Characteristics of partially hydrolyzed egg white and its application on pork meat

A dissertation submitted

for the degree of

DOCTOR OF PHILOSOPHY

Presented by

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Kyoto, Japan, 2019

To my family

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Abbreviations

ANS: 8-anilino-naphthalene-1-sulonate

DH: degree of hydrolysis

EA: emulsifying activity

ES: emulsifying stability

EW: egg white

EY: egg yolk

hEW: hydrolyzed egg white

H_0 : surface hydrophobicity

nEW: native egg white

NMR: nuclear magnetic resonance spectroscopy

OBC: oil-binding capacity

OPA: ortho-*phthalaldehyde*

ORAC: oxygen radical absorbance capacity

pI: isoelectric point

PM: egg white hydrolysate by Protease M®

PNY: egg white hydrolysate by Protin NY100®

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: scanning electron microscopy

T: egg white hydrolysate by Thermoase PC10F®

WHC: water-holding capacity

Chapter I

General Introduction

Hen eggs are considered to be the best source of protein, lipids, vitamins, and minerals. And after it was recognized that egg consumption does not cause high serum cholesterol, consumption of table eggs increased substantially. Moreover, continuous investigation of development of the polyfunctional properties of eggs has led to increase its usage as an ingredient in a variety of processed food (Sunwoo and Gujral, 2014). In fact, around 30% of hen eggs produced in the world are processed (Lomakina and Mikova, 2006), including three most well-known properties of egg as an ingredient: heat-induced coagulation of liquid eggs; foam formation of whipped egg white, as in meringues; and emulsion stabilized by egg yolk lipoprotein, as in mayonnaise (Davis and Reeves, 2002).

1.1 Egg white proteins

Egg white (EW) represents about 60% of the shell egg by weight, and mainly consists of water (88%) and protein (11%), with the remainder made up of carbohydrates, ash, and trace amounts of lipids (1%) (Li-Chan, Powrie *et al*.,1995). It is an excellent source of high quality proteins; over 24 different proteins have been identified and isolated from EW. Table1-1 lists selected properties of the major EW proteins. The composition of EW proteins closely matches human requirements for essential amino acids and has a very high digestibility. The bioavailability of egg protein is about 65% in raw egg and is up to 95% in cooked egg protein (Seuss-baum, 2007).

1.1.1 Ovalbumin

Ovalbumin accounts for 54% of total EW proteins, making it the most abundant. It is also central to EW's functional properties in food (Stadelman and Cotterill, 1994).

Ovalbumin is also known as a phosphoglycoprotein with a molecular mass of about 45 kDa (Warner, 1954) with 386 amino acids, consisting of a single peptide chain molecule with a carbohydrate side chain. Amino acid composition of ovalbumin is unique compared with other proteins (Nisbet, Saundry *et al.*, 1981). Ovalbumin does not have a classical N-terminal ladder sequence (Huntington and Stein, 2001), but has three sites of postsynthetic modification in addition to the N-terminal acetylated glycine (Narita and Ishii,1962) and the C-terminal proline , thus ovalbumin is also known as a glycoprotein. Ovalbumin contains four free sulfhydryl groups and one disulfide bridge (Cys74-Cys121), which are inaccessible in the native state (Doi, Koseki *et al.*, 1987). Furthermore, as shown in Fig 1-1, ovalbumin is a highly structured globular protein. The secondary structure of ovalbumin has various motifs including α-helix (41%), β-sheet (34%), β-turns (12%), and random coils (13%)(Stein, Leslie *et al.*, 1990; Huntington *et al.*, 2001). When heated, ovalbumin undergoes a conformational change from its soluble, serpin structure into an insoluble all-β-sheet structure with exposed hydrophobic regions. This causes the protein to aggregate and cause the solidification associated with cooked EW (Hu and Du, 2000).

1.1.2 Ovotransferrin

Ovotransferrin is a monomeric glycoprotein consisting of 686 amino acids with a molecular weight of 76 kDa (Abeyrathne, Lee *et al.*., 2013) and a pI of 6.1, it is the second most abundant protein in EW, accounting for 12% - 13% of EW proteins. Moreover, ovotransferrin displays multiple activities. As with other transferrins,

ovotransferrin has a strong iron-binding activity. This ability is thought to contribute to antimicrobial properties by depriving microorganisms of the iron necessary for their growth. For example, ovotransferrin has been found to suppress *Pseudomonas* sp., *Escherichia coli*, and *Streptococcus mutans* (Valenti, Antonini *et al.*., 1982). Considering its effect on the treatment of acute diarrhea, ovotransferrin has already been suggested and used as an infant formula ingredient (Del, Leone *et al.*, 1985). Wu and Acero-Lopez (2012) also reported that ovotransferrin has an antioxidant effect on poultry meat by establishing a cellular redox environment.

Table 1-1: Major egg white proteins and selected properties (Mine, 1995)

Protein	Amount (%)	Molecular Weight (kDa)	pI	Characteristics
Ovalbumin	54	45	4.5	---
Ovotransferrin	12-13	77.7	6.0	Binds iron and other metal ions
Ovomucoid	11	28	4.1	Inhibits serine proteinases
Lysozyme	3.4-3.5	14.3	10.7	Lysis of bacterial cell walls
Ovomucin	1.5-3.5	220-270000	4.5-5.0	Interacts with lysozyme

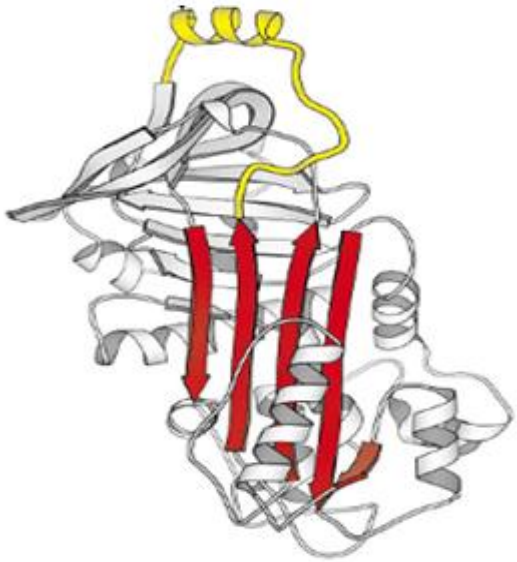

Fig.1-1: The 3-D crystal structure of ovalbumin with the α-helix reaction loop in yellow and main β-sheet A in red (Huntington *et al.*, 2001).

1.1.3 Ovomucin

Ovomucin is a sulphated EW glycoprotein which is composed of two subunits: α-ovomucin with a carbohydrate content of 15% and β-ovomucin with a carbohydrate content of 50%. Two forms of ovomucin exist in EW: insoluble and soluble. Soluble ovomucin is present both in thick and thin albumen, while insoluble ovomucin is found only in thick albumin (Hayakawa and Sato, 1977).

Ovomucin is known to be critical for keeping the high quality and freshness of thick albumen (Wang, Wang *et al.*, 2018). Liu, Oey *et al.*(2017a) suggested that retaining the ovomucin-depleted EW proteins in solution during processing has potential industry applications, for example, protein fortification of drinks with a minimal solution turbidity. Moreover, recently, researchers are paying more and more attention to ovomucin's role as a health-promoting component. For example, Kodama and Kimura (1999) found that ovomucin inhibits colonization of *Helicobacter pylori*.

Ovomucin has similar structures as mammalian mucins: it has a long linear protein chain with a randomly coiled structure, with carbohydrate chains attached to the protein core, in a "bottle brush' configuration (Bansil and Turner, 2006). These structures suggested that ovomucin may possess protein-resistant p properties. This hypothesis was validated by Sun, Huang *et al.* (2018), who found that the strong electrostatic and steric repulsions between protein layers could be attributed primarily to the protein-resistant property of ovomucin. This finding demonstrates that ovomucin has antifouling potential with broad applications in the areas of food processing and biomedical implants.

1.2 Enzymatic hydrolysis of food proteins

Enzymatic proteolysis is a process with mild reaction conditions and avoidance of undesirable byproduct. It has been extensively studied and described over the last 60 years (Aspmo, Horn *et al*., 2005). Van der Plancken *et al*. (2003) studied the effect of heating in the temperature range of 50−92 °C on the susceptibility of ovalbumin and albumen solutions to enzymatic hydrolysis by a mixture of trypsin and α-chymotrypsin at 37 °C and pH 8.0. It was shown that heat treatment resulted in an increase in degree of hydrolysis after 10 min of enzymatic reaction for both ovalbumin and albumen.

Generally speaking, hydrolysis of peptide bonds causes several changes in proteins:

(1) the NH^{3+} and COO^{-} content of the protein increases, increasing its solubility,

(2) the cleavage of peptide bonds, resulting in breakdown of proteins to peptides and amino acids, and

(3) the globular structure of the protein is altered, exposing previously hidden hydrophobic groups (Can-peng, 2005).

Thus enzymatically hydrolyzed proteins possess functional properties, such as low viscosity, increased whipping ability, and high solubility, which make them advantageous for use in many food products (Panyam and Kilara，1996).

Not only the physical functionalities but also the bioactivity of protein hydrolysates have been studied. Dávalos *et al.* (2004) studied the antioxidant activity of peptides produced by enzymatic hydrolysis of crude EW with pepsin. Results showed that four peptides included in the protein sequence of ovalbumin possessed radical scavenging activity higher than that of Trolox. EW hydrolyzed by pepsin for 3 h was previously found to exhibit a strong angiotensin I–converting enzyme (ACE) inhibitory activity in vitro. Mine *et al.* (2004) obtained lysozyme hydrolysate by peptic digestion and

subsequent tryptic digestion, and found that proteolytic hydrolysis broadened the antimicrobial activity of lysozyme to gram-negative bacteria. Hydrolyzed egg yolks (EY) have been shown to inhibit ACE action in vitro and to suppress the development of hypertension in SHRs after oral administration for 12 weeks (Yoshii, Tachi *et al.*, 2001). Enzymatic hydrolysis of protein is a promising method with potential to be widely used in the foods and pharmaceutical industries.

