.4.3.3. Emulsification

The emulsifying properties of EWP increased with an increase in temperature and time. The increase in the flexibility of proteins and surface hydrophobicity due to thermal processing has been accounted for enhanced emulsifying capacity and emulsion stability. With increased exposure of hydrophobic amino acid residues on the egg white protein surface, the absorption in the oil-in water interface improved (Campbell et al., 2003b). The size of oil droplets also decreased during the spray drying process with increased inlet temperature and residence time over liquid egg white. This suggested improved emulsifying properties of EWP over liquid egg white (Ayadi et al., 2008). However, the absorption of the egg white protein in the oil-water interface is also affected by other driving factors such as: pH of the solution, pressure, ionic strength, and presence of other

emulsifiers, Maillard reaction with glucuronic acid-ovalbumin conjugate formation (Hermansson, 1994).

2.4.3.4. Solubility

The solubility of EWP did not significantly change with thermal processing. Even though the hydrophobicity increased at the protein surface, which subsequently improved the gelling, foaming and emulsifying properties, the solubility remained majorly unaffected.

Kato et al. (1995) reported an increase in the gelling property of EWP by four times when subjected to a temperature of 80 ℃ for 10 days, along with increase in foaming and emulsifying properties with respect to time. Such functional changes were explained by the molten structure or mild conformational changes in the protein structure. However, no significant changes in the solubility of EWP have been reported with thermal processing of EWP (Kato et al., 1989).

2.5. RF processing as an alternative

RF processing, a novel technology has been effectively used for the pasteurization of various low moisture products such as spices (Chen et al., 2019; Jeong & Kang, 2014; Kim et al., 2012; Wei et al., 2019), wheat flour (Liu et al., 2018; Villa-Rojas et al., 2017), dry fruits (Mitcham et al., 2004). It has also been commercially used for post-baking of cookies and meat thawing (Dag et al., 2020).

RF processing can be operated between 3 KHz – 300 MHz using dielectric heating mechanism. U.S. Federal Communications Commission has authorized the usage of only three frequencies i.e., 13.56, 27.12 and 40.68 MHz for industrial, scientific and

medical applications (Piyasena et al., 2003). It works on the principle of volumetric heating, in which the electromagnetic waves penetrate deeper into the product and vibrate and orient the polar molecules with the polarity of the electric field generated. The kinetic energy produced as a result of the rapid change in the alignment of polar molecules (27 MHz) colliding with the neighboring molecules (mostly ions) generates heat (Datta & Davidson, 2000; Piyasena et al., 2003; Jiao, Luan, and Tang, 2014). This heat generated within the product ends up rapidly heating the coldest spot, which is often the geometric center, thereby reducing the come-up time. Therefore, when EWP, a low moisture product was preheated with RF processing, the come-up time reduced from 5 days to less than a day (Boreddy et al., 2014; Chen et al., 2019).

RF waves used at 27.12 MHz have shown to reduce the come-up time in low moisture products by 50-80% as compared to HR processing.

It has been observed that the dry heating used during pasteurization of EWP has resulted in improving its functional properties without compromising the solubility (Handa & Kuroda, 1999; Kato et al., 1989; Lechevalier et al., 2007; Ma et al., 2013). Various factors have been identified for the improvement in functionality such as temperature, pressure, alkalinity, moisture content and holding time. With incorporation of RF processing also, the gelling and foaming properties substantially improved at temperatures higher than 80 ℃.

A pretreatment with radiofrequency processing at 90 ℃ followed by holding up to 16 hours in hot air oven at the same temperature, increased the gel firmness of standard EWP (neutral pH, primarily used for foaming functionality) to as high as traditional

thermally pasteurized high-gel EWP (alkaline pH, primarily used for gelling functionality) (Boreddy et al., 2016).

However, RF processing has been associated with non-uniform heating of food products as compared to traditional thermal methods. Non-uniformity could arise because of several factors like container geometry, moisture content of the sample, thermal properties and dielectric properties of the sample. Non-unform heating could also lead to overheating, which in the case of EWP could have an implication on protein conformation and food safety aspects. Subjecting the sample to hot air circulation and mixing during RF wave application are some of the methods suggested to curb the non-uniformity during heating (Liu et al., 2013; Wang et al., 2006).

2.6. Biological functionality of egg white proteins

2.6.1. Effect of thermal processing on the extent of digestion

The in-vitro gastroinestinal (GI) digestion of egg white proteins happen through gastric and intestinal digestion. The acidic pH (1-3) of the stomach is emulated in the gastric digestion, in the presence of the gastric enzyme pepsin. Egg white proteins undergoes unfolding due to pepsinolysis, which is the slowest stage during the digestion process and the rate limiting stage. At the completion of gastric digestion, the pH is raised between pH 5-7, to mimic the conditions in the small intestine. Bile salts and pancreatin are incorporated, and the system is agitated for intestinal digestion (Jones et al., 2019).

The degree of hydrolysis of dry heated EWP depends on the susceptibility of the egg white proteins to the digestive enzymes. However, the protein conformational changes during thermal processing significantly impacts its susceptibility to

gastrointestinal enzymes (Lechevalier et al., 2015). Ovalbumin and lysozyme have particularly lower susceptibility to digestion, however with thermal processing their susceptibility further changes (Mine, 1995). Heat denaturation changes ovalbumin to a more thermally stable S-ovalbumin (Pelegrine & Gasparetto, 2006), making it susceptible to trypsin digestion.

The degree of hydrolysis increases with a controlled increase in the thermal processing temperature and time during gastric digestion of EWP. Lechevalier et al. (2017) reported a decrease in the degree of hydrolysis of EWP heated for 2 days and longer at 60 ℃ and an increase in the degree of hydrolysis for EWP for 5 or 10 days at 70 ℃, 2 days or longer at 80 ℃, and any time at 90 ℃ after gastric digestion. This was in agreement to the observation of Kato et al. (1990), who also reported an increased proteolysis of ovalbumin and ovotransferrin above 80 ℃. Wang et al. (2018) also reported an increase in the in-vitro digestibility from 86% to 89% of EWP post dry heating at 75 ℃ for 12-18 days. However, stronger dry heating (for 2 days and more at 80 or 90 ℃) resulting in increased protein aggregation has shown to form aggregates with limited enzyme accessible sites (Lechevalier et al., 2015; Mine, 1996).

When EWP is thermally processed into a gel, hydrolysis of the protein takes place after the digestive enzymes can access the cleavage sites. The ability of the digestive enzymes to access the cleavage sites depends on their ability to diffuse in the gel network (Nyemb et al., 2014, 2016). Nyemb et al. (2016) reported that the microstructure of the gels formed directly influence the diffusion of digestive enzymes into the gel network, thereby affecting the degree of hydrolysis. Therefore, with increased gel firmness, the extent of digestibility could decrease. The secondary structural changes in the egg white

proteins due to processing also contributed to the extent of digestion. The resistance of the egg white gel towards the action of trypsin during intestinal digestion has been reported to increase with an increase in β-sheeted structure (Nyemb et al., 2016; Soto & Castaño, 1996).

2.6.2. Bioactivities of egg white proteins

Egg white proteins could be potentially used as functional foods or nutraceuticals because of its multitude biological properties. Egg white protein hydrolysates have exhibited antihypertensive, antioxidant, antimicrobial, mineral-binding, antiadhesive, antidiabetic, immunomodulatory and anticancer properties (Yu et al., 2014).

Egg white has been an important source of peptides inhibiting angiotensin-1 converting enzyme (ACE). These peptides catalyze the conversion of angiotensin I to angiotensin II, a vasoconstrictor, which regulates the blood pressure and promotes hypertension (Chang et al., 2018; Yu et al., 2014). Ovalbumin exhibited antihypertensive activity as a result of the isolation of an octapeptide through chymometric digestion called Ovokinin (Mine, 2007). Ovalbumin has shown an increased antimutagenic activity with increased heat denaturation, and antimicrobial properties against both bacteria and fungi (Sharif et al., 2018). Owing to the carbohydrate-rich β-subunit, ovomucin has shown to possess virus hemagluttination inhibition activity, and antiviral activity against newcastle disease virus, bovine rotavirus and human influenza virus (Tsuge et al., 1996). Ovotransferrin is a monomeric glycoprotein, which can reversibly bind two iron ions per molecule. Therefore, it acts as a natural food antimicrobial by scavenging iron delivery from microbes, particularly pathogenic bacteria like *Pseudomonas spp*., *Escherichia coli*, *Streptococcus mutans*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella enteridis*

(Mine, 2007). Ovotransferrin also modulates macrophage and heterophil functions in chickens and inhibits the proliferation of mouse spleen lymphocytes (Mine, 2007; Sharif et al., 2018). Ovomucoid is relatively resistant to the action of heat and digestive enzymes, thereby making it a potent egg white allergen. It is a proteinase inhibitor and improves the oral delivery of insulin. Ovomucoid also exhibits immune modulating activity by inducing T-cell secretion of cytokines interleukin-(IL) and interferon-gamma (IFN-ϒ) (Mine, Y., & D'Silva, I. 2008). Ovomucin produces glycopeptides which demonstrate antitumor, antiangiogenic, antiadhesive, anticancer, antimicrobial, hypocholestrolemic and immune-modulating activities. Lysozyme has been known as an effective antibacterial because of its ability to hydrolyze the β (1-4) linkage between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in the bacterial cell wall. However, lysozyme has been found to exhibit anticancer, anti-inflammatory and immune-modulatory activities (Mine, 2007; Sharif et al., 2018).

2.6.3. Egg white protein allergenicity

Egg white is one of the big 8 food sources (apart from cow's milk, fish, peanut, tree nut, crustaceans, wheat and soybean) for allergies. Egg allergy is caused by an adverse immunological reaction of egg proteins resulting in atopic dermatitis and eosinophilic esophagitis. The allergy is mediated by IgE antibody. The resistance of egg white proteins towards thermal processing and enzymatic digestion, and the IgE-binding epitopes on the protein molecule, affects their ability to exhibit an immune response (Yoshino et al., 2004). The major egg white allergens are ovomucoid, ovalbumin, ovotransferrin and lysozyme. Since thermal processing induces secondary and tertiary conformational changes in specific egg white proteins, it could potentially influence the

allergenicity by destroying, masking or exposing the epitopes (Chang et al., 2018; Rahaman et al., 2016). Thermal processing could also decrease the allergic sensitization of the egg white proteins (Verhoeckx et al., 2015). Thermal processing also affects the allergenicity of egg white proteins by altering their interaction with other ingredients in a food matrix (Rahaman et al., 2016). Increasing the intensity of thermal processing affects the rate of digestion, which can further influence the absorption of egg white peptides, and their allergenic potential (Rahaman et al., 2016).

2.7. Summary

EWP is an essential component in the food industry because of its multifarious functional as well as biological properties. Application of thermal processing has shown to improve the functional properties such as gelling, foaming, emulsification without affecting the solubility. The wide range of biological activities of egg white proteins after thermal processing has not been widely studied so far, even though surplus works have identified the potential bioactive peptides from specific egg proteins for functional food applications. Thermal processing has shown to aid in reducing the allergenicity of egg white proteins. Producing EWP has remained an energy intensive process, because of its low thermal conductivity. RF assisted thermal processing is a promising alternative towards reducing the processing time for EWP and increasing the energy efficiency of the process. Various studies have reported that RF assisted thermal processing could improve the functional properties of EWP over traditional HR processing. However, the processing parameters in RF assisted thermal processing needs to be optimized specific to the application. It is also essential to understand the effect of RF assisted thermal processing on the protein structural conformation as well as biological functionality before it could be widely used.

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Chapter III - Effect of traditional and radiofrequency assisted thermal processing on the gel firmness of egg white powder

Abstract

Radiofrequency (RF) assisted thermal processing is a novel and energy-efficient method used for pasteurization, which heats the product volumetrically as opposed to traditional hot room (HR) pasteurization. RF-assisted thermal processing accelerates the heating rate of egg white powder (EWP), which has a very low thermal conductivity and further affect its gelling properties. In EWP, solubility plays a significant role in the water holding capacity of proteins, which affects the functional properties. The effect of processing methods (RF and HR) due to pH and treatment temperature combination is monitored by subjecting neutral EWP (6.4 pH) to 70 and 80 °C and alkaline EWP (pH 9.9) samples to 70 °C over 0, 5, 10, 15 and 20 d. RF processing substantially improved gelling properties. At 80 °C, the gel firmness was 48.6% higher than HR on the 0th d, indicating RF could potentially improve the functionality as well as reduce the come-up time to less than a day as opposed to the traditional hot room processing used in the industry which takes 5–6 days. The treatment period also significantly affects the gel firmness and insoluble content. With increased treatment severity, the gel firmness substantially increased without changing the soluble protein contents, and insoluble contents.

Keywords

Egg white powder, Gel firmness, Solubility, Dielectric heating, Pasteurization

3.1. Introduction

The diverse applications of egg white in various food-matrix have been widely known for a long time. The functional properties like gelling, foaming, emulsification in egg white helps in controlling sugar crystallization in confectionaries, binding muscle foods better (Lu & Chen, 1999), thickening custard or pudding products (Stadelman & Cotterill, 1995; Bhandari et al., 2013), providing rigidity to the crumb structure. However, for the ease of handling and transportation with longer shelf life, dehydrated egg white or EWP has seen an increase in demand in the food industry (Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007a; Yoshinori Mine, 1995).

Conventionally EWP is processed by spray drying desugarized egg whites (Handa et al., 2001; Lee & Chen, 1999; Ma et al., 2013). Desugarization prevents Maillard browning and coagulation of egg white before pasteurization (Campbell, Raikos, & Euston, 2003; Lee & Chen, 1999). Furthermore, it limits processing induced degradation of the functional properties of egg white (Lechevalier et al., 2007a). After spray drying, the EWP in ~ 9-kg boxes undergo pasteurization in conventional HR set at 58-60 ℃ for 10-14 days (Northolt, Wiegersma, & Schothorst, 1978; Talansier et al., 2009). The pasteurization step has been modified with extended treatment conditions of 60-80 ℃ from 3-30 days, to ameliorate gelling and foaming properties of EWP (Handa et al., 2001).

EWP, being a low moisture product, does not support the growth of microorganisms, but it is generally used with several other ingredients in a food system. If other ingredients have higher water activity, then they can favor pathogen growth (Boreddy et al., 2014) .