1.3 Physical properties of egg white proteins

EW protein is widely utilized as a functional ingredient in the food industry, because of its nutritional and functional properties. Functional properties such as foaming, gelling and emulsifying characteristics can give processed foods unique color, flavor, and texture characteristics. Multiple studies completed in recent decades, showing that many functional properties depend on the exposition of hydrophobic groups in the molecular surface and the interactions of these groups with air (foam), oil (emulsion) or other protein molecules (gels and coagulation) (Li-Chan, 1989).

1.3.1 Foaming property.

Protein molecules act as hydrophilic and hydrophobic groups. The hydrophilic groups are arranged towards the water phase and the hydrophobic groups towards the air phase. During the whipping process air comes into the solution to form bubbles and the hydrophobic regions facilitate adsorption at the interface. Egg albumen has excellent food foaming properties due to its rapidly adsorb on the air-liquid interface during whipping or bubbling and its ability to form a cohesive viscoelastic film by way of intermolecular interactions (Mine, 1995). Having a mixture of proteins allows EW to perform well in foams because each component of EW carries out a different function

(Stadelman and Cotterill，1994), even though each component alone has little or no foaming capacity (Johnson and Zabik, 1981).

Lomakina and Mikova (2006) found that this foaming capacity is significantly affected by protein interactions with ovomucin and lysozyme, while ovomucoid, ovotransferrin and ovalbumin had smaller effects. In food processing, pasteurization of liquid EW near 60°C weakens the foaming capacity of EW liquid; in fact, its foaming property begins to be damaged at temperature as low as 54°C (Cunningham,1965).

The quality of EW is another important factor which affects its foaming capacity. Precisely, the whipping volume of the whole EW has been found to increase slightly with the increasing age of the hen, and storage of egg also has a moderately positive effect on whipping volume (Silversides and Budgell, 2004). This effect was previously studied by Hatta *et al.*(1996), who found that thick egg albumen proportion changes from 50% in fresh hen eggs to 30% after 12 days storage at 25°C, resulting in a decrease in the viscosity of EW, which may explain the influence of the freshness of EW on its foaming capacity. Moreover, Van der Plancken *et al.* (2007) studied the effect of moisture content during dry-heating at 80°C on the foaming properties of freeze-dried EW, and found that the foaming capacity of dried EW increased with longer dry-heating time. A high moisture content of the dried EW contributed to an improvement of foaming capacity rapidly.

1.3.2 Emulsifying property.

Emulsification is the most important process in the manufacturing of many formulated foods. Emulsion is a heterogeneous system of one liquid dispersed throughout another in the form of droplets usually exceeding 0.1 μm in diameter. Food emulsion can be categorized as oil in water (O/W) or water in oil (W/O). The former

emulsion commonly exhibits a creamy texture, while the latter emulsion has greasy textural properties. Due to their capacity to lower the interfacial tension between hydrophobic and hydrophilic components, proteins play a role as effective surface-active agents. Thus, they participate in the formation of O/W and W/O emulsions and stabilize the emulsions that are formed.

Emulsions stabilized by proteins are of great interest. The emulsifying property of proteins basically depend on two effects: (1) a substantial decrease in the interfacial tension due to the adsorption of the protein at the oil-water interface and (2) the electrostatic, structural and mechanical energy barrier to particle association and phase separation, opposing destabilization processes (Izmailova, Yampolskaya *et al.*, 1999). The emulsifying capacity of whole eggs, EY and even EW plays a role in baking and other applications. EW emulsifies due to its albumin protein component, while for EY it is its lipoprotein content. Compared with EY, the emulsifying property of EW is low, hence, in order to broaden the application of EW, many studies have investigated how to improve the emulsifying property of EW. Kato *et al.* (1989) found that after heating at 80°C with 7.5% moisture content for 7 days, the emulsifying properties of EW powder increased with longer heating time, correlating with surface hydrophobicity. Li, Wang *et al.*(2018) indicated that the foaming/emulsifying properties of EW/EY proteins can be manipulated by altering physicochemical characteristics such as charge, surface tension and particle size.

1.3.3 Gelling property.

Gel is an intermediate between solid and liquid, with both flow and elastic characteristics. Gelation is an important commercial process given the number of cooked consumer products, such as desserts, puddings, reformulated meat products, tofu,

and surimi, that rely on protein coagulation, especially the coagulation of egg proteins (Alleoni, 2006). Proteins make gels through coordinate polymerization of molecules, providing a three-dimensional network, and this process occurs by the transformation of the viscous liquid into a viscous-elastic matrix. Both EW and EY have the capacity to form gels upon heating. Gel formation is a two-step process of denaturation followed by aggregation of denatured proteins, as shown in Fig.1-2. In the first step, changes in the conformation (usually induced by heating) or partial denaturation of the protein molecule occur. With denaturation, the dispersion velocity increases as a result of increasing molecular dimensions caused by unfolding of the protein molecule (Ferry 1948). In the second step, a gradual association or molecule aggregations of denatured proteins leads to an exponential increase in viscosity, and to the formation of a three-dimensional network (Hermaneson, 1979; Phillips *et al.*, 1994).

Because of transportation and shelf-life requirements in the egg industry, egg liquid is usually dried and conserved as egg powder, which can withstand high temperatures that allow for the destruction of all pathogens. Kato *et al.* (1990) found that gel strength of dried EW greatly increased by heating in the dry state at 80°C with initiating the Maillard reaction. In France, two types of treatments are used to improve functional properties (whipping and gelling) of dried EW: standard storage at 67°C for about 15 days and storage at 75 to 80°C for 15 days (Baron, Nau *et al.*, 2003). Moreover, Matsudomi *et al.* (2002) improved gelling properties of dried EW by modification with galactomannan through the Maillard reaction.

1.4 Bioactivity of egg white protein.

Egg proteins are nutritionally complete with a good balance of the essential amino

acids that are needed for building and repairing cells in muscles and other body tissues. Enzymatic hydrolysis of proteins releases bioactive peptides and different enzymes have different abilities to release such bioactive fractions (Mine, 2007). Research on egg protein-derived bioactive peptides has progressed during recent decades as shown in Table 1-2. These bioactive peptides are mainly derived from ovalbumin and ovotransferrin which are the two most abundant components of EW proteins. These results also broaden the consumption of eggs, giving an innovative way for the egg industry to update conventional egg products to high-value added products.

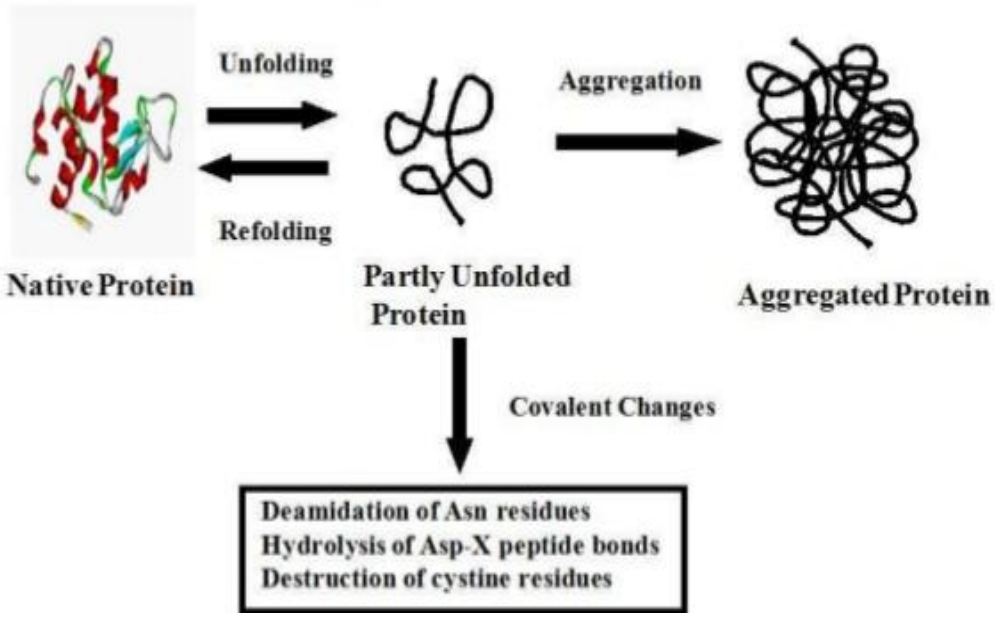

Fig.1-2: Two-step process of gel formation of proteins.

Table 1-2. Examples of bioactive egg white-derived peptides.
(Eckert *et al.*, 2013; Liu *et al.*, 2017b)

Encrypting proteins	Name/remarks/sequence	Effect	Reference
Ovalbumin	AHK, VHH, VHHANEN	Antioxidant	(Takusyoku, 1991; Dávalos, Miguel *et al*., 2004)
Ovotransferrin	Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu	ACE inhibitory and antioxidant	(Majumder and Wu, 2011) (Iwaniak and Minkiewicz 2007)
	Ile-Arg-Trp, Ile-Gln-Trp, Leu-Lys-Pro	ACE inhibitory, Ameliorates Insulin Resistance	(Son *et al.*, 2017)
	OTAP-92	Antimicrobial activities	(Ibrahim *et al.*, 2000)
	Not specified	Anticancer	(Lee *et al.*, 2017)

1.5 Shrinkage of meat

Cooking of meat is essential for sterilizing foodborne pathogens, assuring microbial safety and achieving meat quality (Pathare and Roskilly, 2016). Meat shrinkage and cooking loss have been thought to be the poor meat quality indication by consumers (Barbera and Tassone, 2006). From a nutritional perspective, cooking loss also brings loss of soluble proteins, vitamins and other micronutrients (Yarmand *et al.*, 2013). During cooking, the distinctive meat proteins are heat denatured, resulting in destruction of cell membranes, shrinkage of meat fibers, and aggregation and gel formation of myofibrillar and sarcoplasmic proteins, as well as the shrinkage and solubilization of the connective tissue (Tornberg, 2005; Pathare and Roskilly, 2016).

All meat will shrink in size and weight during cooking. The extent of shrinkage depends on the fat and moisture content of meat, the cooking temperature, and the cooking time. Basically, the higher the cooking temperature, the greater the shrinkage; overcooking draws out more fat and juices from ground beef, resulting in a dry, less tasty product. In order to avoid or decrease shrinkage and cooking loss of meat, many studies have been conducted. Heating temperature has been shown to affect the texture of meat, with a low cooking temperature yielding a tender product with lower cooking losses (Marshall, Wood *et al*., 1960; Penfield and Meyer, 1975). Moreover, cooking method was also shown to have an effect on the physical properties and cooking loss of meat. Domínguez, Gómez *et al*. (2014) proved that microwave cooking resulted in the highest cooking loss of foal meat comparing with other cooking methods (roasting, grilling and frying).