RF assisted thermal processing is a novel thermal processing technology mostly used for low moisture food products. When applied to EWP for pasteurization, RF resulted in enhancing its functionality (Boreddy et al., 2016; Wei et al., 2020). It heats the product volumetrically using high frequency alternating electric field based on the principles of polarization and ionic conductivity (Jiao et al., 2014). Because water molecules and ions are distributed throughout the product, RF waves volumetrically heat the product by vibrating the water molecules and ions that exist throughout the product resulting in more uniform heating. In contrast, traditional thermal processing heats the product from outside resulting in a larger temperature gradient between hot (outside edges) and cold spots (geometric center).

The main factor which affects the heating rate of EWP during conventional pasteurization is its low thermal conductivity. This results in slow heat transfer resulting in a longer come-up time. Come-up time is defined as the time required for the cold spot to reach the target temperature. In the egg industry, HR is used for traditional thermal pasteurization of EWP. In HR, the come-up time for a cubical cardboard box containing ~9 kg of EWP is usually 5-7 days to reach the pasteurization temperature (60 °C). In contrast, RF reduces the come-up time to few hours for the same box depending on RF system power owing to its volumetric heating (Chen et al., 2019). RF heating is more uniform, and the heating uniformity is affected by the electrode configuration of the system, container geometry, surrounding medium and the dielectric properties of the food product (Birla et al., 2004; Tiwari et al., 2011). Using these principles, Boreddy et al., (2014) demonstrated that RF assisted thermal processing of EWP was energy efficient in reducing the pasteurization time from 14 days to less than 1 day.

Gel firmness was the most important functionality for the food industry (Boreddy et al., 2016; Mine, 2002). Boreddy et al. (2016) showed that RF pre-treatment of EWP at higher temperatures improved the functionality in a very short span of time. RF-assisted thermal processing of standard EWP powder (neutral pH, primarily used for foaming functionality) at 90 °C for over 16 hours increased the gel firmness to as high as high-gel EWP (alkaline pH, primarily used for gelling functionality) when compared to traditional thermal processing without affecting foaming. Previous literature reported that severe processing could potentially limit the solubility of EWP due to loss of tertiary structure of proteins (Campbell et al., 2003; Gomes & Pelegrine, 2012). Thus, the goal of this study was to maximize gel firmness without having any detrimental effect in the solubility.

The objective was to maximize the gel firmness of EWP using RF assisted thermal processing of EWP at different temperature, holding time, and pH of EWP. Another objective of the study was to investigate whether RF had any additional non-thermal effect on the gel firmness by comparing RF assisted thermal processing to traditional thermal processing (which is referred to as HR processing in this study) for the same conditions (temperature, holding period, and pH of EWP).

3.2. Materials and methods

3.2.1. Experimental design

Un-pasteurized EWP was procured from Henningsen Foods Inc, Omaha, NE, USA. Three batches of EWP at neutral pH (EWPN) and three batches of EWP at alkaline pH (EWPA) were obtained. EWPN was subjected to a treatment temperature of 70 and 80 °C, while EWPA was subjected only to a treatment temperature of 70 °C. Because gel firmness increased with increased alkalinity, EWPA samples were only subjected to

lower treatment temperature (70 ℃). EWPN at 70 and 80 ℃ and EWPA at 70 ℃ were considered as separate experiments with a factorial-Randomized Complete Block Design (RCBD) structure. The blocks in each of these RCBDs were the batches. The treatment factors in each of these RCBDs were thermal processing method (HR and RF) and holding time (0, 5, 10, 15 and 20 d). The quality parameters being tested were gel firmness, insoluble content and soluble protein content. Each of these RCBDs were separately run with 30 experimental units (2 processing methods x 5 holding time x 3 batches) of 250 g EWP samples.

3.2.2. Proximate analysis

EWP samples (untreated samples at neutral and alkaline pH with 3 replicated per batch) were analyzed for moisture content (AOAC 935.29), ash content using Inductively coupled Plasma emission spectroscopy (AOAC 985.01), crude protein content using the combustion method (AOAC 992.15), fat content using modified Mojonnier ether extraction method (AOAC 989.05), and carbohydrate content by subtracting the cited main components as cited under AOAC methods.

3.2.3. Processing methods

3.2.3.1. Hot room (HR) processing

The traditional HR processing of EWP was carried out in a lab-scale process simulating the industrial process by using a forced air convection oven (VWR force air oven, 104 L, VWR International, USA) set at the given treatment temperature (70 and 80 ℃) in order to simulate the conditions of traditional HR treatment. EWP sample (250 g) was uniformly distributed in a cylindrical polypropylene container (190 mm diameter, 23 mm height and 6 mm thickness). Excess loss of moisture was prevented by covering the

exposed top surface of the sample container with aluminum foil. At this stage, the samples were placed inside the forced convection oven. The come-up time required by the EWP to reach the treatment temperature was monitored using thermocouples, which were inserted into the cold spot (geometric center) of the sample container inside the forced convection oven (Boreddy et al., 2014). The beginning of the treatment period was measured from the time at which the coldest spot reached the treatment temperature. EWPN from the 3 production lots were subjected to a treatment temperature of 70 ℃ and 80 ℃ respectively. EWPA samples from the 3 different production lots were subjected to a treatment temperature of 70 ℃. The holding time i.e., the amount of time the sample was held at the treatment temperature inside the hot room was maintained between 0 to 20 d for all the samples, with samples being drawn out on every 5th day to analyze their functional properties.

3.2.3.2. Radiofrequency (RF) assisted thermal processing

The RF-assisted thermal processing of EWP was accomplished by subjecting the EWP to radio waves before holding it in forced convection oven for equivalent time intervals as that of HR processed samples. A 6-kW parallel plate configured RF oven operating at 27.12 MHz (Model SO-6B, Monga Strayfield Pvt. Ltd., Pune, India) was used for preheating the samples to target temperatures with a shorter come-up time. The EWP sample (250g) was placed inside the RF oven, and the gap between the parallel electrode plates was adjusted at 105 mm. The system was turned off as soon as the cold spot reached the target temperature of 70 and 80 ℃ as per the experiment. The heating duration in the RF was ~5 min. The sample was then covered with a sheet of aluminum foil and placed inside the forced convection oven, which was already set at the target

treatment temperature. The samples were further held in the forced convection oven following the same method as HR treatment (section 3.2.3.1) to analyze the effect of the treatment period. For brevity purposes, the RF-assisted thermal processing will simply be called as RF in the following sections.

3.2.4. Gel preparation

Egg white gels were prepared by reconstituting 50 g of egg white in 350 mL of distilled water in a mixer-blender (700 power watts, Oster 10 blade mixer, United States) at low speed without whipping. The reconstituted egg white solution (200 mL) without foam was poured into Viscofan fiber-reinforced cellulose casings of 30 mm diameter and 250 mm length. The sealed casings containing egg white solution were slow-cooked in a water bath at 80 ℃ for 35 min and thereafter cooled in an ice-water bath for 5 min and stored at 4 ℃ for 12 h. The egg white gels were brought to room temperature before testing the gel firmness. Each gel was cut into cylinders (30 mm height) for gel strength measurement.

3.2.5. Gel firmness

The gel firmness was measured using Texture Analyzer (TA.XT. plus, Stable microsystems, Texture Technologies Corporation, Hamilton, MA, 01982), using an 8 mm diameter uniaxial penetration ball probe. The maximum force (50 kg) exerted by the probe into the gel at a penetration speed of 2 mm sec-1 up until a penetration depth of 25 mm with a double penetration was analyzed to give the gel firmness of the egg white samples.

3.2.6. Insoluble fraction

The insoluble fraction determined the extent of the solubility of egg white powder in water. EWP solution was made at 10% concentration mimicking real egg white by reconstituting EWP in water in pre-weighed 15 mL centrifuge tubes and centrifuging the solution at 10000 g x 30 minutes at 20 ℃. The supernatant was collected, and the weight of the retentate in the original pre-weighed tube was recorded. The ratio between the amount of retentate in the sample to the amount of retentate in the control (untreated sample) gave the relative insoluble fraction due to treatment. Furthermore, soluble protein content in the sample was analyzed as an effect of treatment as discussed in the Section 3.2.7 (Nicorescu et al., 2011).

3.2.7. Soluble protein content

The soluble protein content was measured using the soluble content (supernatant) obtained after centrifuging the sample (10% EWP solution). Lowry's method of protein content determination was used to assess the protein content in the sample, using Chicken Serum Albumin (Sigma Aldrich, sterile filtered, 98% pure) as the standard (Lowry & Rosebrough, 1951). The absorbance values were read using a microplate reader (Synergy H1, Hybrid Multi-mode reader, Biotek Instruments, Winooski, VT, USA) at 750 nm (Waterborg, 2009).

3.2.8. Statistical analysis

A combined analysis of variance was conducted for EWPN at 70 and 80 ℃ to evaluate the effect of treatment temperature on the processing method (RF vs HR) and holding time. Because the batches in EWPN at 70 ℃ and EWPN at 80 ℃ were common, the effect of batch (block) and its interaction with the treatment factors were also evaluated.

Furthermore, a combined analysis of variance was conducted for EWPN and EWPA at 70 ℃ to evaluate the effect of pH on the processing method and holding time. The batches considered as blocks were different in EWPN and EWPA, therefore unlike the previous case, it was not possible to test the main effect of batch and its interaction with treatment. For both the combined analyses, it was assumed that a 250 g EWP sample was the experimental unit for each treatment level (temperature or pH), with a specific processing method and holding time. The response variables were gel firmness, soluble and insoluble protein content. Separate analysis of variances for each of EWPN at 70 ℃, EWPN at 80 ℃, and EWPA at 70 ℃ were conducted to evaluate the effect of thermal processing method and holding time on the gel firmness alone, as gel firmness was the most important quality factor in this study.

3.3. Results and discussion

3.3.1. Proximate analysis

Table 3.1. summarizes the proximate composition of EWP samples averaged over 3 production batches. There were two categories of EWP samples, one with a neutral pH denoted as EWPN (pH 6.4) and the other with an alkaline pH denoted as EWPA (pH 9.9). As observed from Table 3.1, the moisture contents, ash contents, lipid contents, and protein contents did not vary between EWPN and EWPA, which was in agreement with previous works (Chang et al., 2020; Olayemi et al., 2016). However, the carbohydrate content in EWPN was nearly two-fold higher than that of EWPA (p-value = 0.0423), suggesting that the alkaline treatment might be releasing the N or O-linked carbohydrates moieties from the glycoproteins (i.e. Ovomucin, Ovomucoid) in egg white (Argade, Daves, Van Halbeek, & Alhadeff, 1989; Offengenden, Fentabil, & Wu, 2011).

3.3.2. Functional properties

Table 3.2. summarizes the combined ANOVA results due to the treatment temperature on EWPN. RF processing substantially improved the gel firmness at both the treatment temperatures. The gel firmness significantly increased with an increased treatment temperature and holding time and their interaction. The insoluble content was significantly affected by holding time; however, it did not show a specific trend (discussed in detail in Section 3.3.2.2). Similar to the insoluble content, the soluble protein content also was significantly affected by treatment temperature and holding time; however, it did not show a specific trend (discussed in detail in Section 3.3.2.3).

Table 3.3 summarizes the combined ANOVA results due to the difference in the pH of EWP treated at 70 °C. RF processing significantly increased the gel firmness. Gel firmness also increased with increased alkalinity and holding time, and their interactions. Contrary to insoluble content, the soluble protein content underwent significant changes only with respect to holding time; however, these changes did not follow a specific trend (discussed later in section 3.3.2.3). The insoluble content was not affected by any of the treatment factors (Table 3.3).

An increased pH increased the ability of the proteins to unfold and aggregate because of the change it induced on the net charge of the protein. Furthermore, the gel firmness was enhanced as a result of an accelerated SH-SS interchange reaction (Beveridge & Arntfield, 1979; Chen et al., 2015; Renkema et al., 2002). This was corroborated in the present study, where the gel firmness increased significantly with increased pH. RF had a significant effect on functional properties of EWP, when compared to HR.

3.3.2.1. Gel firmness

Table 3.4. summarizes the effect of thermal processing methods used (HR and RF) and holding time (0, 5, 10, 15 and 20 d) for EWPN at 70 and 80 ℃ and EWPA at 70 ℃. The interaction effect was not significant and main effects (processing and time) were significant.

Hermansson (1994) reported that the gel firmness in EWP was majorly affected by protein concentration, heating rate used while cooking the gels, gelation temperature and pH. The protein concentration, heating rate, and gelation temperature were kept constant in this study. As processing intensity (temperature and holding time) increased, the gel firmness also increased.

RF processing significantly increased the gel firmness for neutral pH EWP at both the treatment temperatures. However, the processing method had borderline significance at alkaline pH EWP (Table 3.4). Holding time significantly improved gel firmness for all samples.

Figure 3.1. summarizes the gel firmness at 70 and 80 ℃ for EWPN samples. The gel firmness increased from 0th d until 15th d, and then it declined subsequently for EWPN at 70 ℃ and plateaued for EWPN at 80 ℃. The effect of RF processing on each of the holding times revealed that, on the 10th and 15th d of EWPN at 70 ℃, the gel firmness was substantially higher (Figures 3.1 and 3.2).

For EWPN at 80 ℃, a significant increment of 48.6% in the gel firmness was observed with RF processing over HR processing ($p < 0.05$) on the 0th d. With increased holding time up to 5 d, the gel firmness was still higher with RF processing even though

it was not statistically significant. Boreddy et al. (2016) showed that the gel firmness of RF processed EWP at 90 ℃ (for 16 hours) was 60% higher than traditional HR processed (at 60 ℃) EWP samples obtained from the industry (Boreddy et al., 2016). In the present study as well, RF significantly increased the gel firmness on the 0th d at higher treatment temperature (80 ℃).

Figure 3.2. illustrates the effect of RF over HR processing for EWPN and EWPA samples treated at 70 ℃ for 20 days of holding time. The gel firmness of EWPA also followed the same trend as that of EWPN at 70 ℃ from 0th d to 15th d, and then it plateaued over the next five days. A 25.5% increase in the gel firmness was observed on the 10th d with RF processing of EWPA at 70 ℃ (Figure 3.2).

Boreddy et al. (2016) had reported that the gel firmness of EWP at neutral pH matched the gel firmness at alkaline pH when both were subjected to RF processing at higher temperatures (90 ℃) and held for over 4 hours (Boreddy et al., 2016). However, in the present study the gel firmness of EWPA with RF processing had a higher gel firmness over EWPN with RF processing at 70 ℃ when the holding times were constant.

A combination of faster volumetric heating followed by holding at higher treatment temperature for elongated time and elevated pH (alkaline as compared to neutral) resulted in maximum gel firmness (555.98 g at 70 ℃ for EWPA) as shown in Figure 3.2. This was in accordance with previous works, which had shown increased gel firmness over increased pH and heating time and a reduction in free-amino residues as a subsequent effect (Mine, 1996).

3.3.2.2. Insoluble fraction

Table 3.5. summarizes the insoluble protein content obtained relative to the insoluble content of the untreated EWP sample in each case over processing methods (HR and RF) and 20 days of holding time. The insoluble fraction decreased with an increased intensity of treatment temperature and pH (p-value = 0.0001). It is commonly known that egg white protein loses its solubility at pH values closer to isoelectric point (pH 4.5) and elevated treatment temperatures (above 55 ℃) (Gomes & Pelegrine, 2012; Van der Plancken, Van Loey, & Hendrickx, 2006). Thus, there was no significant effect of RF and increased holding time on the insoluble content of EWP.

When EWP was at alkaline pH (pH 9-11) which was further away from its isoelectric point (pH 4.5), it showed higher increased water holding capacity. An increased solubility was also observed at alkaline pH. As the EWP approached pH 5-7, which was nearer to the isoelectric pH, the solubility of EWP declined, thereby causing a decline in the water holding capacity as well (Handa, Takahashi, Kuroda, & Froning, 1998). This was in agreement with the current work, where lower insoluble content was observed in alkaline pH samples.

With a change in either pH, temperature, protein concentration, moisture content or salt concentration of the EWP solution, the ratio of hydrophilic to hydrophobic residues present also changed (Campbell et al., 2003; Sheng et al., 2018; Van der Plancken, Van Loey, & Hendrickx, 2005; Van der Plancken et al., 2006). Hydrophobic groups, which facilitated protein-protein interaction, were often embedded in the protein core. Thus, it was mostly the number of hydrophilic groups on the surface that determined the extent of protein solubility (Campbell et al., 2003). The chromatographic

study of native egg white and spray dried egg white revealed that the solubility of the globulin proteins was most sensitive to spray drying process (Galyean & Cotterill, 1979). EWP did not significantly change on a protein structural level after passing through a spray drier, but it initiated most of the changes in the solubility of rehydrated EWP and significantly affected the functional properties thereafter (Galyean & Cotterill, 1979; Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007b).

3.3.2.3. Soluble protein content

Table 3.6 summarizes the soluble protein content relative to untreated EWP sample in EWPN at 70 ℃ and 80 ℃ and EWPA at 70 ℃ for 20 days of holding time. It was significantly affected by the holding time (Tables 3.2 and 3.3) and treatment temperature (Table 3.2) used. However, there was no specific trend observed in the soluble protein contents for EWPN at 70 and 80 ℃ and EWPA at 70 ℃.

It was stipulated that the soluble protein content did not undergo discernible changes due to the thermal processing method used indicating that processing did not significantly affect protein conformational changes in EWP. This was in agreement with a previous study on EWP which reported that the soluble protein content varied greatly at lower temperatures (40-60 ℃) between neutral and alkaline pH. At higher temperatures (90-100 ℃) though, there was no significant difference on the soluble protein content even with a substantial difference in pH values (Gomes & Pelegrine, 2012).

Earlier works also reported that the removal of glucose from EWP and maintaining the moisture content of EWP at 6.8-7% had a similar impact on solubility. No significant difference in soluble protein content and insoluble content was reported

due to thermal processing method used (Van der Plancken et al., 2007; Kijowski et al., 1995; Boreddy et al., 2016).

3.4. Conclusion

Overall, RF processing significantly increased the gel firmness of EWP at elevated treatment temperature and pH. The soluble protein content and insoluble content were not affected by thermal processing method used. Holding time caused some fluctuations in the soluble protein content, but no specific trend howsoever was observed. The gel firmness also substantially increased with an increased treatment intensity (temperature, pH and holding time). RF processing was highly advantageous for EWPN, because a huge enhancement in gel firmness (48.6%) was observed on the 0th d at 80 ℃. An increase of 11-12% in the gel firmness with RF processing of EWPN was also observed at 70 ℃. Maximum gel firmness (555.98g) was found at EWPA treated with RF at 70 ℃ for 10 d holding time. From the industrial application perspective, RF also has the advantage of reducing the come-up time to less than 1 d when compared to a week of come-up time in traditional thermal processing. Thus, RF can be vital for improving the gel firmness in EWPN without affecting the solubility and making the pasteurization process energy efficient.

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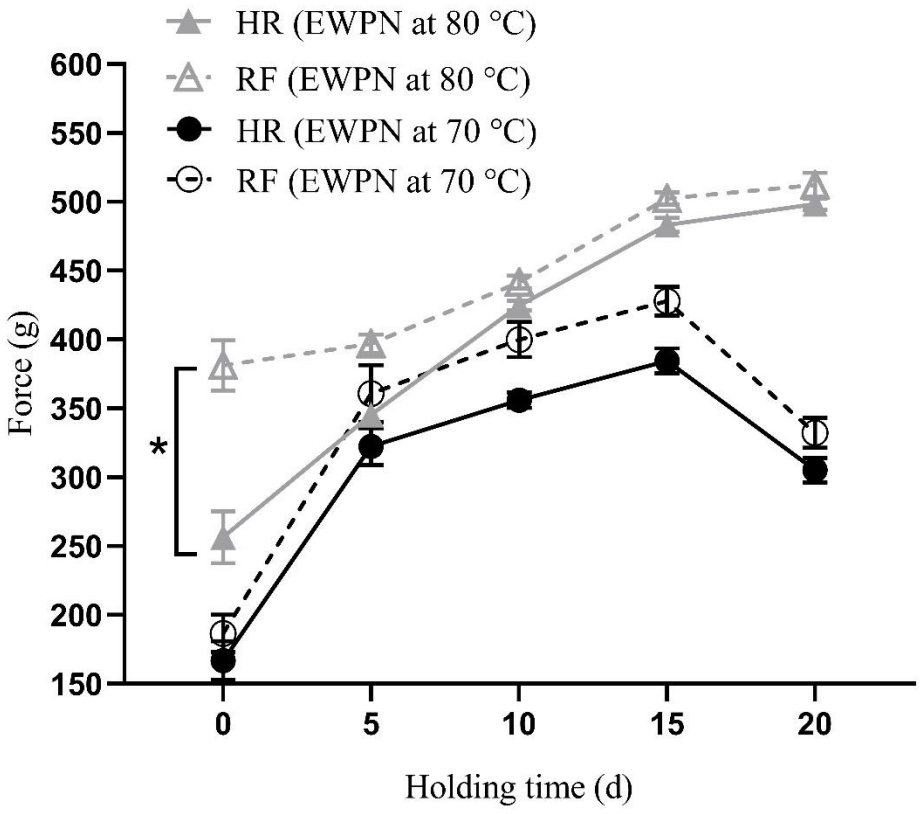

Figure 3.1. Effect of treatment temperature and processing methods (HR and RF) on the Gel firmness (Force in (g)) values of EWPN at treatment temperatures 70 and 80 ℃ analyzed using least square means and '*' indicates significant difference.

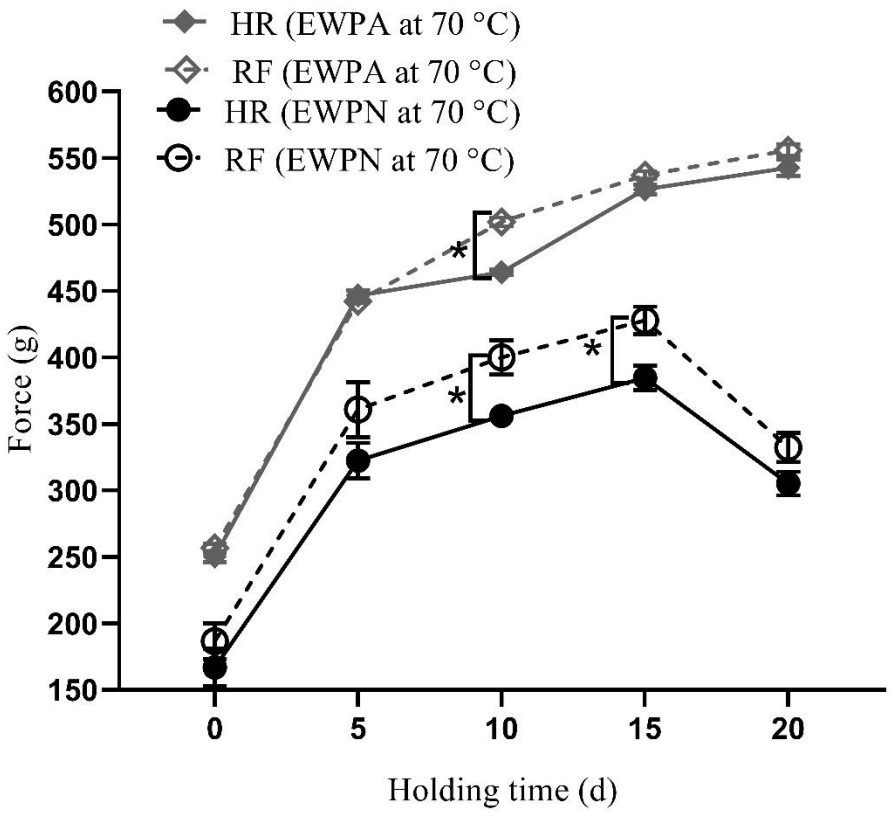

Figure 3.2. Effect of pH and processing methods (HR and RF) across holding times on the gel firmness (Force in (g)) values of EWPN and EWPA at a treatment temperature of 70 ℃ analyzed using least square means and '*' indicates significant difference.

Table 3.1. Proximate analysis (in dry basis, g/100 g) and pH of untreated EWP samples from three production lots (mean ± standard deviation)

Sample	pH	Moisture	Ash	Lipid	Protein	Carbohydrate
EWPN	6.4 ± 0.10	7.8 ± 0.26	4.7 ± 0.22	< 0.5	83.2 ± 0.29	4.1 ± 0.28
EWPA	9.9 ± 0.05	7.6 ± 0.17	4.6 ± 0.09	< 0.5	85.2 ± 0.58	2.6 ± 0.39

Table 3.2. Effect of temperature, processing method, and holding time on the gel firmness, soluble protein content and insoluble content of EWPN.

	p-value		
	Gel firmness	Insoluble content	Soluble protein content
Processing[$]	<.0001	0.6331	0.424
Temp	0.0356	0.0513	0.0321
Time	<.0001	0.0002	0.0083
Processing x Temp	0.5568	0.0071	0.4625
Time x Processing	0.4311	0.7139	0.6233
Temp x Time	<.0001	0.1672	0.0064
Temp x Time x Processing	0.1411	0.0567	0.8357
Batch	0.1658	0.1918	0.1802
Batch x Temp	0.0167	0.0272	0.5211

$Processing refers to the processing method used - HR and RF.

Table 3.3. Effect of pH, processing and holding time on the on the gel firmness, soluble protein content and insoluble content for EWPN and EWPA at 70 ℃

	p-value		
	Gel firmness	Insoluble content	Soluble protein content
Processing[$]	0.0001***	0.1204	0.874
pH	0.015**	0.191	0.25
Time	<.0001***	0.9892	0.0036**
pH x Processing	0.0565	0.9941	0.9298
Time x Processing	0.5427	0.9478	0.5642
pH x Time	<.0001***	0.2104	0.0185
pH x Time x Processing	0.8199	0.1942	0.9203
Batch(pH)	<.0001***	<.0001***	0.0171**

[$]Processing refers to the processing method used – HR and RF.

Table 3.4. Separate ANOVA results for the gel firmness due to processing methods used and holding time for EWPN at 70 ℃, EWPN at 80 ℃ and EWPA at 70℃

	p-value		
	EWPN 70 ℃,	EWPN 80 ℃,	EWPA 70 ℃,
Processing[$]	0.0014**	0.0096**	0.0559
Time	<0.0001	<0.0001***	<0.0001***
Processing x time	0.8903	0.1725	0. 3276

[$]Processing refers to the processing method used - HR and RF. Statistical significance is categorized as p-value < 0.05 symbolized with *, *p*-value < 0.005 symbolized with ** and *p*-value < 0.0005 symbolized with ***.

Table 3.5. Effect of processing method across holding time on EWPN at 70 ℃ and 80 ℃, and EWPA at 70 ℃ on the relative insoluble content. The mean values with the same letter (superscript) are not significantly different at α=0.05. The first letter in the superscript describes the mean comparison between columns in each row and the second letter describes the mean comparison between rows in each column. Tukey's Honest significant difference has been used for multiple comparison.

Time (d)	EWPN at 70 ℃		EWPN at 80 ℃		EWPA at 70 ℃	
	HR	RF	HR	RF	HR	RF
0	1.38^{aa}	1.13^{aa}	0.83^{aa}	1.00^{aa}	1.15^{aa}	1.22^{aa}
5	1.28^{aa}	1.24^{aa}	0.98^{aa}	1.11^{aa}	1.13^{aa}	1.13^{aa}
10	1.29^{aa}	1.44^{ab}	1.10^{aa}	1.18^{aa}	1.16^{aa}	0.92^{aa}
15	1.45^{aa}	1.31^{aa}	1.15^{ab}	1.30^{ab}	1.08^{aa}	0.97^{aa}
20	1.36^{aa}	1.28^{aa}	0.95^{aa}	0.99^{aa}	1.18^{aa}	1.10^{aa}

[$]Standard errors were calculated over 3 batches which were considered as triplicates. The standard error values for EWPN at 70 ℃ = 0.0686; EWPN at 80 ℃ = 0.06429; EWPA at 70 ℃ = 0.1233

Table 3.6. Effect of processing method across holding time on EWPN at 70 ℃ and 80 ℃; EWPA at 70 ℃ on the relative soluble protein content. The mean values with the same letter (superscript) are not significantly different at α=0.05. The first letter in the superscript describes the mean comparison between columns in each row and the second letter describes the mean comparison between rows in each column. Tukey's Honest significant difference has been used for multiple comparison.