In addition to cooking method, different treatments have been studied to enhance

the appearance and flavor of meat products. Polyphosphates are known to change pH value, increase the amount of bound water, decrease weight losses from cooking, improve texture and sensory properties (tenderness, juiciness, color and flavor), extend shelf-life, etc. Therefore, polyphosphates have been widely used in meat processing industry (Long, Gál *et al*., 2011). However, as cardiovascular morbidity and mortality have been associated with high intake of phosphate additives, the use of polyphosphates faced to criticism (Ritz *et al*., 2012; Glorieux *et al*., 2017).

Proteins are very important for sensory properties and quality of meat products. Omana *et al*. (2012) thought that lean meat content (protein content) should be sufficient to stabilize emulsion and gel formation during heating. Cereal and legume proteins can be added to meat products to help reduce formulation costs and cooking loss, to improve nutritional value, and to improve in emulsifying property (Correia and Mittal, 2000). Among all the cereal and legume proteins, soybeans are the most commonly used in processed meat products due to their low cost and functional properties (Omana *et al*., 2012). Soy protein isolate (SPI) as a binder is also widely used in processed meat, resulting in reduced costs and water loss (Homco-Ryan *et al*., 2003). However, the U.S. Department of Agriculture set a limit of 3.5% for cereal-based materials incorporated in the meat formulations, and SPI is limited to 2% (Homco-Ryan *et al*., 2003).

In addition to cereal and legume protein, animal protein like EW is also used in cooked sausages such as frankfurters, due to its ability to form a stable and heat-irreversible gel, which positively contributes to the firmness of low-cost emulsified sausages. The addition rate of EW varies widely; high inclusion levels result in an egg flavor within the finished product (Keeton and Osburn, 2001).

1.6 Objectives of the present study

EW has been widely used in the food industries because of its nutritional value and functional properties, such as foaming and gelling. Based on these properties, many foods with different textures have been developed in the past years. Enzymatic hydrolysis has proved to be a moderate and environmentally friendly method to modify protein characteristics to improve the physical properties of proteins. As well, several bioactivities like antioxidative capacity and antimicrobial activity could be reserved in the hydrolysates (certain peptides).

Of the physical properties of EW, the most applied in the food industry are gelling and foaming capacity. Even as a protein with both hydrophilic and hydrophobic sites, however, EW protein shows a quite weak emulsifying property which limits its application in both food and cosmetic industries. In our previous study, the emulsifying activity and emulsifying stability were ameliorated by partial hydrolysis by a thermo-stable enzyme –Protin NY100®, Thermoase PC10F®, Protease M®, combined with a heat treatment at 90°C. Both the enzymatic hydrolysis and heat treatment were proved to be indispensable to obtain an excellent emulsifying property which could even be comparable to that of EY.

In our present study, we tested another two kinds of enzymes, to test their potential and possibilities to be used to improve properties of EW.

Thus, the objectives of this research are:

1) to examine characteristics and functional properties of EW hydrolysates by using three different enzymes.
2) to study the effect of hydrolysis on an antioxidative capacity of EW

hydrolysates, and its potential to suppress color change in pork meat during storage.

3) to investigate cooking loss and shrinkage rate of pork meat slices soaked in different EW hydrolysates solutions.

Chapter II describes how EW proteins were hydrolyzed by three enzymes-Protin NY100®, Thermoase PC10F®, and Protease M®, followed by heat treatment at 90°C, and characteristics and functional properties of these obtained hEWs were examined, including solubility, water-holding capacity (WHC), oil-binding capacity (OBC) and emulsifying capacity in relation to the enzyme types used in the hydrolysis process. Furthermore, in Chapter II, the antioxidative capacity of hEWs were also studied. To study EW's potential to be used as a natural preservative in meat processing, pork meat slices were soaked separately in each hEWs solution. The effect of hEW on suppressing color change and myoglobin proportions of meat slices was also studied. Meat shrinkage and cooking loss are also key factors of meat quality for consumers. In the last part of this project, meat shrinkage and cooking loss were evaluated for pork meat slices treated by soaking in each hEWs solution. In order to understand better, the microstructures of these treated meat slices were also examined.

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Chapter II

Properties of partially hydrolyzed egg white

2.1 Introduction

Egg white (EW) is a significant protein source of dietary protein, accounting for about 58% of the entire mass of an egg, with a protein content of about 10% (Abeyrathne *et al*., 2013; Kovacs-Nolan *et al*., 2005). It is also known as a desirable ingredient for many foods such as bakery goods, meringues, and meat products in which it is mainly used because of its excellent gelling and foaming properties (Mariotti *et al*., 2012). However, for some applications, it could be useful to improve and to diversify EW properties. In particular, increasing the emulsifying properties of EW could be an innovative way to obtain a pure protein emulsifier, which is a fat-free functional ingredient compatible with "light food" claims.

Enzymatic modifications are efficient for modifying protein functionality (Panyam *et al*., 1996). Especially, proteolysis has been suggested as an efficient way to improve functional properties by (Lqari *et al*., 2005). These authors showed that lupin protein and α-conglutin hydrolyzed by alkaline protease (alcalase) had better emulsifying activity (EA) than native lupin protein and α-conglutin, respectively. Although the emulsifying stability (ES) of hydrolysates of lupin protein and α-conglutin decreased relative to the native proteins, lupin protein hydrolysates were still thought to be potential to be used as ingredients in emulsion-based food formulations such as salad dressing and mayonnaise. Furthermore, thermal treatments that are usually used for inactivating the enzymes have also been shown to affect protein structure (Sanchez and

Fremont , 2003), which should be related to protein functionality.

In the present study, physical properties of partially hydrolyzed EW protein by partial hydrolysis were measured, including emulsifying properties, water-binding capacities and oil-binding capacity.

2.2 Materials and Methods

2.2.1 Preparation of egg white hydrolysates.

Three kinds of enzymes: Protin NY100®, Thermoase PC10F®, Protease M®, which were provided by Amano Enzyme Inc. (Japan) were used in this study, EW hydrolysates obtained using these three enzymes were named: PNY, T and PM, respectively. Optimal pH and temperature for each enzyme were listed in Table 2-1.

Hen eggs were obtained from a local supermarket (Kyoto, Japan) and were manually broken and separated yolk from EW. EW was mixed using a hand mixer (National MK-210, Japan) at a rotational speed of 540 rpm for 3 s, then filtered by passing through a stainless mesh (sieve size 0.60 mm), any foam was removed. The pH of EW was adjusted to each optimal working pH as shown in Table 2-1 with 10% (w/v) citric acid solution before using for the experiment.

The enzyme was added at a concentration of 0.4% (w/w) after EW being warmed up to 50°C. Enzymatic treatments were conducted as follows: 10 min at 50°C, then adjusted to its corresponding optimal working condition, and maintained for 30 min before inactivation. Inactivation of the enzyme was achieved by holding the resulting hydrolysates at 90°C for 8 min, before homogenization by a mechanical homogenizer (IKA T18 basic, Germany) at Dial 5 (15,000 rpm) for 60 s. To ensure enzyme was completely inactivated, x-ray films (Fuji Film, Japan) were used. The surface of x-ray

film is covered with thin gelatin film, which is hydrolyzed by the possible remained active enzyme, leading to the appearance of transparency of films.

EW hydrolysates were then freeze-dried (FreeZone Plus 12 Liter Cascade Console Freeze Dry System, Labconco, Japan) and stored as powder at -30°C in a Biomedical Freezer (MDF-U539-PJ, Panasonic, Japan).

2.2.2 Reference emulsifying peptide.

Runpep® (Pharma Foods International Co. Ltd, Japan) is a mixture of EW peptides with molecular weight lower than 10 kDa (as reported in the product description), it was used as a reference for emulsifying properties. Runpep (80% proteins) was dissolved in distilled water at a concentration of 100 mg (protein) / mL as a reference sample, which was then stored at 4°C until use.

2.2.3 Determination of total protein content.

Determination of total protein content in hEW, nEW, Runpep, and EY was conducted by modified Lowry method (Lowry *et al*., 1951; Markwell *et al*., 1978).

2.2.4 Determination of hydrolysis degree.

EW hydrolysate powder was dissolved in distilled water at a concentration of 100 mg/mL before determination of the degree of hydrolysis (DH). Free amino groups were quantified using the *o-phthalaldehyde* (OPA) micromethod described by Church *et al*., (1983) and with modifications by Darrouzet-Nardi *et al*., (2013).

OPA reagent was mixed as follows: 25 mg OPA were dissolved in 2.5 mL methanol; then 2.5 mL SDS 20% and 50 µL β-mercaptoethanol were added, and the solution was filled up to 100 mL with 20 mM potassium tetraborate. The reagent was covered with aluminium foil to protect from light.100 µL OPA reagent were thoroughly mixed with 50 µL hydrolysate samples and incubated at room temperature for 10 min

before reading the absorbance at 340 nm by a spectrophotometer (Infinite M200, TECAN). A standard curve was previously prepared using methionine solutions concentration from 9.4 to 53.6 µg / mL).

Total acidic hydrolysate of EW was used as a reference for complete hydrolysis (DH = 100%). It was prepared by adding 2 mL 6 N HCl to 2 mg EW protein powder before the mixture was heated at 110°C for 18 h. After then, vacuum concentration was used in order to remove the remaining HCl in the hydrolysate, and the hydrolysate volume was adjusted to the original EW sample volume with distilled water.

For each enzymatic hydrolysate, DH was calculated as follows:

$$\%\,\mathrm{DH} = \frac{L_t - L_0}{L_{tot}} * 100$$

Where L_t is the amount of liberated free NH_2 at time t min, L_0 is the amount of the free NH_2 at 0 min, and L_{tot} is the maximum amount of the free NH_2 obtained after complete acidic hydrolysis.