Time	EWPN at 70 ℃		EWPN at 80 ℃		EWPA at 70 ℃	
(d)	HR	RF	HR	RF	HR	RF
0	1.10^{aa}	1.17^{aa}	0.91^{aa}	0.98^{aa}	1.00^{aa}	1.04^{aa}
5	1.12^{aa}	1.14^{aa}	1.05^{aa}	1.05^{aa}	1.04^{aa}	1.07^{aa}
10	1.06^{aa}	1.02^{aa}	1.00^{aa}	1.06^{aa}	1.05^{aa}	1.06^{aa}
15	0.98^{aa}	0.98^{aa}	0.97^{aa}	1.02^{aa}	1.06^{aa}	1.01^{aa}
20	1.21^{ba}	1.17^{aa}	1.03^{aa}	1.00^{aa}	1.08^{aa}	1.07^{aa}

[$]Standard errors were calculated over 3 batches which were considered as triplicates. The standard error values for N70 = 0.0483; N80 = 0.0416; A70 = 0.0341.

Chapter IV - Effect of radiofrequency assisted thermal processing on the structural conformation and bio-functional properties of egg white proteins

Abstract

The effect of RF processing with respect to HR processing on the structural and bio-functional properties of egg white proteins was investigated. The secondary structure of egg white protein was evaluated using surface hydrophobicity, circular dichroism spectroscopy, and Raman spectroscopy for EWP processed at 80 ℃ and 0 d. RF processing conferred no significant differences in the exposure of buried hydrophobic residues, and egg white protein secondary structures as compared to HR processing. The bio-functional properties due to RF processing were evaluated on the hydrolysates from invitro gastrointestinal digestion of egg white gels obtained from EWP processed at 80 ℃ and 0 d. Minor differences were observed in the digestibility due to RF processing. However, an increasing trend was recorded in the bioaccessibility and bioavailaibility of peptides from the soluble fraction of egg white gel hydrolysates. An in-silico analysis of the peptides in the soluble fraction of egg white gel hydrolysates demonstrated the generation of unique peptides with different cleavage sites, from Ovalbumin and Ovotranferrin. The structural integrity and viability of epithelial cells (Caco-2 cells) used in the bioavailability study was not compromised by RF processing.

Keywords

Secondary protein structure, Egg white gel hydrolysate, structure-function relationship, invitro gastrointestinal digestion, Bioaccessibility

4.1. Introduction

EWP is well-known for its gelling properties with various applications in the bakery, confectionery, and meat products (Handa et al., 1998; Lu & Chen, 1999). These applications have been broadly associated with protein denaturation and aggregation, causing gel network formation (Gosal & Ross-Murphy, 2000). The water-protein interaction and protein-protein interactions in gelation result from exposure of the buried hydrophobic groups from the protein core. These groups aggregate and form fine stranded networks or amorphous particulate gels. For heat-induced globular protein gels as egg white gel, there is a rather small effect on the actual protein size and shape. Increased gel firmness is characterized by the conversion of α-helix structure into β-sheeted structure and increase in the random coil structure in the secondary protein conformation (Gosal & Ross-Murphy, 2000; Haskard & Li-Chan, 1998; Kato & Nakai, 1980; Painter & Koenig, 1976). There are a host of implicit variables contributing to egg white gelation such as pH, temperature, pressure, treatment time, ionic strength, protein concentration, and amino acid sequence (Croguennec et al., 2002; Gosal & Ross-Murphy, 2000; Handa et al., 1998, 2001; Handa & Kuroda, 1999). Dry heating or thermal processing coupled with holding time has been generally used for pasteurizing EWP. The gelling properties have shown a substantial rise with higher dry heating temperature and longer holding times (Mine, 1995, 1996, 1997). However, because of the low thermal conductivity of EWP, the come-up time in dry heating used for pasteurization (at 60 °C) is almost 5 days (Boreddy et al., 2016). Therefore, RF assisted thermal processing, a dielectric heating technique coupled with dry heating was introduced to reduce the come-up time in the pasteurization of EWP from 5 days to less than a day (Ahmed et al., 2007;

Awuah et al., 2015; Kar et al., 2020; Wei et al., 2019). Interestingly, RF processing reported an improvement in gelling, foaming, and emulsifying properties over traditional dry heating in hot rooms (Boreddy et al., 2014, 2016). Our earlier study (Kar et al., 2020) observed a 48% increase in the gel firmness of EWP processed at 80 ℃ without further exposure to dry heating. Furthermore, with a holding time of 10 days at 70 ℃ after RF processing, a 12% increase in the gel firmness over the traditional hot room (HR) dry heating was recorded. However, the implication of RF processing on the egg white protein structure, which is further causing an improvement in gel firmness, remains unknown.

Egg white protein has also been deemed significant as a functional food and associated with many beneficial health outcomes (Wu et al., 2010). Since ingested proteins are always digested in the gastrointestinal tract, any biological effects observed are due to peptide fragments, amino acids, or their metabolites produced after gastrointestinal digestion. However, heat treatment and gelation could potentially reduce the accessibility of the digestive enzymes and reduce the bioaccessibility of peptides and amino acids. Gel microstructure has shown to significantly affect the bioaccessibility and eventually bioavailability of egg white proteins (Farjami et al., 2021). Therefore, effective processing of EWP for improving gelling properties would entail not just the mechanism of dry heating with other implicit variables, but also its subsequent impact on the biological functionality. Since RF processing has a significant effect on the egg white gel firmness, it could also aid further differences in the biological functionality. These overlapping factors could majorly impact the broader usage of RF processing as an alternative pasteurization method.

The structure-function relationship in egg white proteins, and the effect of gel microstructure on biological functionality due to dry heating has been somewhat explored. However, the effect of dielectric heating using RF assisted thermal processing on egg white protein has remained largely unknown. The effect of dielectric heating on the egg white protein secondary conformation which further enhances the gel firmness is also unclear. Furthermore, the extended effect of improved gelation due to RF assisted thermal processing on the biological functionality, has yet to be explored.

Therefore, the present study aimed to investigate the impact of RF assisted thermal processing on the structural conformation of egg white proteins as compared to traditional dry heating or HR processing. This study also examines the effect of RF processing on the biological properties such as bioaccessibility and bioavailability of the resulting egg white gels-derived peptides through quantitative and qualitative methods.

4.2. Materials and methods

4.2.1. Experimental design

EWP (Egg white powder at neutral pH) procured from Henningsen Foods Inc, Omaha, NE, USA was processed in traditional HR and RF assisted thermal processing at 70 and 80 ℃ for 0 and 15 d holding time based on Kar et al (2020). Three production batches of EWP were used which served as biological replicates for all the experiments. The soluble protein content and peptide content was measured to analyze the effect of treatment temperature, holding time, and processing method. The effect of processing was analyzed using surface hydrophobicity, circular dichroism spectroscopy, and Raman spectroscopy on processed EWP at 80 ℃ and 0 d (where significantly high gel firmness was observed). To further analyze the biological functionality, egg white gels from

processed EWP at 80 ℃ and 0 d were subjected to simulated gastrointestinal digestion. The insoluble and soluble egg white gel hydrolysates were analyzed for bioaccessibility, insilico analysis, structural analysis, and bioavailability.

4.2.2. Soluble protein content

Lowry's protein estimation assay was modified according to Kar et al., (2020) and Rao et al., (2020) for soluble protein content estimation in HR and RF processed EWP at 70 and 80 ℃ for 0 d and 15 d. The sample preparation was as follows: 100 mg/mL of EWP solution was prepared in double distilled water and centrifuged in TX-400 rotor at 4696g for 30 min at 20 ℃. The supernatant was filtered using a 0.45 µm nylon syringe and serially diluted with double distilled water to obtain 1 mg/mL solution of EWP. The complex forming alkaline reagent was prepared using 2% (w/v) Sodium carbonate, 2% (w/v) Sodium potassium tartrate, and 1% (w/v) Copper sulphate solution at 200:1:1. The sample (1mg/mL of EWP solution) and the complex forming reagent were rigorously mixed at a ratio of 1:10 and incubated at 37 ℃ for 10 min. Folin's reagent was added to the sample-reagent mixture at 0.1 N and incubated at 37 ℃ for 30 min for color development, which was further quantitated using 800 TS microplate reader (Bioteck, Winooski, VA, USA) at 750 nm. The standard curve was developed using 0.1 mg/mL to 1 mg/mL solution of Chicken Serum Albumin (Sigma Aldrich, CS405-100mL, sterile filtered, 98% pure).

4.2.3. Peptide content

Pierce^{TM} quantitative fluorometric peptide assay (23290, Thermo Scientific, Waltham, MA) was used for estimating the peptide content in the HR and RF processed EWP at 70 and 80 ℃ for 0 d and 15 d. The sample was prepared to a concentration of 1

mg/mL using the same method as used in the Lowry's protein estimation assay (as described in Section 4.2.2). Further, it was diluted with double distilled water to a concentration of 125 μg/mL. The fluorometric peptide assay buffer and the fluorometric peptide assay reagent were loaded on the samples and incubated for 5 min. The fluorescence was measured at an excitation wavelength of 390 nm and an emission wavelength of 475 nm using 800 TS microplate reader (Bioteck, Winooski, VA, USA).

4.2.4. Surface hydrophobicity

The surface hydrophobicity measurements were determined on 1 mg/mL aliquots of treated EWP dispersions diluted with 0.1 M phosphate buffer to obtain the final concentrations of 0.03125 mg/mL, 0.125 mg/mL, and 0.5 mg/mL for each sample. Fluorescence intensity was measured using the fluorescence probe 1-anilino-8-napthalen-sulfonate (ANS, Sigma-Aldrich Inc., St Louis, MO, USA) according to the method described by Kato & Nakai (1980) and modified by Arzeni et al. (2012). The ANS probe was added to the samples at a ratio of 1: 200 and incubated for 5 minutes. The fluorescence was measured at an excitation wavelength of 390 nm and an emission wavelength of 468 nm at a scanning speed of 10 nm/s, before and after addition of ANS, using 800 TS microplate reader (Bioteck, Winooski, VA, USA).

4.2.5. Raman spectroscopy

The Raman spectra of the samples were performed using XploRA ONE™ 785 nm Raman Spectrometer System (HORIBA, Ltd., Kyoto, Japan) with a 785 nm near-infrared diode laser. A small portion of sample (1-2 mg) was mounted on a gold-silicon microscope slide and was uniformly compressed on the surface using a glass slide. The spectra were plotted as intensity (arbitrary units) versus the Raman shift in wavenumber

(cm^{-1}) with a detection range from 400 to 2000 cm^{-1}. An objective lens of 50x magnification and a 10% filter was used for detection. Three random spots were selected for each sample and three time points of Raman spectra were collected for each of the three spots, which was equivalent to 9 technical replicates. Raman spectra was acquisitioned for egg white powder after RF and HR processing and egg white gel retentate powder after completion of in-vitro gastrointestinal digestion. The spectral data were baseline corrected using protein phenylalanine peak at 1003±1 cm^{-1}. The analysis was conducted on processed EWP and insoluble egg white gel hydrolysates.

4.2.6. Circular Dichroism

The secondary protein structures of processed EWP were measured using CD spectropolarimeter (Jasco Co J-720., serial number 9078142, Tokyo, Japan). EWP were diluted in 50 mM Phosphate buffer to obtain 0.1 mg/mL solution for which the CD spectra were acquired from 250-200 nm, at 100 nm/min scan speed, 0.5 s response time, 0.5 nm bandwidth and standard sensitivity. The measurements were performed using a circular quartz cell of 0.1 cm path length at 25 °C. The curve fitting was performed using the main model spectra, consisting of α-helix, β-sheet and coil in the PEPFIT program, according to Reed & Reed (1997).

4.2.7. Simulated gastrointestinal digestion

Simulated gastrointestinal digestion was performed for egg white gel samples (Kar et al., 2020) made from EWP processed at 70 and 80 ℃ for 0 d and 15 d. The digestion was performed for oral, gastric, and intestinal phase using the INFOGEST protocol (Brodkorb et al., 2019), with minor modification in the preparation of digestion fluids from Minekus et al (2014). The simulated digestion fluids for each phase were

freshly prepared (the compositions are listed in Table 4.1 and Table 4.2). For the oral phase, 50 g of freshly prepared gel was minced in a manual mincer (Kitchen Basics, ASIN: B00JX0ENHE) to simulate chewing action in the mouth. It was further stomached with 9 mL of simulated salivary fluid and 1 mL of α-amylase (750 U/mL) tempered at 37 °C for 6 minutes in a Seward Stomacher^{R} (Seward; BA6040/CLR). The gastric and intestinal phase were performed in a 250 mL jacketed beaker clamped onto Titrando 902 pH-stat coupled with an 800 dosino device (Metrohm AG, Herisau, Switzerland) used in recording the pH and the volume of titrand used in adjusting the pH. The oral bolus was mixed with 60 mL of simulated gastric fluid, and the pH was adjusted to 3.00. Next, 3.2 mL of gastric porcine (25,000 U/mL) solution was added, and the gastric digestion was run for 3600 s, maintained at 37 °C. At the end of gastric digestion, 20 mL of gastric digested sample was collected for further analysis and to the remaining 100 mL of digest, 90 mL of simulated intestinal fluid was added, and the pH was adjusted to 7.00. Thereafter, 10 mL of pancreatin solution (800 U/mL) was added intestinal digestion was run for 3600 s, maintained at 37 °C. The digestion was terminated by lowering the pH to 6.00. The intestinal digesta was then centrifuged at 10000g for 30 minutes and the supernatant was frozen at -80 °C, for freeze drying later, and further analysis.

The degree of hydrolysis (DH) was calculated using pH-stat method and Mitscherlich Model.

Degree of hydrolysis in the gastric phase,

$$DH_{gastric} = 100 \times \frac{V(HCl) \times M\,(HCl)}{m\,(protein) \times htot} \times \frac{1}{1-\alpha\,(COOH)}$$

Degree of hydrolysis in the intestinal phase,

$$DH_{intestinal} = 100 \times \frac{V\,(NaOH) \times M\,(NaOH)}{m\,(protein) \times htot} \times \frac{1}{\alpha(NH2)}$$

V is the titrant volume (mL), M the molarity of the titrant (M), m is the protein mass (g), h_{tot} is the number of peptide bond per gram of protein (7.67 for egg), α (COOH) the mean degree of dissociation of carboxylic acid (0.09), and α(NH2) the mean degree of dissociation for the amino groups (0.44).

4.2.8. Peptide profile analysis

The reverse phase separation was performed for soluble egg white gel hydrolysate after HR and RF processing, and the apical and basal layer obtained from the transport study using these soluble egg white gel hydrolysates. For separation of peptides, 10 mg of sample was resuspended in water to a final concentration of 20 µg/µL. The larger peptides were separated using a 2-times diluted sample, run by reverse phase ACCQ-TAG ULTRA C18 1.7 um (2.1 x 100 mm, Waters) column using a Vanquish (Thermo) HPLC at 40°C and at a flow rate of 300 µL/min with a gradient of A (0.1% formic acid in 100% LC-MS grade water) and B (0.1% formic acid in 100% acetonitrile) as follow: 2% B for 2 min, 2% to 35% B in 11 min, 35% to 90% B in 2 min, held at 90% B for 1 min, and then at 2% B for 0.5 min. The QE-HF was run in a data-dependent acquisition mode triggering on peptides with charge states 1 to 3 using a mass range of 100 to 1000 m/z at 60,000 resolutions, with an AGC target of 3e6 and a maximum ion time of 50 ms. The isolated ions were further fragmented by HCD using isolation window of 1.6 m/z and scanned at a resolution of 15,000.

The acquired data from RP separation were analyzed separately using Progenesis QI (Waters, v 2.4). For accurate quantitative analysis, the chromatograms were aligned,

and the peaks were detected using a comprehensive set of algorithms, including isotope and adducts deconvolution. NIST MS/MS v1.0 was used for library search. The compounds were filtered using a score of at least 30, a mass accuracy <5ppm and isotopic similarity of at least 90. In addition, the RP data was also analyzed using Proteome Discoverer 2.4 (ThermoFisher Scientific). The database search was performed using Mascot 2.6.2 with no enzyme specificity and Uniprot_UP000000539_Gallus. Mascot was searched with a fragment ion mass tolerance of 0.060 Da and a parent ion tolerance of 10.0 PPM. Peptide validation was done by Percolator with a 0.01 posterior error probability (PEP) threshold. The peptides were quantified using the precursor abundance based on intensity. The peak abundance was normalized for differences in sample loading using total peptide amount. The normalization factor used is the factor of the sum of the sample and the maximum sum in all files.

4.2.9. *In-silico* analysis

Protein Data Bank (RCSB PDB) was used to identify the peptide sequences obtained from simulated gastrointestinal digestion of egg white gels. For the major egg white proteins identified, the peptide sequences were segregated basing on their relative abundance in HR or RF processing methods. The unique peptides which showed abundance in either HR or RF processed samples were then identified and the two major egg white protein (Ovalbumin and Ovotransferrin) with higher abundance of these unique peptides were then selected for further analysis. The sequence (in FASTA) of the major egg white proteins Ovalbumin (*accession no: P01012*) and Ovotransferrin (*accession no: P02789*) was obtained from National Center for Biotechnology Information protein database. Furthermore, an *in-silico* proteolysis was performed on these major egg white

proteins with gastrointestinal enzymes pepsin and trypsin, using Expasy Peptide Cutter. The cleavage sites in the unique peptides were compared with the cleavage sites in the *in-silico* proteolysis resulted peptides from the parent egg white proteins. The cleavage sites in the unique peptide sequences that were different across the two processing methods (HR and RF), and different from the parent protein cleavage sites were considered as unique cleavage sites. Furthermore, the 3-D structure of the parent proteins were constructed using Jmol molecular modelling software using the PDB protein structures. The unique peptides, unique cleavage sites and other structural information were illustrated in these models for visualization and identification of their position within the protein structure.

4.2.10. Cell culture

Caco-2 cells (ATCC® HTB-37™, Manassas, VA, USA) were grown in Eagle's Minimum Essential Medium (EMEM; ATCC® 30-2003™, Manassas, VA, USA) supplemented with 20% Fetal Bovine Serum (FBS; Gibco, 10437028) and 1% Penicillin-Streptomycin (Gibco, 1514022, Waltham, MA, USA) at 37 °C and 5% CO_2 in a humidified environment. The cells were grown until 80% confluency, after which they were sub-cultured for further experiments.

Caco-2 cells (between passages 30-35) were seeded in a Transwell® 24 well permeable supports with 0.4 μm pore polyester membrane (Corning, #3470, Birmingham, UK) at a density of 20,000 cells/insert. They were differentiated in EMEM for 16 days, ensuring the culture media was replaced in the apical side (0.1 mL) and basolateral side (0.6 mL) every 2 days. The integrity of the cell monolayer was ensured by measuring the Transepithelial Electrical Resistance (TEER) with a EVOM3 paired with STX2-Plus

electrode (World Precision Instruments; Sarasota, FL, USA). The cell monolayers with TEER greater than 400 Ohm cm^2 were only used for the transport study.

4.2.10.1. Peptide transport study

On the day of the experiment, the EMEM growth medium in the basal layer was replaced with 0.6 mL of HBSS (pH 7.4, pH adjusted with 25 mM HEPES), while the apical layer was replaced with 0.1 mL HBSS (pH 6.5, pH adjusted with MES). The cells were incubated for 15 min at 37 °C and 5% CO_2, after which their TEER values were measured (before experiment). Following that, the HBSS buffer in the apical layer was replaced with 0.1 mL of 1 mg/mL hydrolyzed egg white gel powder dissolved in HBSS, pH 6.5, and the plate was set in an orbital shaker (200 rpm) at 37 °C for 2 h to allow the transport of the egg hydrolysates across the Caco-2 cells. After 2 h, the TEER values of the cells were measured, and the apical and basal layers were collected for the peptide analysis. After collecting the apical layers, 100 μL of RIPA lysis buffer (Thermo Fischer Scientific, #89900, Waltham, MA) were added onto the cells and the inserts were kept on ice for 5 min, following which the cells were harvested for the analysis of the tight junction proteins via western immunoblotting.

4.2.10.2. Western immunoblotting

The total protein concentration of the harvested Caco-2 cell lysates, from the inserts, were quantified using Pierce™ 660nm Protein Assay Reagent (Thermo Scientific, #22660, Waltham, MA) according to the manufacturer's protocol. Cell lysates were then mixed with 2x Laemmli Sample Buffer (Bio-Rad, #1610737, Hercules, California, USA) such that only 30 μg protein was loaded into each well. The samples were boiled for 5 min and the proteins were then loaded into a 4-20% gradient gel (Bio-

Rad, #4561096) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separate proteins were then transferred onto a nitrocellulose membrane using a Trans-Blot Turbo Transfer system (Bio-Rad, # 1704150). The membranes were blocked for 1 h using Odyssey blocking buffer (Li-Cor, #927-50000, Lincoln, Nebraska, USA) on a shaker, followed by an overnight incubation with the tight junction antibodies, namely, anti-Claudin-1 (Invitrogen, #37-4900), anti-Occludin (Invitrogen, #33-1500), anti-Zonula Occludens-1 (ZO-1) (Invitrogen, #61-7300), and the loading control, anti-β-Actin (Sigma, #A5316) at their company recommended dilutions. Next day, the membranes were washed with Tris buffered saline with Tween 20 (three times, 5 min each), and incubated in dark with the respective secondary antibodies, i.e., IR Dye 800CW goat anti-rabbit IgG and/or IR Dye 680RD goat anti-mouse IgG, depending on the host of the primary antibody used, at a concentration of 1:10,000 at room temperature for 1 h. Following incubation, the membranes were washed with TBST (three times, 5 min each) and the proteins were detected using the fluorescent red and green channels in an Odyssey CLx imaging system (Li-Cor Biosciences). The protein expression was quantified using Image Studio software from Li-Cor, and the expression was normalized using β-actin as the loading control.

4.2.10.3. Cell viability

The cell viability assay was performed using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent) assay kit (Abcam, #ab211091) following the manufacturer's guidelines. Caco-2 cells were seeded in a clear bottom 96-well black plate (VWR, #29444-008) at 40,000 cells/well and grown for 15 days in EMEM media (20% FBS and 1% Pen-Step) to promote differentiation of the cells. The cell toxicity of 1

mg/mL egg hydrolysates were measured after incubating the different hydrolysates on the Caco-2 cells for 2 h. After the incubation, the cell media was discarded from each of the wells, which was then replaced with 50 µL of the MTT reagent and 50 µL of serum-free EMEM media, along with a background control, containing no cells. The plate was incubated for 3 h at 37 ˚C, after which the MTT-supplemented media was replaced with 150 µL of MTT solvent in each of the wells. The plate was incubated in the dark for 15 min on an orbital shaker (200 rpm, 37 ºC) (MaxQTM 4450, Thermo Scientific™, USA), following which the absorbance of the plate was read at 590 nm (Synergy H1 microplate reader, BioTek®, USA). The background control readings were subtracted from each of the wells and the cell viability percentage (%) was calculated with the formula:

Cell viability % = (sample/control) x 100,

where sample is the 1 mg/mL egg hydrolysate treated cells and control is cells without the egg hydrolysates.

4.2.11. Statistical analysis

All the assays were performed in triplicates with 3 biological replicates from 3 production batches of EWP and 3 technical replicates for each biological replicate. The data was analyzed using a two-way analysis of variance (ANOVA) with Tukey's post hoc test, and student's t-test using Graphpad Prism 8 software.

4.3. Results

4.3.1. Effect of processing on solubility and protein breakdown

4.3.1.1. Soluble protein content

Figure 4.1.a. demonstrates the soluble protein content after HR and RF processing on 0th and 15th days at 70 and 80 ℃, where significantly high gelling properties were observed in our preceding work (Kar et al., 2020). The soluble protein content was not affected by an increase in temperature, holding time and RF processing.

4.3.1.2. Peptide content

Figure 4.1.b. describes the processing induced changes in the partial hydrolysis of proteins to peptides. Similar to the outcome in soluble protein content (in section 4.3.1.1), the peptide content was also unaffected by RF processing. The initial peptide content in EWP was normalized before gelation and further functional analysis.

4.3.2. Processing induced structural changes in EWP

4.3.2.1. Surface hydrophobicity

The surface hydrophobicity was measured from the slope of the fluorescence intensity curves with ANS fluorescence probe (as described in Figure 4.2.a.). RF processing did not affect the exposure of the buried hydrophobic residues to the protein surface.

4.3.2.2. Circular dichroism spectroscopy

The CD spectroscopy has been widely used to study the spatial structure of proteins influencing the physicochemical properties and functionality (Kato et al., 1989; Lechevalier et al., 2007; Li et al., 2018; Mine, 1995, 1996, 1997; Wang et al., 2020).

Figure 4.2.b. described that RF processing did not have a significant effect in changing the secondary structure of proteins. The random coil structure increased subtly with thermal processing in general, by 1.8% in HR processing and 1.9% in RF processing. The α-helix structure decreased with thermal processing (17.8% in EWP before thermal processing), with slightly higher content in HR processing (16.4%) than RF processing (15.1 %). However, these differences in the protein structure owing to thermal processing as well as dielectric heating were not significant and did not explain for improved gelling properties as observed in dielectric heating.

4.3.2.3. Raman spectroscopy

To elucidate the enhanced gel firmness due to RF processing at 80 °C and 0 d, the secondary protein structure was further investigated using Raman spectroscopy (Figure 4.2.c.). The wavenumbers of EWP for amide I were recognized between 1706-1656 cm^{-1} and amide III bands were recognized between 1330-1230 cm^{-1} according to Hu & Du, (2000); Li-Chan, (1996); Painter & Koenig, (1976). Raman spectra did not show a significant difference between HR and RF processed EWP. Table 4.3. describes the intensity ratios of the major peaks observed at amide I and amide III bands with respect to the Phenylalanine peak at 1003 cm^{-1}. There was no significant difference in the ratio of intensities observed with HR and RF processed EWP for both amide I and amide III bands. Amide I corresponded to anti-parallel β-sheeted structure and amide III corresponded to disordered structure in the egg white proteins of HR and RF processed EWP.

4.3.3. Effect of processing on the bioaccessibility of egg white gel

The bioaccessibility study was performed on egg white gels prepared from HR and RF processed EWP at 80 °C and 0 d. The two-tier effect of thermal processing, i.e., thermal processing (using HR and RF) and gelation on the protein-protein and protein-water interaction was evaluated.

4.3.3.1. Degree of hydrolysis and rate of digestion

The DH of the egg white gel was measured after simulated gastric and intestinal digestion as demonstrated in Figure 4.3.a and Figure 4.3.b. Based on the calculated DH values from pH-stat method (as described in section 4.2.7), it was observed that RF processing did not significantly change the pepsin and pancreatin susceptibility to egg white proteins between HR and RF processing. The rate of digestion curve and digestibility during gastric phase in Figure 4.3.g (volume of titrant with respect to time) demonstrated that RF processing showed a trend of increasing the gastric digestion between 20-60 min (p-value = 0.06). It could be implied that RF processing contributed to some changes in the functional moieties of cleavage sites for pepsin during the gastric digestion of egg white gels. However, such an effect of RF processing was not observed during the intestinal digestion (Figure 4.3.h). Therefore, it could be further implied that the peptides generated during simulated gastrointestinal digestion of egg white gels, could have possible qualitative differences rather than quantitative differences.

4.3.3.2. Protein content after simulated gastrointestinal digestion

As described in Figure 4.3.c and Figure 4.3.d, the protein content (μg/g) of HR and RF processed egg white gels after the simulated gastrointestinal digestion, remained unaffected. A higher protein content was observed after intestinal digestion than gastric

digestion. The protein content increased by by 2.5% in HR and 3.6% in RF processing between the gastric phase and intestinal phase.

4.3.3.3. Peptide content after simulated gastrointestinal digestion

The peptide content (μg/g) of HR and RF processed egg white gels after gastric and intestinal digestion demonstrated a similar trend as the protein content (as described in section 4.3.3.2). RF processing did not affect the proteolysis of the egg white proteins in the gel matrix. A higher peptide content was detected at the end of intestinal digestion over gastric digestion because of the cumulative action of gastrointestinal enzymes on the egg white protein hydrolysis. The rise in the peptide content was almost 11% in both HR and RF processing from gastric phase to intestinal phase, as described in Figures 4.3.e, and 4.3.f respectively.

4.3.4. Effect of processing on the egg white gel hydrolysates

At the end of simulated gastrointestinal digestion, two fractions of egg white gel hydrolysates were obtained, one was the soluble fraction with shorter peptide chains (less than 3 KDa), and the other was the insoluble fraction with longer peptide chains (more than 3 KDa). Both the fractions were analyzed separately to identify the possible structural differences induced by HR and RF processing. Furthermore, the soluble egg white gel hydrolysates were investigated for their bio-functional properties induced by the two thermal processing methods used.