2.2.5 Surface hydrophobicity.

Samples were diluted with phosphate buffer (0.01 M, pH 7.0) before centrifuging at 10,000g for 10 min, and supernatant of each sample was stored at 4°C for further analysis. Protein surface hydrophobicity (H_0) was measured using fluorescence probe 1-anilinonaphthalene-8 sulfonic acid (ANS). ANS solution (45 µl, 8 mM) was added to 3 ml sample solution. ANS fluorescence intensity was measured at 470 nm with excitation at 390 nm. Excitation and emission slits were 2.5 nm. The slope of the plots of fluorescence intensity versus protein concentration (0, 0.05, 0.1, 0.15, 0.25 mg/ml) was calculated by linear regression and used as a measurement of H_0.

Table 2-1 Working condition of enzymes.

Enzyme name	Proteolytic activity of enzyme	Optimal Temperature and pH	Enzyme Inactivation temperature /T°C
Protin NY100®	900,000 U/g	50°C; pH 7.0	65-70°C
ThermoasePC10F®	700,000 U/g	65°C; pH 7.5	85-90°C
Protease M®	40,000 U/g	50°C; pH 6.0	60-70°C

2.2.6 Determination of emulsifying properties.

Emulsifying properties were measured according to the turbidimetric method developed by Pearce *et al*. (1978) with slight modifications. Briefly, colza oil, hEW (or EY, Runpep, nEW) and water were homogenized with a weight ratio of 3:2:1 by a mechanical dispenser (Polytron PT-MR2100, Switzerland) at 25,000 rpm for 1 min, then 200 μl of emulsion was pipetted from the bottom of the container immediately (T_0) and 2 hours (T_{2h}) after homogenization. Each aliquot was diluted 1,000 times with SDS solution (0.1%, w/v). Absorbance of these diluted emulsions (A_0 and A_{2h}, respectively) were measured at 500 nm by a spectrophotometer (Unico S1205, USA). A_0 indicated emulsifying activity (EA). Emulsifying stability (ES) was calculated as follows:

$$ES = A_0 / (A_0 - A_{2h})$$

2.2.7 Particle size measurement.

hEW (PNY, T and PM) or native egg white (NEw) was diluted to the final protein concentration of 2% (w/v) with denionized water. Then the protein solutions were mixed with colza oil at a volume ratio of 9:1, followed by pre-homogenizing for 2 min at 13,000 rpm using a homogenizer (Polytron PT-MR2100, Switzeland) equipped with a 5 mm diameter head. The resulting emulsions were sealed and stored at 4°C until analysis. Droplet size distribution profiles of various freshly prepared emulsions were obtained with a laser diffraction particle size analyzer (SALD-2200, Shimadzu, Japan). Droplet size measurements were reported as the volume-average droplet size, $d_{3,2} = (\sum n_i d_i^3 / n_i d_i^2)$, where n_i is the number of droplets with diameter d_i (Chang, Niu *et al*., 2016). All determinations were conducted on individual sample in triplicates.

2.2.8 SDS-PAGE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was

performed according to Laemmli (1970). Pre-cast gels of 5-20% acrylamide (C-520L, Atto Corporation, Tokyo, Japan) and protein ladder (WSE-7020, Atto Corporation, Tokyo, Japan) with the molecular weight from 10 kDa to 245 kDa were used.

2.2.9 Solubility.

Solubility was determined using method described by Snyder and Kwon (1987) with slight modifications. An aqueous solution (1.0%, w/v) of samples in deionized water was stirred magnetically for 30 min. Then it was centrifuged at 13,500 rpm for 30 min at 4°C (CFRXII, Hitachi, Japan). After an appropriate dilution with deionized water, protein content of the supernatant was determined by the method of Markwell *et al*. (1978). The soluble protein percentage was expressed as (protein content of the supernatant) / (sample protein content) ×100.

2.2.10 Water-holding capacity.

Water-holding capacity (WHC) was determined as described by D'appolonia (1977). Samples (1 g) added to centrifuge tubes (15 mL) containing distilled water (9 mL). Tubes were shaken at room temperature for 2 h. Samples were then centrifuged at 9,000 rpm (CFRXII, Hitachi, Japan) for 30 min at 20°C. Then tubes were inverted and allowed to drain for 10 min, supernatant was decanted, drained weight was determined. WHC was determined as percent of water retention.

2.2.11 Oil-binding capacity.

Oil-binding capacity (OBC) was determined using a modified method of Homco-Ryan *et al*. (2003) and Seguchi (1985). Sample (0.3 g) was combined with colza oil (3 mL) in a 15-mL plastic test tube. Tubes were shaken vigorously by a mechanical shaker for 1 min before standing at room temperature for 1h. Then samples were centrifuged at 3,500×g for 25 min at 20°C. Tubes were inverted and allowed to drain for

30 min, drained weight was determined. OBC was calculated as: OBC = oil bound (g) / sample (g) × 100.

2.2.12 Statistics analysis.

All experiments were carried out in triplicates. The data were subjected to multifactor analysis of variance (ANOVA), followed by the Least Significant Difference (LSD) test to determine the significant difference between samples at $p < 0.05$ using the software SPSS V.16.

2.3 Results and Discussion

2.3.1 Degree of hydrolysis.

When analyzed by SDS-PAGE, EW proteins presented a wide range of molecular masses and concentrations. The main EW proteins: ovalbumin (44.5 kDa), ovotransferrin (77.7 kDa), ovomucoid (28 kDa) and lysozyme (14.3 kDa), constitute 54, 12, 11 and 3.4% of the total EW proteins, respectively (Abeyrathne, Lee *et al*. 2013). As observed in Fig.2-1, Lane I-6 represented band of NEw, the bands for ovotransferrin (around 75 kDa) and lysozyme (around 15 kDa) could be observed easily, but the bands for ovalbumin and ovomucoid were connected to each other. Unlike NEw, the band around 75 kDa for sample PNY, T and PM disappeared completely, which suggested that ovotransferrin could be hydrolyzed easier by Protin NY100®, Thermoase PC10F® as well as Protease M® than ovalbumin. Moreover, Peptide S was hydrolyzed the most, with a DH of 26.0% (Fig.2-2) and an average molecular weight less than 10 kDa shown in Fig.2-1 I. After passing through 0.45 μm of the filter, most bands between 35 kDa to 45 kDa were still visible. Most bands for EW hydrolysates T disappeared after filtration, that means the percentage of water-soluble protein in these three samples are low, which

is in accordance with the result of solubility measurement shown in Fig.2-2.

2.3.2 Solubility.

In this study, pork meat slices were planned to be soaked in the hEW solutions thus, solubility of hEW was one of important factors to evaluate its possible application on meat slices. Degradation of proteins by a proteolytic enzyme was widely used to increase the solubility and retain the nutritional values of proteins. According to Table.2, solubility did not differ between NEw and Peptide S, solubility of hEW (PNY, T and PM) decreased compared with NEw. This inconsistence may have occurred due to high temperature (90°C) used for inactivation of enzymes during the preparation of hEW, which led to the appearance of some insoluble aggregates, while freeze drying didn't not affect the high solubility of NEw. The order of solubility of hEW was as follows: PNY > PM > T.

2.3.3 Surface hydrophobicity.

Surface hydrophobicity was reported to have great significance in elucidating the protein functions (Kato and Nakai, 1980). Fig.2-3 shows the fluorescence intensity of the EW hydrolysates prepared by various enzymes and NEw solutions, as the wavelength changes. It was observed that the solution prepared by T showed the highest fluorescence intensity compared to other samples. Result of surface hydrophobicity was shown in Fig.2-4. Except for small peptide S, solutions prepared by hEW microparticles showed an increase of surface hydrophobicity compared to solution prepared by NEw, this result was in accordance with our former study (Wang *et al*., 2018), partial hydrolysis contributed to an increase of surface hydrophobicity. High surface hydrophobicity indicating a better molecule flexibility and higher expansion degree of

proteins, thus resulted in a better adsorption capacity onto oil/water interface (Chang *et al*., 2016a; Chang *et al*., 2016b).

2.3.4 Average droplet size.

The average droplet size, difference between the maximum and minimum diameter of droplets of the dispersed phase and the degree of their dispersion are considered as the significant parameters characterizing a given emulsion (Dajnowiec *et al*., 2016). The droplet size distribution influences the properties of emulsion in aspects such as degradation rates, long-term stability, texture and optical appearance (Fernandez *et al*., 2004; Jurado *et al*., 2007).

In the current study, oil droplet particle size was used to evaluate the emulsifying properties of hEWs by using different enzymes. Mean diameters of oil droplets ($d_{3,2}$) in the emulsions stabilized by different hEWs were shown in Table 2-2. The largest particle size was found in Peptide S, referring to lower emulsifying property of Peptide S. The average particle sizes of hEW samples (PNY, T, PM) were observed less than that of NEw and Peptide S. It was reported that smaller particle size allowed the protein to coat the fat or water droplets more efficiently as there were more particles available to form a monolayer (Homco-Ryan *et al*., 2003).

Droplet size distribution curves were also shown in Fig.2-5. According to the shape of curves, EY and Runpep exhibited single peaked droplet size distribution, the amount of small droplets size (between 0.1 and 1.0 μm) was found to be the most in EY. The smallest and the largest particle size were found in EY and Runpep respectively, referring to the low emulsifying property of Runpep compared to EY. This result is in accordance with that obtained by the former turbidimetric method. Regarding hEWs,

shape of droplet size distribution curves became complicated than EY and Runpep, a peak at the point of size less than 10 μm was found for all the hEW samples.

2.3.5 Emulsifying properties.

The ability of a protein to aid the formation of an emulsion is related to its ability to attach to and stabilize the oil-water interface, the more the interfacial area that can be coated by the available protein, EA should be higher (Day *et al*., 2009). Due to the formation of smaller droplets during emulsification, more light scattering resulted in higher turbidity, and the turbidity increase indicates an increase in EA (Van Vliet *et al*., 2002). Similarly, the maintenance of a high turbidity value during the storage of an emulsion indicates high ES, while a turbidity decrease indicates instability of the emulsion.