4.3.4.1. Raman spectroscopy of insoluble egg white gel hydrolysate

Raman spectroscopy was performed on the insoluble egg white gel hydrolysate fraction obtained from simulated gastrointestinal digestion to elucidate the concomitant effect of processing (described in Figure 4.4.a). Similar to earlier observation in EWP (in

section 4.3.2.3), there was no significant difference in the Raman spectra of egg white gel retentate fraction between HR and RF processing. A sharp doublet band corresponding to exposed Tyrosine residues between 850-830 cm^{-1} concurred in HR and RF according to Tu, (1986). Amide I and amide III bands were observed between 1701-1630 cm^{-1} and 1340-1210 cm^{-1}, respectively. There was no significant difference in the intensity ratios of HR and RF processing with respect to the Phenylalanine peak at 1003 cm^{-1} (Table 4.3). Both amide I and amide II bands corresponded to disordered random coil structure in egg white proteins.

4.3.4.2. *In-silico* analysis of soluble egg white gel hydrolysates

The 3D structures of Ovalbumin (Figure 4.4.b and 4.4.c) and Ovotransferrin (Figure 4.4.d and 4.4.e) were visualized to identify the position of unique peptides generated during HR and RF in egg white gel hydrolysate. Ovalbumin was identified as the parent protein for 19 unique peptides from HR processing, and 17 unique peptides from RF processing, respectively (Table 4.3). Ovotransferrin was identified as the parent protein for 4 unique peptides in HR processing, and 7 unique peptides from RF processing. respectively (Table 4.3).

Furthermore, the unique specific sites of accession by pepsin and trypsin in Ovalbumin and Ovotransferrin to generate these unique peptides were identified and denominated as unique cleavage sites (described in Figure 4.4.b, 4.4.c, 4.4.d, and 4.4.e).

4.3.5. Effect of processing on the transport of soluble egg white gel hydrolysates

To further investigate the qualitative changes attributed by processing in the soluble egg white gel hydrolysates, and its implication on the biological functionality, an absorption study was performed on differentiated Caco-2 cells.

4.3.5.1. Qualitative analysis of peptides from soluble egg white gel hydrolysates

Figures 4.5.a and 4.5.b describe the structure of peptides identified in the apical layer of Caco-2 monolayer when subjected to the soluble egg white gel hydrolysates. The amino acid positions and the side chain characteristics of the resulting peptides had minor differences due to HR and RF processing (Figures 4.5.a and 4.5.b). The above results imply that RF processing did not substantially affect the functional groups (Figures 4.5.a and 4.5.b) in the amino acid sequences of the unique peptides (Table 4.3) generated. However, the differences in the peptide chain lengths between HR and RF processing could confer to possible differences in the absorption of these unique peptides.

4.3.5.2. Transport study of soluble egg white gel hydrolysates

The Caco-2 cells were differentiated until the TEER values exceeded 400 Ω cm^2 before being used in the absorption study of soluble egg white gel hydrolysates.

Figure 4.5.c describes the absorption of hydrolyzed egg white gel from 0 to 2 hrs. There was no significant difference between the TEER values of the basolateral layer between HR and RF processing.

Figure 4.5.d describes the increase in the peptide content of the basolateral layer after the 2-hour absorption period for both HR and RF processing. However, no significant difference in the absorbed peptide content was observed due to RF processing. RF processing did not confer significant differences in the absorption of peptides from soluble egg white gel hydrolysates in the Caco-2 monolayer. However the subsequent implications on the structural integrity of tight junction proteins in the epithelial cells, and the cell viability also requires investigation to elucidate on the biological risks associated with RF processing.

4.3.6. Effect of radiofrequency processing on the tight junction proteins and cytotoxicity

The resulting harvested Caco-2 cell lysates at the end of the absorption study were further analyzed for the integrity of tight junction proteins Zonnula Occludens-1 (ZO-1) and Claudin-1 contents, structures, and the overall cell viability, to determine the safety concerns related with using RF processing.

4.3.6.1. Western immunoblotting

Figures 4.6.a and 4.6.b represent the effect of processing on the tight junction proteins ZO-1 and Claudin-1, respectively. The expression of tight junction proteins was neither affected by HR and RF processing at 80 ℃ and 0 d, nor by a 2 h absorption of these peptides in the Caco-2 cells.

4.3.6.2. Cell viability

Figure 4.6.c demonstrates processing induced changes in the egg white gel peptides to induce cytotoxicity in Caco-2 cells. It was observed that HR and RF processed egg white gel peptides at a concentration of 1 mg/mL did not decrease the cell viability after a 2 h absorption period. This suggested that HR processing could be potentially replaced with RF processing to enhance the functionality without influencing cytotoxicity.

4.4. Discussion

RF processing at 80 ℃ and 0 d increased the gel firmness of EWP over HR processing by 48.6 % without affecting the solubility. The soluble protein content and peptide content of EWP after thermal processing (via HR and RF), and before gel formation revealed no significant differences. The surface hydrophobicity and secondary

protein structural conformations were also not affected significantly by RF processing. These results were indicative of two possibilities: one, the effect of either dielectric heating or faster rate of heating in RF processing, which was improving the gel firmness, was nascent. Two, the effect of either dielectric heating or faster rate of heating in RF processing on EWP was more prominent on the water-protein interaction in the gel matrix, rather than just protein conformational changes in EWP.

Therefore, bioaccessibility study was performed on the egg white gels rather than egg white powder to elucidate on the water-protein and protein-protein interactions and their subsequent effects. A minor increase in the gastric phase was observed between 20 and 60 minutes with RF processed egg white gels, however, such an effect was not observed in the intestinal phase. There were no significant differences in the degree of hydrolysis (calculated using pH stat method), and peptide content after gastric as well as intestinal digestions. To evaluate the effect of RF processing on the quality of peptides generated, the egg white gel hydrolysates were analyzed for structural differences using RP-LC-MS/MS (for the soluble fraction) and Raman spectroscopy (for the insoluble fraction), as well as functional differences using an absorption study on the Caco-2 cells using the soluble fraction of egg white gel hydrolysates.

The current findings in this study confirmed that RF processing did not affect the protein aggregation and partial hydrolysis of EWP, which have been associated with protein solubility and protein functionality. The soluble protein content observed in this study corresponded with the earlier works by Lechevalier et al. (2017); Baron et al. (2003); Desfougères et al. (2008); Kato et al. (1989) in which dry heating temperature and heating time did not significantly affect the soluble protein content in EWP.

Dry heating has been associated with ameliorating the functional properties of proteins by partial unfolding and exposing the buried aromatic amino acids (Haskard & Li-Chan, 1998) to the protein surface to interact at air and water interface (Damodaran & Parkin, 2017; Kato & Nakai, 1980; Kim et al.,2006). Earlier works have reported an increase in the surface hydrophobicity with increased treatment temperature, holding time, and alkalinity, which have concomitantly improved foaming, gelling, and emulsifying properties of egg white proteins (Handa et al., 2001; Kato & Nakai, 1980; Lechevalier et al., 2007; Stănciuc et al., 2016; Van der Plancken et al., 2007; Wang et al., 2018). However, almost negligible difference in the surface hydrophobicity between HR and RF processing could have two implications: one, RF processing did not significantly expose the hydrophobic amino acids to the surface; two, it reburied them in the interior of the protein molecule via aggregation (Wang et al., 2018).

The functional properties associated with EWP have been frequently attributed to changes in the secondary structural conformations (Clark et al., 1992; Handa et al., 2001; Li-Chan, 1996; Mine, 1995; Van der Plancken et al., 2007). Clark et al. (1992) and Mine (1996), have demonstrated a decrease in the α-helix content with increased dry heat treatment, dielectric heating and irradiation. Kato et al. (1989, 1990) and Lechevalier et al. (2007), have also reported minor changes in the protein conformation between dry heated and non-heated egg white powder, all of which support the findings in this study. The effect of dielectric heating in microwave processing has shown minor changes in the random coil and α-helix structure of egg white proteins (Li et al., 2018; Wang et al., 2020). This substantiated the minor structural changes observed due to RF processing as a means of possible dielectric heating in this study. The minor increase in random coil

structure, and the concomitant decrease in the α-helix structure were indicative of partial protein unfolding and aided in increasing the egg white protein's flexibility towards water-protein interaction. Therefore, increased gel firmness observed due to RF processing could be partly explained by the improved water-protein interaction, which was caused by an increase in the α-helix structure transforming into random coil structure.

EWP proteins have commonly shown characteristic prevalence of amide I and amide III bands in the regions between 1670-1600 cm^{-1} and 1300-1200 cm^{-1} respectively based on the peptide backbone exposure to targeted-functionality based processing (Li-Chan et al., 1994; Li-Chan, 1996; Painter & Koenig, 1976; Wong et al., 2009; Zhao et al., 2020; Zhao et al., 2004). In the current study, Raman spectroscopy revealed no interdependent relationship between increased gel firmness and secondary protein conformation due to RF processing. A strong amide I band at 1665-1680 cm^{-1} and amide III band at 1238-1245 cm^{-1} in both EWP and the insoluble egg white gel hydrolysate fraction indicated a high β-sheet structure (Li-Chan et al., 1994). This was also corroborated by the results from CD spectroscopy of EWP (section 4.3.2.2) which revealed over 50% β-sheeted structure in both HR and RF processing. A sharp peak at 864 cm^{-1} with a shoulder peak at 843 cm^{-1} corresponded to exposed tyrosine residues in egg white gel retentate, according to (Li-Chan et al., 1994; Tu 1986). The mole fraction (N) of buried and exposed tyrosine groups was calculated using the equations by Tu, (1986) with the intensity ratio (I864/843) in HR and RF:

$$N_{buried} + N_{exposed} = 1$$

$$0.5N_{buried} + 1.25N_{exposed} = I_{864/843}$$

HR processing showed 92% exposed tyrosine groups, while RF processing resulted in 97% exposed tyrosine groups. The tyrosine groups tend to subsequently expose the hydroxyl groups to interact more strongly with the polar solvent, while simultaneously acting as a hydrogen bond acceptor, and donor (Burmeister, 2000; Gitlin et al., 1989; Wang et al., 2018). Hence, a slightly higher exposed tyrosine could possibly explain the water-protein interaction causing enhanced gel firmness due to RF processing.

Therefore, two interpretations could be proposed based on the protein structural information. Firstly, the improvement in the functional properties of RF processed EWP is not entirely attributed to protein-protein interactions, as well as changes in the secondary protein conformations. Secondly, RF processing has some implications on the water-protein interaction, which could possibly cause improved functionality, and impact the biological functionality of the protein.

The digestibility of egg white proteins in this study has a multiplicative effect of protein conformational changes due to dry heat processing as well as gelation. Damodaran (2005, 2017) has elucidated that dry heat processing amounted to milder conformational changes in proteins, thereby having a minor effect on the protein digestibility. However, Croguennec et al. (2002) has demonstrated that gelation of proteins reduces their surface area to volume ratio as they form spherical aggregates, and that eventually reduces their digestibility. Earlier works by Luo et al. (2015); Nyemb et al. (2014, 2016) have shown that the digestibility of protein gels was substantially affected by the gel microstructure. Overall, processing affects the steric hinderance of the immobilized protein in a gel structure against the ingress of gastrointestinal enzymes, and

the egress of peptides (Farjami et al., 2021). Thus, the digestion kinetics explained by the rate of digestion in this study was influenced by the extent of protein aggregation, the degree of protein unfolding and the surface area to volume of the aggregates (Dupont & Nau, 2019).

The digestibility showed an increased trend with RF processing of egg white gels during the gastric phase. However, such an increase was not observed during intestinal phase. Since the specificity of pepsin hydrolysis has been identified as aromatic amino acids such as phenylalanine, tryptophan, and tyrosine, therefore an increased exposure of these amino acids could explain for the increased digestibility during the gastric phase (Wang et al., 2018). The earlier results from Raman spectroscopy of insoluble egg white gel hydrolysates (Section 4.3.4.1) revealed a subtle increase in the exposure of tyrosine residues with RF processing. This could suggest two possible implications: one, an increase in the polar interaction between the hydroxyl groups of tyrosine residues with water, leading to an improved gel firmness with RF processing. Two, a possible increase in the activity of pepsin due to more exposed tyrosine residues, thus resulting in an increased trend in the gastric digestion. It has also been reported that most of the aromatic amino acid residues including the exposed tyrosine groups are cleaved from Ovalbumin during dry heating (Burmeister, 2000; Gitlin et al., 1989). The unique peptides observed in the current study also stemmed majorly from Ovalbumin, and 6 out of 17 unique peptides (Table 4.4) were cleaved by pepsin in RF processed soluble egg white gel hydrolysate.

However, the peptide content did not differ significantly between HR and RF processing after gastric digestion, suggesting that the increased trend in gastric digestion

did not result in a significant change in the quantity of peptides released. The peptide content increased from gastric to intestinal digestion in both HR and RF processing. Pepsin penetrated the gel matrix to access the immobilized proteins and initiated proteolysis (Luo et al., 2015). It also unfolded protein moieties for pancreatin to access, and eventually the activity of pancreatin increased in the presence of alkaline pH. Consequently, the peptide content increased at the end of intestinal digestion, however it remained comparable between HR and RF processing. It could be implied that a higher rate of gastric digestion with RF processing could have led to qualitatively and functionally different peptides, but the quantity of peptides at the end of gastrointestinal digestion remained unaffected.

The *in-silico* analysis of soluble egg white gel hydrolysates demonstrated the generation of 44 unique peptides from Ovalbumin and Ovotransferrin due to HR and RF processing. The generation of unique peptides suggested possible differences in the qualitative attributes of the peptides. The peptide profile analysis on the apical layer of Caco-2 cells treated with soluble egg white gel hydrolysates revealed that there were subtle differences in the amino acid sequences of the peptides between HR and RF processing. These minor qualitative differences could explain the increased trend in the absorption of egg white gel hydrolysates (soluble fraction) due to RF processing. The expression of tight junction proteins ZO-1 and Claudin-1 were not affected by RF processing. The cytotoxicity assay demonstrated that the cell viability remained unaffected by RF processing. These results indicated that RF processing could be a promising novel technology which could replace the traditional HR processing to improve the gelation in EWP, without impacting the other crucial bio-functionality.

4.5. Conclusion

The presently reported study shows that RF processing does not induce significant secondary conformational changes in the egg white protein structure as compared to HR processing, despite improving the gelling property significantly. Inhibitions regarding the bioavailability of food products processed with electromagnetic waves, and their deleterious effects on intestinal cells have restricted the broader use of RF processing. However, the current study demonstrate that RF processing does not impact the integrity of the tight junction proteins and does not reduce the cell viability. RF processing also shows a trend towards improving the bioaccessibility and absorption of egg white peptides as compared to HR processing. Therefore, RF processing could replace HR processing as not just an energy efficient alternative to pasteurize egg white powder, but it can also improve the gelling functionality without adversely affecting the biological functionality associated with EWP.

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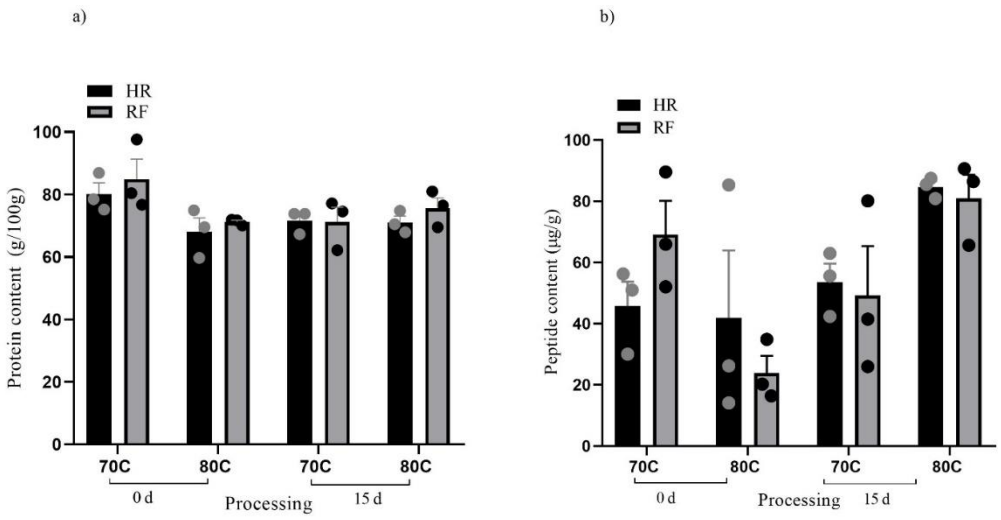

Figure 4.1. Effect of thermal processing, holding time, and temperature on the protein content and protein breakdown in EWP (a) Protein content (b) Peptide content

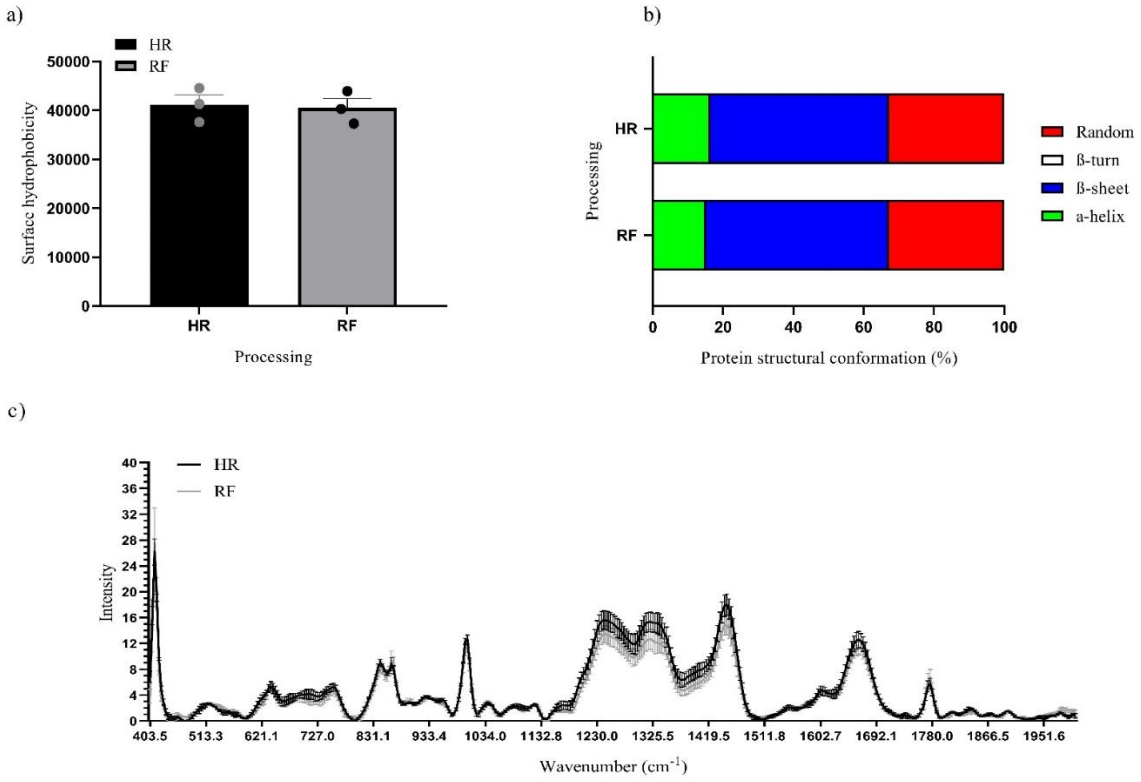

Figure 4.2. Effect of processing method used at 80 ℃ and 0 d on the structural properties of egg white proteins from EWPN (a) Surface hydrophobicity using fluorescence probe 1-anilino-8-naphathalene-sulfonate (b) CD spectra (c) Raman spectra with baseline correction, amide III (1330-1230 cm^{-1}), and amide I (1706-1656 cm^{-1})

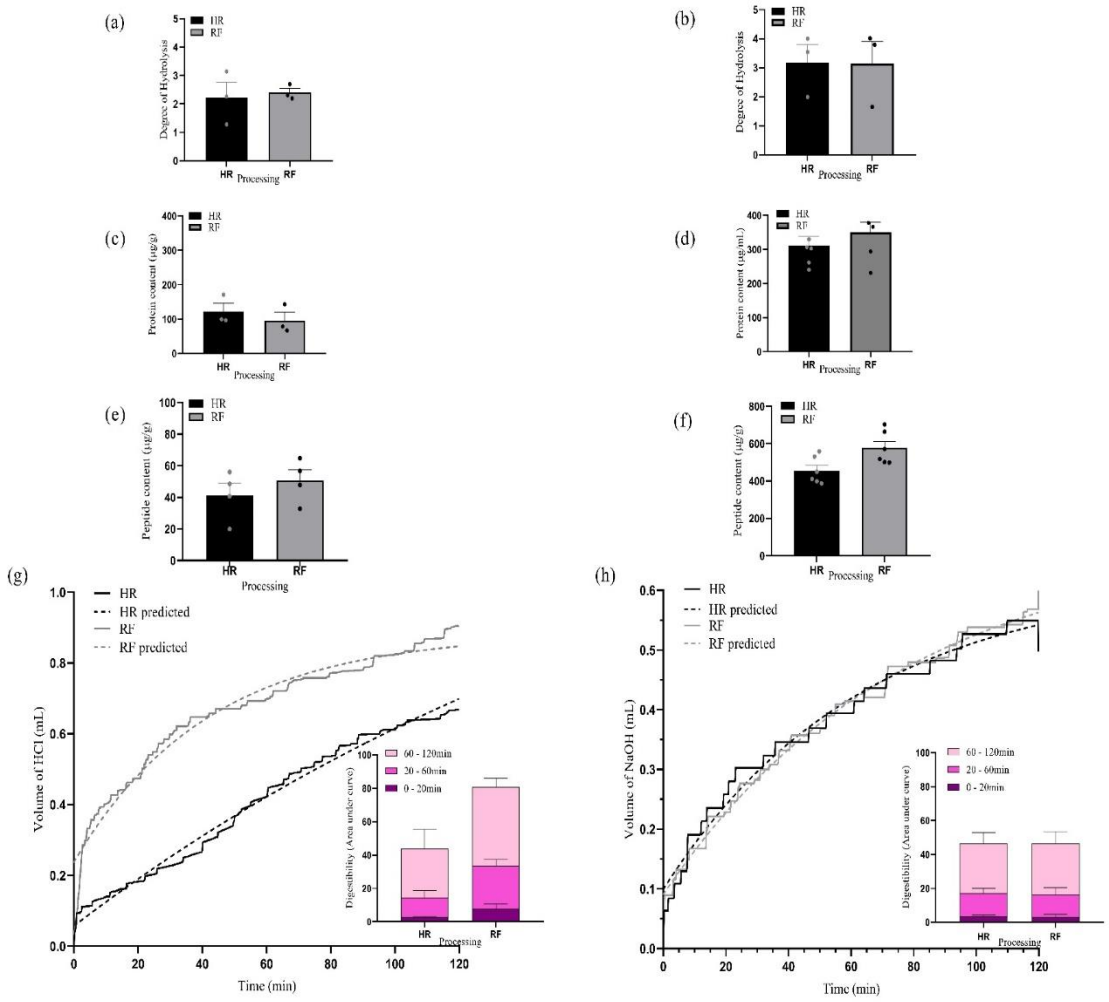

Figure 4.3. Effect of processing method used at 80 ℃ and 0 d, on the digestibility and bio-accessibility of egg white gels from EWPN (a) Degree of hydrolysis (by pHstat method) as affected by gastric digestion (b) Degree of hydrolysis (by pHstat method) as affected by intestinal digestion (c) Protein content after gastric digestion (d) Protein content after intestinal digestion (e) Peptide content after gastric digestion (f) Peptide content after intestinal digestion (g) Digestibility during gastric phase (with Hydrochloric acid as the titrant for digestion) using Mitscherlich model (h) Digestibility during intestinal phase (with Sodium Hydroxide as the titrant for digestion) using Mitscherlich model

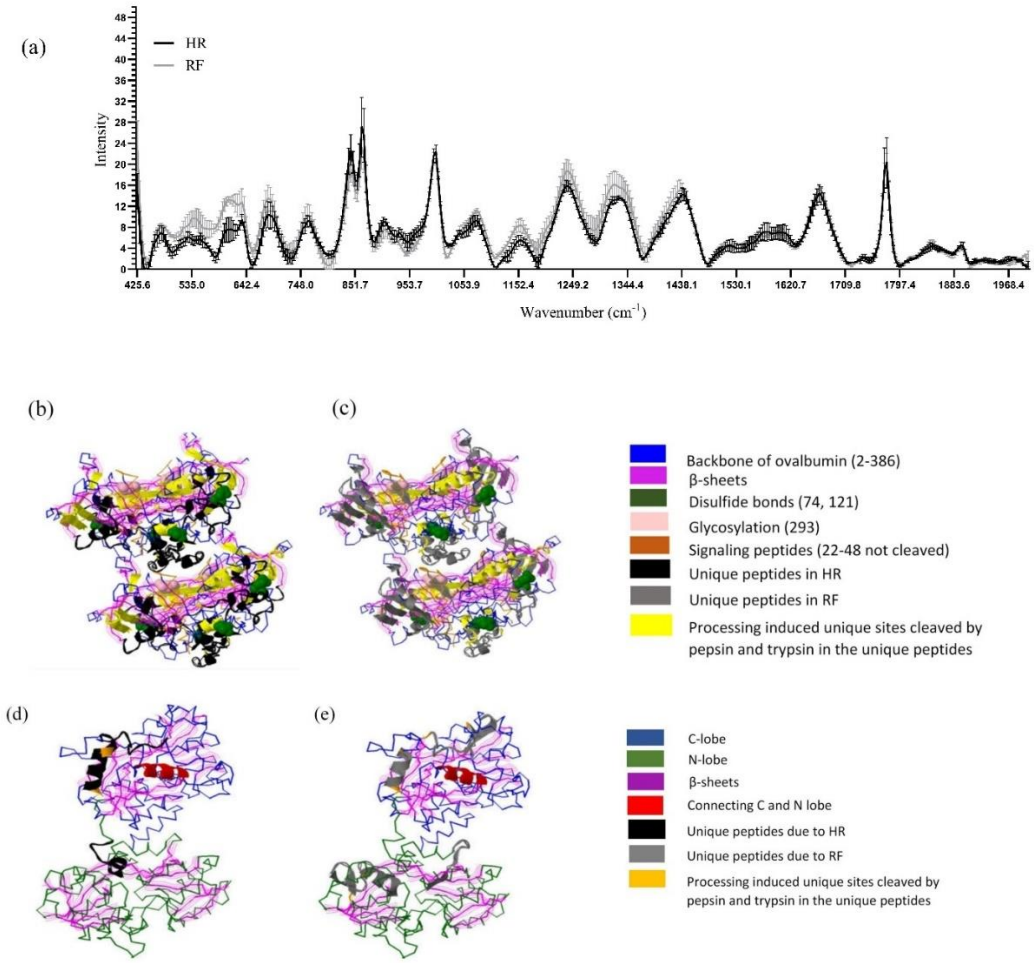

Figure 4.4. Effect of processing method used at 80 ℃ and 0 d on the structural changes of egg white proteins after simulated gastrointestinal digestion of egg white gels (a) Raman spectra of the insoluble egg white gel hydrolysate, with baseline correction, amide III (1240-1350 cm-1), and amide I (1670-1800 cm-1) (b)*In-silico* model of Ovalbumin as affected by to HR (c) *In-silico* model of Ovalbumin as affected by RF (d) *In-silico* model of Ovotransferrin as affected by HR (e) *In-silico* model of Ovotransferrin as affected by RF