Turbidity measurements of emulsions stabilized by different hydrolysates were performed immediately after emulsification (T_0) and after 2h of storage (T_{2h}). Absorbance (500 nm) observed at T_0 was used as an index of EA, ES was calculated by using the equation in the method. Results of EA and ES were shown in Fig. 2-6: among all the hydrolysates, EW hydrolyzed by Thermoase resulted in the best EA and ES, which was comparable to that of EY and much higher than that of NEw. It is noticeable that, regarding EA, almost all the hEW samples were better than NEw, which that partially hydrolysis of egg white contributed to the improvement of EA and ES. Peptide S showed a similar EA with that of PNY and PM, however, ES of Peptide S was such a small value (close to 1), that means turbidity of emulsions after 2h became almost 0, emulsions separated completely. This could suggest that the higher emulsifying properties are obtained for moderate proteolysis. And the highly hydrolyzed products-

like Peptide S offered an excellent EA but low ES.

It is well known that protein hydrolysates can be attached to the oil-water interface more efficiently compared to proteins, because of molecular size. However, protein hydrolysates are more difficult to form a network structure due to fewer hydrophobic binding sites (Pokora *et al.*, 2013), resulting in a relative worse ES of protein hydrolysates. Because the complex, folded and coiled protein molecules were cut down into separate units by the previous hydrolysis treatment, the hydrolysate after heating at 90°C (enzyme inactivation temperature) was unable to form a well ordered tertiary network or matrix, resulting in a creamy texture, without causing any gelling or coagulation even heated at 90°C.

2.3.6 Water-holding capacity and oil-binding capacity.

Results of WHC and OBC were shown in Table 2-2. Significant WHC difference existed among hEW (PNY, T and PM), sample T was able to retain nearly 90% of the water that it absorbed. WHC of NEw and Peptide S were fairly low, almost 0, which means that NEw and Peptide S could not retain any water that they absorbed. Regarding OBC, all samples were quite low. Furthermore, OBC decreased after hydrolysis.

2.4 Conclusion

The effects of enzyme kind on the degree of hydrolysis, surface hydrophobicity, and emulsifying properties of EW proteins were evaluated in this part. By using three kinds of enzymes, we could prepare egg white hydrolysates that were all more efficient than native egg white considering emulsifying activity and stability. The optimal enzyme to obtain best emulsifying properties was Thermoase 10F, which is a thermo-stable enzyme. Higher hydrolysis (in the case of Peptide S) resulted in peptides

which are with an excellent emulsifying activity but low emulsifying stability. Surface hydrophobicity was found to be an important factor related to the emulsifying activity and stability of hydrolyzed egg white proteins. Moreover, solubility did not differ between NEw and Peptide S, solubility of hEW (PNY, T and PM) decreased compared with NEw. Hydrolysate-T was able to retain nearly 90% of the water that it absorbed, but regarding OBC, all samples were quite low.

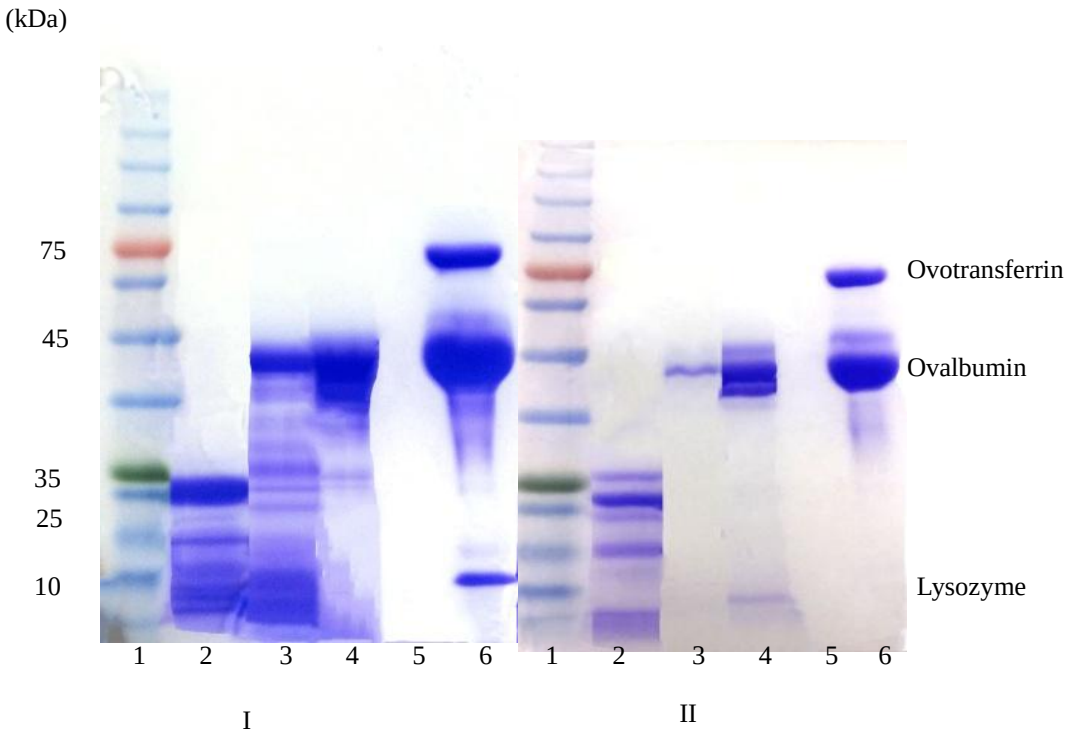

Fig.2-1 SDS-PAGE for various egg white hydrolysates and native egg white.

I: mixture of water-soluble fraction and water-insoluble fraction. II: water-insoluble fraction. Lane 1: molecular size markers. Lane 2: egg white hydrolyzed by Protin NY100®; Lane 3: egg white hydrolyzed by Thermoase PC10F®; Lane 4: egg white hydrolyzed by Protease M®; Lane 5: highly hydrolyzed commercial egg white peptides; Lane 6: native egg white.

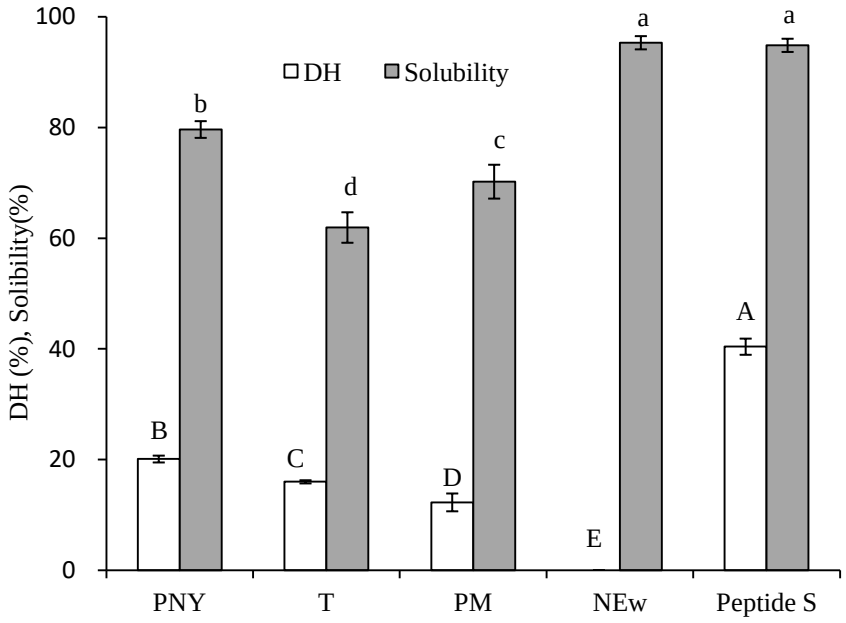

Fig.2-2. Solubility and degree of hydrolysis (DH) of egg white hydrolysates. Means within column with no common superscript differ significantly ($p < 0.05$).

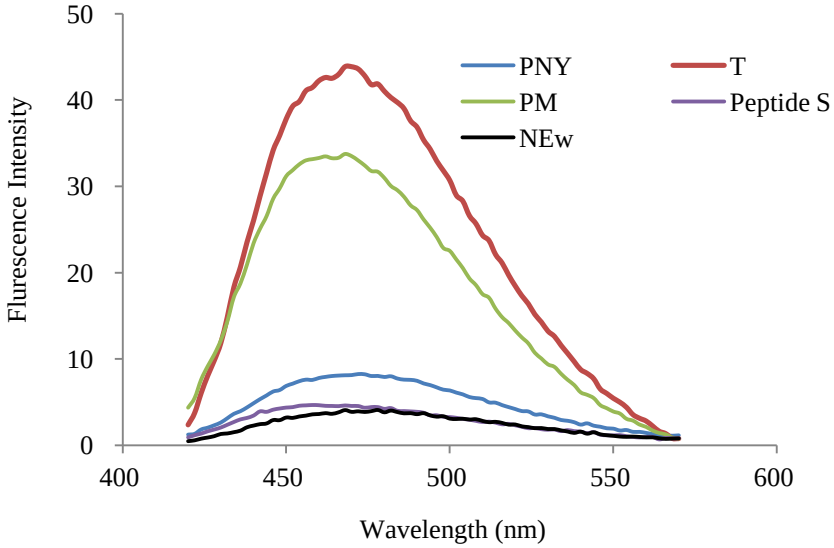

Fig.2-3. Fluorescence emission spectra of ANS in the presence of egg white hydrolysates and native egg white.

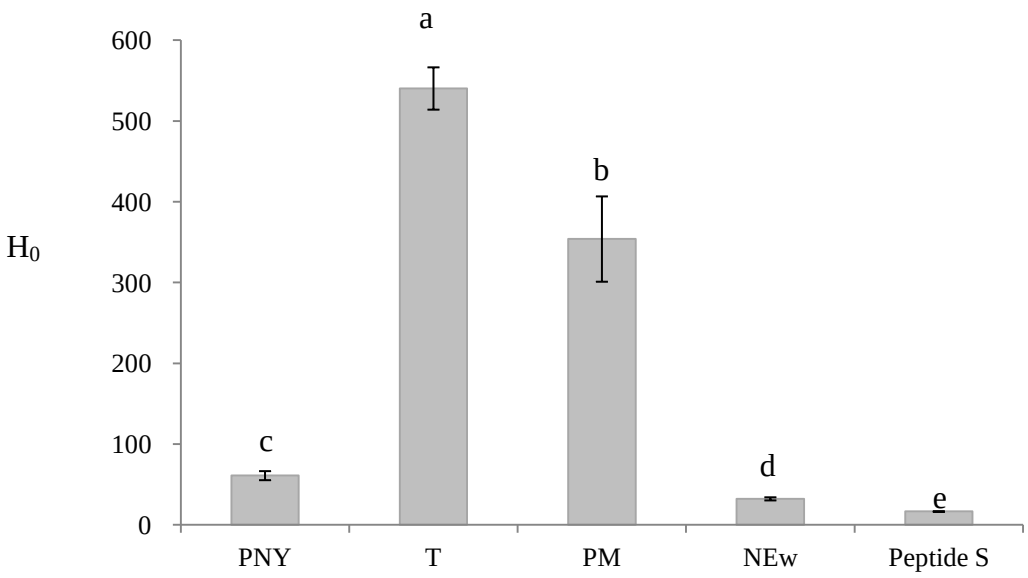

Fig.2-4. Surface hydrophobicity (H_0) of hydrolyzed EW samples. The same letters denote the lack of significant differences ($p < 0.05$).