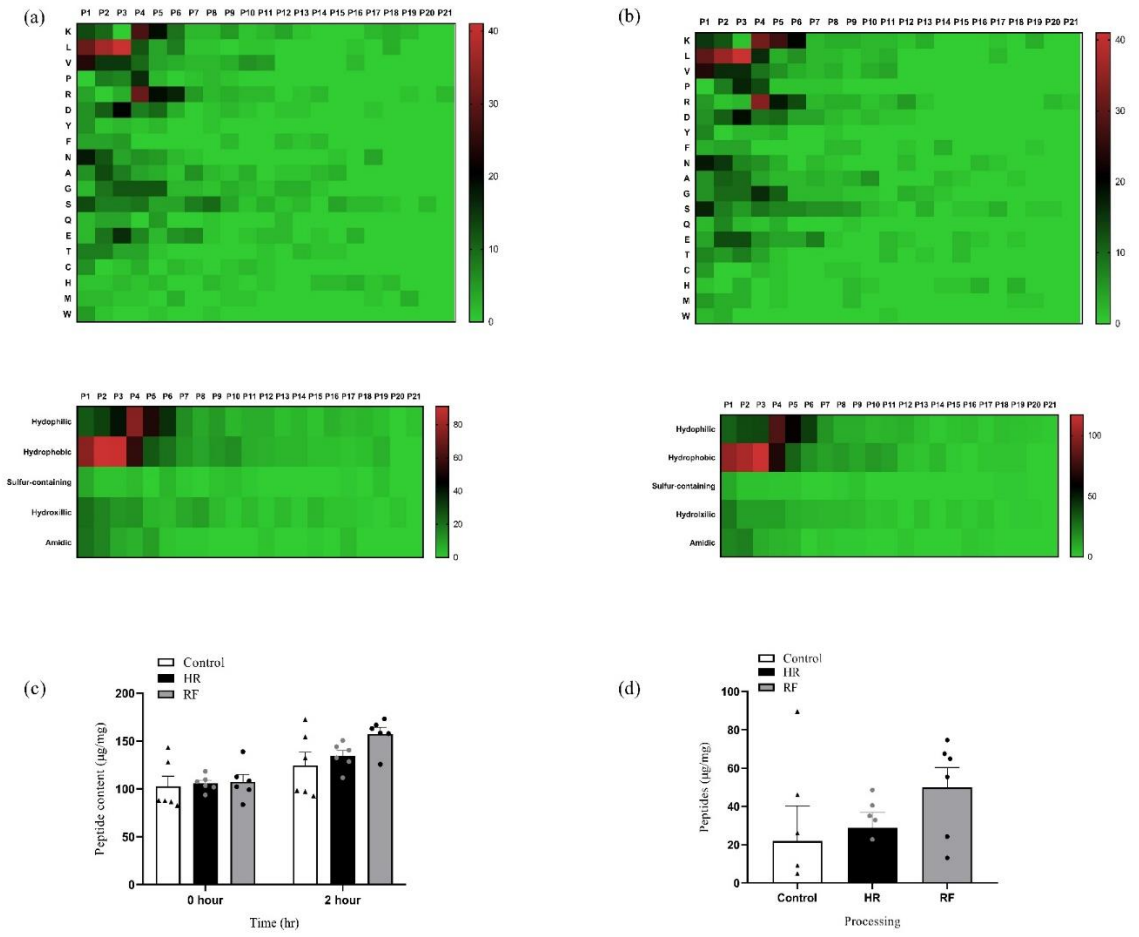

Figure 4.5. Effect of processing methods HR and RF used at 80 ℃ and 0 d on the transport of egg white gel hydrolysates in the Caco-2 (a) Heat map demonstrating the position of amino acids, their prevalence, and structural characteristics in the apical layer of Caco-2 monolayer cells as an effect of HR (b) Heat map demonstrating the position of amino acids their prevalence, and structural characteristics in the apical layer of Caco-2 monolayer cells as an effect of RF monolayer (c) Peptide content in the basal layer of Caco-2 monolayer cells at 0 and 2 hours of absorption study (d) Peptide content absorbed in the basal layer of Caco-2 monolayer cells at the end of absorption study

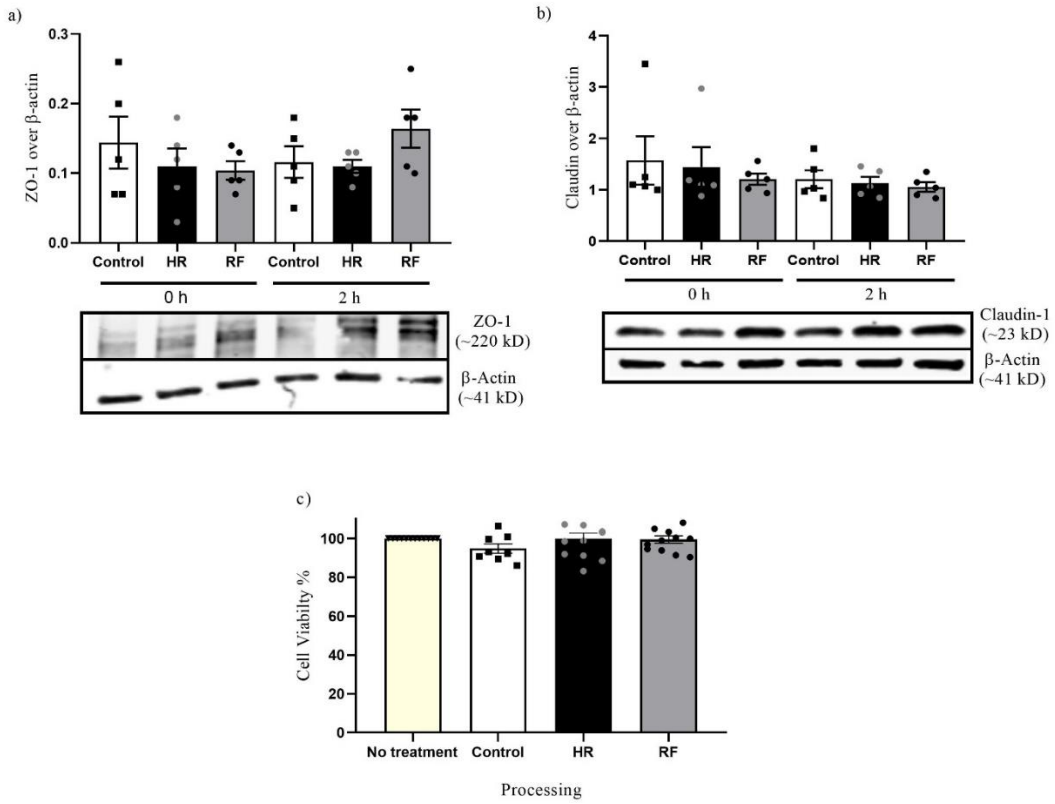

Figure 4.6. Effect of processing methods HR and RF used at 80 ℃ and 0 d on the structure of key tight junction proteins and cytotoxicity: (a) ZO-1 content and structure over β-actin standard as a result of permeability of egg white gel soluble peptides in the Caco-2 cells (b) Claudin-1 content and structure over β-actin standard as a result of permeability of egg white gel soluble peptides in the Caco-2 cells. Control sample received egg white gel hydrolysate before HR and RF processing. (c) Cytotoxicity of egg white gel soluble peptides processed by HR and RF at 80 ℃ and 0d at Caco-2 cells. No treatment refers to the cytotoxicity of Caco-2 cells without egg white gel soluble peptides. Control refers to the cytotoxicity of egg white gel hydrolysate before HR and RF processing on the Caco-2 cells.

Table 4.1. Composition of Simulated Digestion Fluids for 1.25X stock

	SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution added	Vol. of stock added to make 400 mL of 1.25X (mL)	Final salt conc. in SSF (mM)	Vol. of stock added to make 400 mL of 1.25X (mL)	Final salt conc. in SGF (mM)	Vol. of stock added to make 400 mL of 1.25X (mL)	Final salt conc. in SIF (mM)
KCL	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	3.7	3.7	0.9	0.9	0.8	0.8
$MgCl_2$	2.3	13.6	12	72.2	20.6	123.4
$(NH_4)_2CO_3$	0.2	0.15	0.1	0.1	0.3	0.33
NaCl	0.1	0.06	0.5	0.5	-	
H_2O	378.6	-	379.6	-	371.5	-

Table 4.2. Final composition of reagents and Simulated Digestion Fluids

	Oral Phase	Gastric Phase	Intestinal Phase
Sample	50 g	60 mL (from oral phase)	100 mL (from gastric phase)
SSF	7 mL	-	-
$CaCl_2$	0.05 mL	0.01 mL	0.08 mL
DI Water	1.95 mL	1.39 mL	2.62 mL
Amylase	1 mL	-	-
SGF	-	55 mL	-
Pepsin	-	3.2 mL	-
1M HCl	-	0.4 mL	-
SIF	-	-	82 mL
Pancreatin	-	-	10 mL
Bile	-	-	5 mL
1M NaOH	-	-	0.3 mL
Total Vol.	60 mL	120 mL	200 mL

Table 4.3. Raman intensity ratio (against Phenylalanine band at 1003 cm^{-1}) as affected by HR and RF processing on EWP and the insoluble egg white gel hydrolysates.

		Wavenumber	Secondary structure	Functional group	HR (I/I_{Phe})	RF (I/I_{Phe})
Amide I	EWPN	1670	Anti-parallel β-sheet	C=O stretch, N-H wag	0.944	0.944
	Egg white gel retentate	1667	Disordered structure	C=O stretch, N-H wag	0.645	0.721
Amide III	EWPN	1241	Disordered structure	N-H in-plane bend, C-N stretch	1.242	1.178
	Egg white gel retentate	1241	Disordered structure	N-H in-plane bend, C-N stretch	0.707	0.885

Table 4.4. Unique peptides in the soluble fraction of egg white gel hydrolysates as a result of processing.

Parent protein	Unique peptides			
	HR processing		RF processing	
	Sequences	Cleavage Position	Sequences	Cleavage Position
Ovalbumin (P01012)	GTSVNVHSSLRDILNQITKPNDV	f75-97	LGAKDSTRTQ	f44-53
	NVHSSLRDILNQITKPN	f79-95	NVHSSLRDILNQITKPNDV	f79-97
	NVHSSLRDILNQITKPNDVYS	f79-99	VHSSLRDILNQITKPNDV	f80-97
	RDILNQITKPNDVY	f85-98	RELINSWVESQ	f143-153
	QTAADQARELINSWVESQTNGIIR	f136-159	ELINSWVESQTNGIIRN	f144-160
	RELINSWVESQTNGIIRN	f143-160	INSWVESQTNGIIRNVL	f146-162
	ELINSWVESQTNGIIR	f144-159	NSWVESQTNGIIRNV	f147-161
	IIRNVLQPSSVDS	f157-169	WVESQTNGIIRNVL	f149-162
	RVASMASEKMKIL	f219-231	VESQTNGIIRNVL	f150-162
	RVASMASEKMKILELPFASGTM	f219-240	QTNGIIRNV	f153-161
	KLTEWTSSNVM	f264-274	PFRVTEQESKPVQ	f198-210
	KLTEWTSSNVMEERKIKV	f264-281	KILELPFASGTM	f229-240
	FSSSANLSGISSAES	f307-321	VLLPDEVSGLEQLESIIN	f244-261
	SGISSAESLKISQAV	f314-328	KLTEWTSSNV	f264-273
	AAHAEINEAGRE	f330-341	SGISSAESLKISQ	f314-326
	AHAEINEAGREVVGSAEAGVDA	f331-352	HAEINEAGREVVGSAEAGVDA	f332-352
	AHAEINEAGREVVGSAEAGVDAA	f331-353	GREVVGSAEAGVD	f339-351
	AEINEAGREVVGSA	f333-346		
	EINEAGREVVGS	f334-345		
Ovotransferrin (P02789)	KRVPSLMDSQL	f327-337	FVKHTTVNENAPDQKDE	f226-242
	NLAEVPTHAV	f604-613	GPPGKKDPVL	f305-314
	NLAEVPTHAVVVRPE	f604-618	HSTVEENTGGKNKADWA	f561-577
	NKIRDLLERQE	f621-631	NLAEVPTHA	f604-612

Chapter V: Conclusion and suggestions for future work

5.1. Conclusion

The overall goal of this dissertation was to optimize the usage of radiofrequency (RF) assisted thermal processing to enhance the gel firmness in egg white powder (EWP) and further evaluate the impact on the egg white protein conformations and the bio-functionality.

Chapter III evaluated the effect of processing methods i.e., traditional hot room (HR) processing and RF assisted thermal processing due to pH (neutral and alkaline), treatment temperature (70 and 80 ℃) and holding times (0, 5, 10, 15, and 20 d) on EWP to maximize the gelling properties. A factorial-Randomized Complete Block Design (RCBD) structure was used in this study, wherein EWP at neutral and alkaline pH were considered as separate experiments. At neutral pH, the EWP was subjected to 70 and 80 ℃ and at alkaline pH, the EWP was subjected to only 70 ℃. The thermal processing method and holding times were the treatment factors in each of these RCBDs. The batches (3 batches of EWP) were blocks in this study. Gel firmness increased with increase in holding time with both neutral and alkaline pH EWP. RF assisted thermal processing increased the gel firmness in neutral pH EWP by 48.6% when subjected to 80 ℃ at 0 d, and by 12% when subjected to 70 ℃ for 10 d over traditional HR processing. RF assisted thermal processing at 70 ℃ and 10 d for alkaline pH EWP increased the gel firmness by about 25%. The soluble protein content and the insoluble content were not affected by RF assisted thermal processing. Therefore, RF assisted thermal processing proved to be an effective alternative against traditional HR processing to reduce the come-up time, and significantly improve the gelling properties. Even though processing

did not perturb the solubility and soluble protein content, the qualitative aspects pertaining to the egg white proteins causing the improvement in the gelling properties was not clear at this stage.

Chapter IV investigated the effect of RF processing on the secondary protein conformation, and biological functionality such as bioaccessibility and bioavailability. A comparative analysis between traditional HR processing and RF processing was performed at various stages of analysis. The secondary protein conformations for neutral pH EWP processed at 80 ℃ and 0 d showed no significant difference between the two processing methods. An in vitro digestion of processed egg white gels showed an increasing trend of gastric digestion with RF processing; however no significant difference was observed in the intestinal digestion. An in-silico analysis of the soluble egg white gel hydrolysates revealed Ovalbumin and Ovotransferrin as the parent proteins for the generation of unique peptides as a result of HR and RF processing. Furthermore, Raman spectroscopy of the insoluble egg white gel hydrolysates revealed a greater exposed tyrosine group due to RF assisted thermal processing. This partly explained for improved gel firmness observed in RF assisted thermal processing due to the exposure of more hydroxyl groups in the tyrosine residues to interact with water. The peptide content of the soluble egg white gel hydrolysates did not show a significant difference. An absorption study on the Caco-2 cells revealed that RF processing showed an increasing trend towards the absorption of peptides. In addition, RF processing did not induce cytotoxicity and affect the expression of tight junction proteins ZO-1 and Claudin-1 in the epithelial cells. The study bolstered the findings from Chapter III and proved that RF

processing could be an efficient alternative, which would not compromise the bio-functionality of egg white proteins.

5.2. Suggestions for future research

While this dissertation focused on optimizing RF processing for EWP to enhance its gel firmness and demonstrating the structural and bio-functional properties that were affected in the process, there are several other interesting research opportunities that could be tapped in the future.

Although this study illuminated on the fact that RF processing could significantly improve the gelling properties, the applications where this property could further be utilized has not been explored. It is possible to optimally formulate products using the gelling functionality of EWP in a way that would be cost efficient as well as nutritionally enriching.

The egg white gel hydrolysates could be further explored to evaluate if RF processing could modulate any specific bioactive property of egg white proteins. The differences in the transport mechanism of these peptides would also be useful to evaluate their pathway of absorption. Such findings could further help in the development of value-added functional foods.