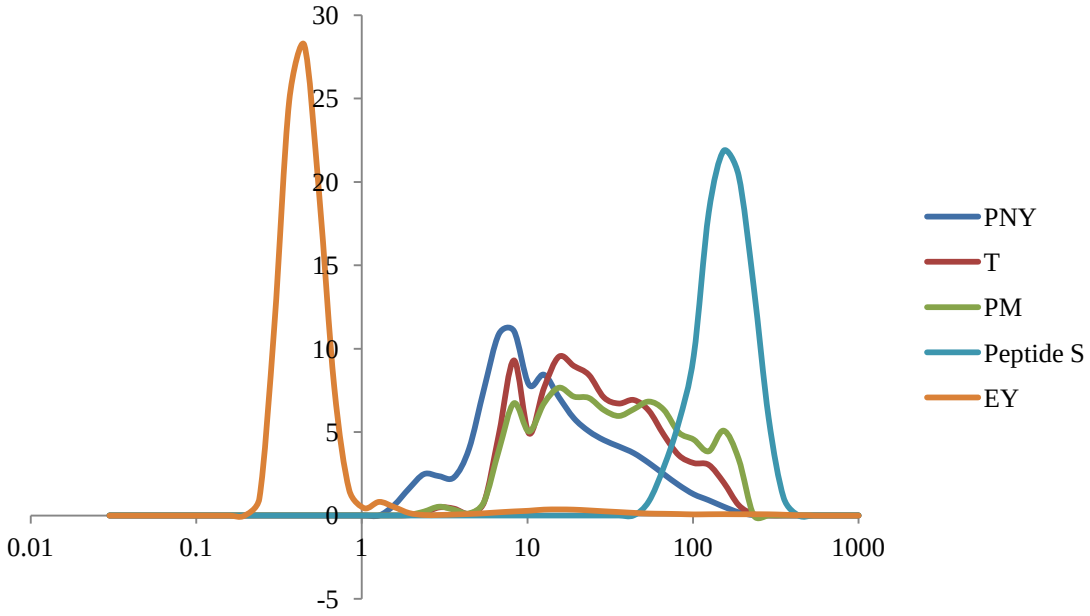

Fig.2-5. Particle size distribution of emulsion containing 10% oil and different emulsifiers.

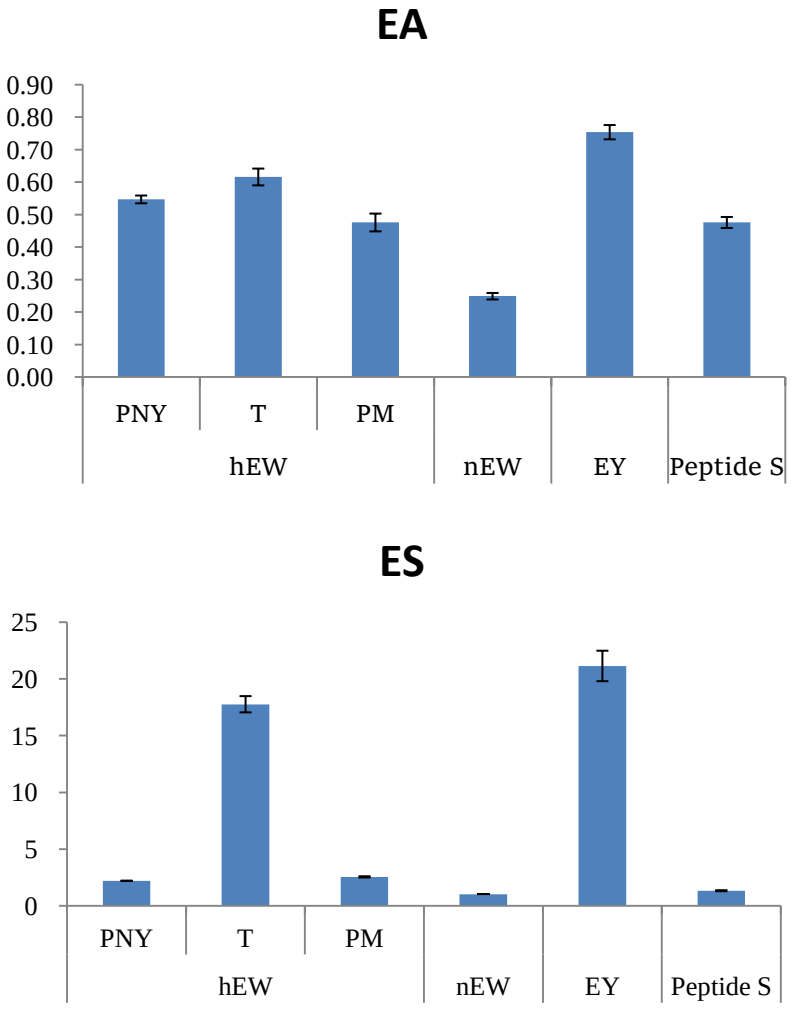

Fig. 2-6. Comparison of emulsifying activity (EA) and emulsifying stability (ES).

Table 2-2 Mean diameter of oil droplets ($d_{3,2}$) in the emulsions stabilized by different egg white hydrolysates**,** water-holding capacity (WHC) and oil-binding capacity (OBC) in different egg white hydrolysates and native egg white.

	PNY	T	PM	Peptide S	NEw
$d_{3,2}$	42.5± 0.50^{c}	27.3± 0.40^{e}	34.1±0.80^{d}	116.8 ±1.12^{a}	106 ±0.30^{b}
WHC	14.67 ±3.21^{c}	89.28 ±8.60^{a}	77.63 ±10.68^{b}	0.02 ±0.02^{d}	0.01 ±0.01^{d}
OBC	4.15 ±0.12^{b}	2.69 ±0.27^{c}	4.51 ±0.09^{b}	5.60 ±0.72^{b}	8.98 ±0.61^{a}

Means ± SD are shown (n=3). In each tested parameter, different superscript letters indicate significant differences between means in the same row ($p < 0.05$).

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Chapter III

Effect of egg white hydrolysates on color stability of pork meat slices

3.1 Introduction

Lipid oxidation is of great concern to the food industry, because it is a major cause of meat food deterioration, affecting color, flavor, texture and nutritional value (Mansour and Khalil, 2000). It was reported that 74% of consumers regarded that meat color, accelerated by lipid oxidation, as one of the major quality parameters affecting their decisions to purchase meat, because they viewed bright red color as a sign of freshness (Lynch *et al*., 1986; Ismail *et al*., 2009). It is known that the red color of meat depends on the concentration of myoglobin and its derivatives (Hood, 1980; Faustman *et al*., 1992). Metmyoglobin is the state when the iron has oxidized and appears tan or brown in color.

Application of antioxidants is the best strategy to prevent oxidation reactions in food (Shahidi, 2000). Antioxidants are added to different meat products for prevention of lipid oxidation, retarded development of off-flavors and improved color stability, including BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate) and TBHQ (tert-*butyl hydro* qui-none) (Kumar *et al*., 2015). But due to adverse attention received by synthetic antioxidants, and also due to the worldwide trend to avoid or minimize use of artificial (synthetic) food additives, many researchers around the world have been evaluating the potential of natural antioxidants for preventing lipid oxidation in food products (Gahruie *et al*., 2017).

Nasri *et al*. (2013) concluded that incorporation of protein hydrolysates prepared from Goby fish (*Zosterisessor ophiocephalus*) could delay lipid oxidation in turkey

meat sausage. Kim *et al*. (2013) assessed the antioxidant efficacy of 10 leafy edible plants and investigated the effects of butterbur and broccoli extracts on lipid oxidation in ground beef patties, the beef patties formulated with the selected plant extracts showed significantly better color stability ($p \leq 0.05$) than those without antioxidants. Mansour and Khalil (2000) proved that freeze-dried extracts from ginger rhizomes and fenugreek seeds were more effective than potato peel extract in controlling lipid oxidation and color changes during cold storage of beef patties.

Egg proteins are nutritionally complete with a good balance of the essential amino acids needed for building and repairing the cells in muscles and other body tissues. Nowadays, many researchers have uncovered the hidden biological functions of peptides hidden in egg proteins such as anti-hypertensive (Miguel and Aleixandre, 2006), anticancer (Ibrahim *et al*., 2009; Moon *et al*., 2013) or exaggerated antimicrobial activities (Ibrahim, Sugimoto *et al*., 2000). EW is also known as a desirable ingredient in foods such as bakery products, meringues and meat products in which it is mainly used for its excellent gelling and foaming properties.

In this study, we assessed the antioxidant efficacy of hydrolysates of EW proteins by using four different-sized distributions of EW hydrolysates, and evaluated their potential as natural antioxidants for meat preservation, especially for preventing or reducing color changes in pork meat stored at 4°C.

3.2 Materials and Methods

3.2.1 ORAC-FL assay.

The ORAC-FL assay was based on the method proposed by Ou *et al*.., (2001) and was modified as previously described by Dávalos *et al*., (2004). Samples prepared in 75

mM phosphate buffer (pH 7.4) were mixed with 50 µL of fluorescein (200 nM) and incubated at 37°C for 10 min. Trolox (2-16 µM) was used as the standard. Then 50 µL of 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (80 mM) as an oxygen radical generator was added to each well using an automated dispenser and the plate was automatically shaken for 5 s. Fluorescence was recorded using a fluorometric microplate reader (Infinite M200, TECAN, Switzerland) at 1 min interval for 60 min at excitation and emission wavelengths of 485 and 538 nm, respectively.

All reaction mixtures were prepared in triplicate, and at least three independent runs were performed for each sample. Measurements of fluorescence were normalized to the curve of the blank (no antioxidant). The net area under curve (AUC) of the sample was calculated by subtracting the AUC of the blank. ORAC-FL values were expressed as Trolox equivalents by using the standard curve calculated for each assay. Final ORAC-FL values were expressed as micromoles of Trolox equivalent per gram of protein for each hEW.

3.2.2 Treatment of pork slices by hydrolyzed egg white.

Approximately 8 cm×6 cm of lean pork (leg meat) slices with a thickness of 4 mm were supplied by a local butcher shop (Kyoto, Japan), 24 h postmortem. Fresh meat pH was determined as described by Pigott *et al.*, (2000). Each piece of pork was weighed, then soaked individually in the same weight of EW hydrolysate solution (protein concentration of 10%, w/w) in a zipper freezer bag. It was then stored in a fridge at 4°C for 24h before further analysis.

3.2.3 Color measurement.

Objective measurements of color were performed using a CR 400 colorimeter (Minolta, Osaka, Japan). Each slice of meat was cut and the color of the slices was

measured three times for each point. A portable colorimeter was used to measure meat color, with the settings: pulsed xenon arc lamp, 0 viewing angle geometry and aperture size 8 mm, in the CIELAB space (L: lightness; a: redness; b: yellowness,). Before each series of measurements, the instrument was calibrated using the reference white ceramic tile.

Total color change and % color change were calculated according to the following formula (Ünal *et al.*, 2014):

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

3.2.4 Relative proportions of myoglobin redox forms.

Pigments in pork samples were extracted according to the method of Lee *et al*., (1999) with some modifications (Viriyarattanasak *et al*., 2011). The sample (2 g) was first minced in a pre-cooled mortar and then placed into a 50-ml polypropylene centrifuge tube, and 20 ml ice-cold phosphate buffer (pH 6.8, 40 mM, 4°C) was added. The mixture was homogenized with a mechanical homogenizer (IKA T18 basic, Germany) at Dial 5 (15,000 rpm) for 20 s. The homogenized sample was centrifuged at 6,000 g for 30 min at 4 °C (CFRXII, Hitachi, Japan). In order to avoid any turbidity of the extracts, the supernatant was filtered with a filter paper (Number 2, Advantec, Toyo, Japan). The absorption at 525, 545, 565 and 572 nm of Mb derivatives was measured using a UV-vis spectrophotometer (U-2001, Hitachi, Japan).

The relative concentrations of metmyoglobin were calculated using the following equations. Measurements were performed in triplicate.

% metmyoglobin $=(-2.514R_1 + 0.777R_2 + 0.800R_3 + 1.098) \times 100$

While $R_1 = A_{572\ nm} / A_{525\ nm}$, $R_2 = A_{565\ nm} / A_{525\ nm}$, $R_3 = A_{545\ nm} / A_{525\ nm}$.

3.2.5 Statistical analysis.

All experiments were carried out in triplicates. The data were subjected to multifactor analysis of variance (ANOVA), followed by the Least Significant Difference (LSD) test to determine the significant difference between samples at $p < 0.05$, level using the software SPSS V.16.

3.3 Results and Discussion

3.3.1 Antioxidant activity (ORAC value) of egg white hydrolysates.

ORAC value was expressed as micromoles of Trolox equivalent per gram of protein. Egg proteins are a source of biologically active peptides. In the current study, after the breakdown of protein, EW hydrolysates except for PM showed relatively higher ORAC values compared to NEw, suggesting that, hydrolysis using certain enzymes may be one effective way for EW protein to obtain antioxidative capacity. Moreover, as shown in Fig.3-1, PNY and Peptide S exhibited relatively higher ORAC values compared with the others. Combined with the results shown by SDS-PAGE, as well as DH data, Peptide S was the most hydrolyzed among all the samples. Chen *et al*. (2012) investigated antioxidative activities of EW protein hydrolysate prepared with trypsin at different degrees of hydrolysis, and found that the fractions with molecular weight lower than 3 kDa by ultrafiltration of the hydrolysate (DH of 12.4%) exhibited the highest antioxidant capacity. It was also found thatEW hydrolysates with different DH have different bioactive and functional properties;therefore, EW hydrolysates created by controlled hydrolysis may be useful ingredients in food and nutraceuticals, with potential bioactive properties.

3.3.2 Profiles of color characteristics of different samples.

Meat color is one of the most important indexes defining the quality of meat (Lindahl *et al*., 2001). The measurement of color is usually presented as the resultant of three different values – *L* (lightness), *a* (redness), *b* (yellowness). Changes in *L*, a and b of pork meat slices are shown in Table 3-1. Photos of meat before and after the soak in different solutions are shown in Fig.3-2a and Fig. 3-2b, respectively.

Several researchers have already studied the effect of natural anti-oxidative extracted compounds on ground beef or pork patties. For example, Park *et al*.(2010) found that the addition of garlic extracts to pork patties decreased the pH, redness, and thiobarbituric acid reactive substances (TBARS) values.

In Japan, and even worldwide, grilled pork meat is a quite popular meal, so in the current study, we chose to test pork. Pork meat slices were soaked in a solution which was with or without EW hydrolysates. After soaking in the solution for 24 h, samples were compared with the control (soaked in water), and samples soaked with NEw or hEWs showed lower ΔE. The lowest ΔE was observed in samples soaked in Peptide S solution (see in Table 3-1), suggesting that Peptide S has the greatest potential to maintain color stability. Samples soaked in water (control sample) and NEw samples showed the lowest redness.

The color of meat products is influenced by the percentage of metmyoglobin in muscle tissue. The myoglobin changes into oxymyoglobin (light pink color), which could result in brighter red meat, and then oxymyoglobin is oxidized into metmyoglobin during storage (Zhang *et al*., 2016). As myoglobin is a water-soluble protein in meat, when meat was soaked in the control (only with water), the loss of myoglobin led to the most color change (△E=10.6). A significant increase in L values indicated the light

color reduction of meat shown in Table 3-1. Combined with results shown in Fig. 2-2 and Fig. 2-3, peptide S was found to possess a higher ORAC value and a quite good solubility; the small size of peptide could be absorbed easily into the center of meat fiber, protecting the remaining myoglobin from oxidation.

3.3.3 Relative proportions of metmyoglobin of pork soaked in different EW hydrolysate solutions.

Krzywicki's equations have been widely used for estimating the relative proportions of myoglobin redox forms in aqueous solution (Viriyarattanasak *et al*., 2011). Relative proportions of metmyoglobin of pork soaked in different EW hydrolysate solutions are shown in Table 3-2. Myoglobin has three natural colors (deoxymyoglobin, oxymyoglobin and metmyoglobin). Depending on its exposure to oxygen and the chemical state of the iron, metmyoglobin is a compound formed from myoglobin by oxidation of the ferrous to the ferric state with essentially ionic bonds. It was obvious to observe that after 24h, the relative proportion of metmyoglobin increased, especially in the control sample that was soaked in only water, demonstrating that oxidation of myoglobin still occurred even after being soaked in solutions. Results showed that the lowest metmyoglobin proportion was found in the pork treated by the small EW peptide, i.e. Peptide S, which showed a relatively higher ORAC value than the other samples. Regardless of the presence of ovotransferrin shown by SDS-PAGE, PM and NEw showed a similar effect on the proportion of metmyoglobin in the pork. In general, the result of metmyoglobin measurements are is in good agreement with the ORAC values shown in Fig.3-1, suggesting that EW hydrolysates can potentially be used as preservatives in meat products.

3.4 Conclusion

This work aimed to study the potential of egg white hydrolysate as a natural antioxidant for the preservation of pork meat slices. Different egg white hydrolysates samples were prepared, and different characteristics such as solubility, molecular size and ORAC values were evaluated. Meat slices were soaked in egg white hydrolysates stored at 4°C for 24h. Small peptide - Peptide S (with molecular weight lower than 10 kDa) showed the best ORAC value (389 µmol TE/g) and excellent water solubility, and could inhibit the color change in treated pork slices. Moreover, it was demonstrated that egg white hydrolysates were generally better than the control (water) in improving color stability during storage time. Therefore, it can be concluded that egg white hydrolysates can be used as a natural antioxidant in meat. This study may have potential implications for developing enhanced meat products, since egg white proteins are the nutritionally complete with a good balance of essential amino acids.

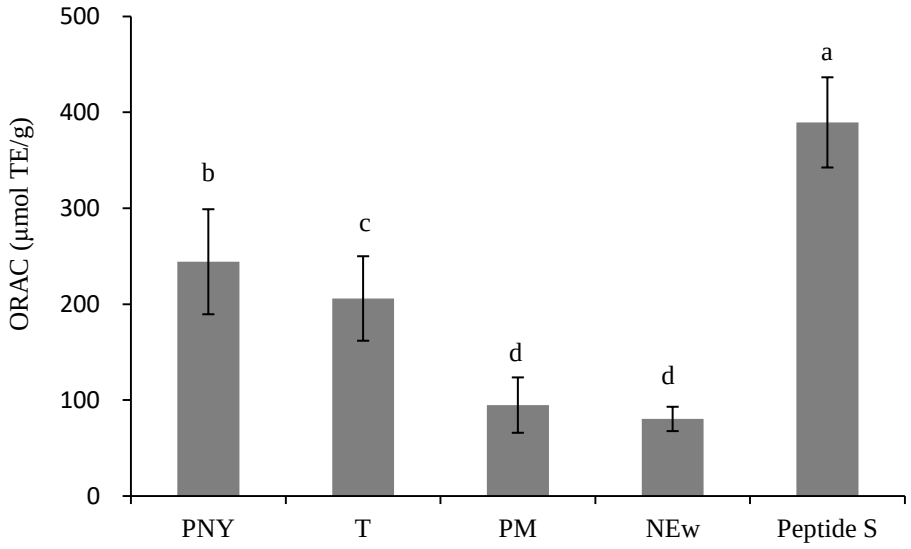

Fig.3-1 ORAC values of different samples. Means within column with no common superscript differ significantly ($p < 0.05$).

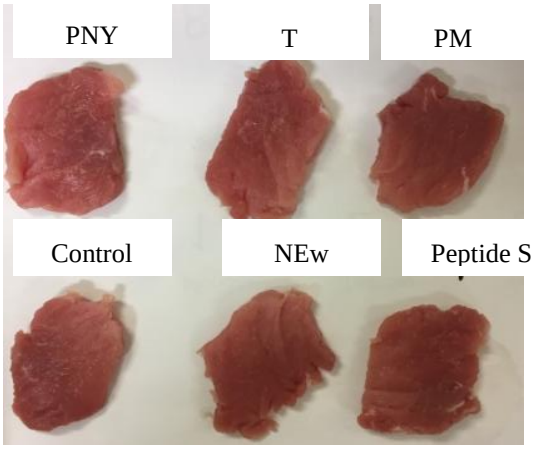

Fig.3-2a. Original Meat

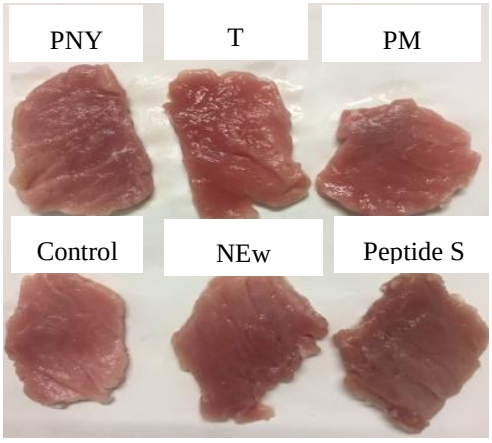

Fig. 3-2b. Meat after soaking for 24h

Fig.3-2. Photos of meat before soak (a) and after soak (b) in different solutions.

Table 3-1 Profiles of color characteristics of pork slices treated by different samples.

Sample	PNY	T	PM	Control	NEw	Peptide S
Original Meat						
L			35.92 ± 0.18			
a			11.27 ± 0.45			
b			6.86 ± 0.33			
E			**38.27**			
After Soak						
L	38.33±0.15	38.83±0.81	39.33±0.88	45.76±2.23	38.04±0.06	36.74±0.31
a	9.33± 0.58	8.97± 0.77	8.96±0.62	7.52± 1.37	7.84± 0.05	9.30 ±0.44
b	4.31± 0.08	5.24±1.20	5.25± 0.32	5.97±0.54	4.72± 0.37	5.05± 0.38
△E	**3.46**	**4.05**	**4.42**	**10.57**	**4.56**	**2.80**

Means ± SD are shown (n=3). Control: meat soaked in only water.

Table 3-2 Relative proportions of metmyoglobin in meat slices soaked in different egg white hydrolysates.

Sample	Original Meat	PNY	T	PM	Control	NEw	Peptide S
MetMb (%)	30.49±0.83^{d}	33.24±0.53^{c}	34.34±0.25^{b}	35.49±1.25^{a}	37.40±0.50^{a}	35.92±0.84^{a}	32.47±0.78^{c}

Means ± SD are shown (n=3). Means within column with no common superscript differ significantly ($p < 0.05$).

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Chapter IV

Effect of egg white hydrolysates on the shrinkage, cooking loss and texture of pork meat slices

4.1 Introduction

Color and texture are among the key factors that influence consumers' acceptance of food products (Costell, 2010). Meat shrinkage and cooking loss during cooking has often been thought by consumers to be an indicator of poor meat quality (Barbera and Tassone, 2006). Non-meat ingredients especially vegetable ingredients, are often introduced into meat processing for economic reasons. However, the majority of studies focused on the application of non-meat ingredients to meat emulsion products, such as hams and sausages. Until now, non-meat ingredients used in meat emulsion products have included milk products (non-fat dry milk, sodium caseinate, milk coprecipitates, whey and whey products), soy proteins and isolated soy protein, oilseeds (sunflower and others), and cereal products (carbohydrate-rich products, wheat flour, pea, chickpea flours and textured navy bean protein concentrate) (Mittal and Usborne, 1985; Correia and Mittal, 2000).

Pork meat is mainly composed of water, protein, and fat. In meat emulsion products, foreign proteins like soybean proteins can also act as emulsifying agents, due to their amphiphilic structure. The addition of foreign protein thus results in a more homogeneous texture and more stable products, and can also sometimes reduce product cost. However, very few researchers have focused on the application of non-meat ingredients on non-emulsified meat products. Grilled pork meat is one of the most

popular foods in Japan,as well as worldwide, high cooking losses and shrinkage have reduced consumer satisfaction.

In the last part of our research, described in Chapter III, EW proteins were partially hydrolyzed, resulting in a smaller molecular size. Results showed that EW after hydrolysis had a better emulsifying property than nEW. In this chapter, the potential of using partially hydrolyzed EW as a natural meat additive to decrease cooking loss and meat shrinkage will be studied.

4.2 Materials and Methods

4.2.1 Treatment of pork slices with egg white hydrolysates.

Approximately 8 cm × 6 cm of lean pork (leg meat) slices with thickness of 4 mm were supplied by a local butcher shop (Kyoto, Japan). Visible fats and connective tissues were trimmed. Fresh meat pH was determined as described by (Pigott *et al*., 2000) (data not shown). Each piece of pork slice was weighed (W_0), the shape of each slice was copied using a printer (DCP-9020CDW, Brother, Japan), paper was cut carefully by following the outline of each copied meat, and the cut paper was weighed (W_0^*). Then meat slices were soaked individually in the same weight of hEW solution (protein concentration of 10%, w/v) in a zipper freezer bag, was and stored at 4°C for 24 h before further analysis (Pigott *et al*., 2000).

4.2.2 Absorption rate, shrinkage and water loss rate of pork slices soaked in different egg white hydrolysate solutions.

Treated meat slices were slightly rinsed with distilled water and then placed on a paper towel for 1 min to remove extra moisture. Each pork slice was weighed individually (W_1), the shape of each slice was copied and cut as mentioned before, and

the cut paper of meat after soaking in hEW was weighed (W_1^*). Meat was cooked using a hotplate (EA-XC45-HW, Zojirushi, Japan). The highest temperature of the hotplate (about 250°C) was used. Each side of meat was roasted at the same location on the hotplate for 30 s. Each cooked meat slice was weighed (W_2), the shape of each cooked meat slice was copied and cut as mentioned before, and the cut paper of cooked meat was weighed (W_2^*). Absorption rate, shrinkage and water loss rate were calculated as follows:

$$\text{Absorption rate (\%)} = (W_1 - W_0) / W_0 \times 100$$

$$\text{Cooking loss (\%)} = (W_0 - W_2) / W_0 \times 100$$

$$\text{Shrinkage (\%)} = (W_0^* - W_2^*) / W_0^* \times 100$$

4.2.3 Water content of cooked pork slices treated by hydrolyzed egg white.

Cooked meat slice was cut down to about 1×1cm in cubed pieces, then the meat was dried at 120°C for 24h using a drying oven (DS600, Yamato Scientific, Japan). Water content of cooked meat was calculated as

Water content = (Initial weight - final drying weight) / Initial weight × 100.

4.2.4 Texture evaluation.

Tests were performed with a Texo-Graph (Japan Food R&D Institute, Japan). A compression test with a cylindrical plunger of 0.5 cm^2 was performed to determine textural characteristics of meat slices after cooking. The probe extruded on the surface of cooked meat at a rate of 0.8 mm/s and the force exerted on the probe was automatically recorded as a load deformation curve which corresponded to textural characteristics of hardness (and toughness) and elasticity. Measurements were developed four times for each sample.

4.2.5 Micro structure.

Cooked meat was cut down to about 1×1cm, then freeze-dried overnight (FreeZone Plus 12 Liter Cascade Console Freeze Dry System, Labconco, Japan) . Thin silk of the dried specimens was mounted on metal stubs with double-stick tape, coated with gold (JEC- 3000 FC Auto Fine Coater, JOEL, Japan) and observed under a scanning electron micro-scope (JSM-7100, JOEL, Japan). Images were calibrated to determine magnification (Zuckerman *et al.*, 2013).

4.2.6 Statistical analysis.

All experiments were carried out in triplicates. The data were subjected to multifactor analysis of variance (ANOVA), followed by the Least Significant Difference (LSD) test to determine the significant difference between samples at $p < 0.05$ level using the software SPSS V.16.

4.3 Results and Discussion

4.3.1 Cooking loss and shrinkage.

Data for hEWs in meat model system are shown in Table 4-1. Pork meat slices with a thickness of 4 mm and a uniform size were soaked in hEW or NEw solutions. The weight change percentage of each meat slice soaked in each corresponding dispersion was expressed as absorption rate (%).Table 4-1 showed that absorption rates of all hEW as well as NEw samples were significantly higher than that of the control (meat slice soaked in only water). The existence of proteins exposed at the interface contributed to the penetration of liquid into the inside of the meat. Peptide S showed the same absorption rate with all three hEW samples (PNY, T and PM), which suggests that the absorption rate is not associated with the particle size of proteins; pH may be an additional consideration.

In meat proteins, the majority of water is held in myofibrils. Water content within the meat myofibrils in the narrow channels between the filaments changes as meat shrinks within the tissue matrix, resulting in cooking loss with heating. (Huff-Lonergan and Lonergan, 2005). Cooking loss was calculated as the percent weight difference between fresh and cooked meat samples (Chiavaro *et al*., 2009). Both nuclear magnetic resonance (NMR) and cooking studies have shown that the water lost during cooking of minced hamburger muscle is similar to that of intact meat muscle (Bertram *et al*., 2004; Tornberg, 2005). Meat cooking shrinkage is the difference between the raw and cooked areas of the meat sample, expressed as a percentage of the raw area (Barbera and Tassone, 2006). In this study, meat shrinkage was measured by archiving the image of raw and cooked meat samples. Results showed that when a pork meat slice was immersed in only water (control), about 38.0% of weight was lost after cooking and the shrinkage rate was about 22.7%. Similarly, Tornberg (2005) reported a considerable shrinkage of meat: 7-19% on area basis. Furthermore, it is clear from the results that cooking loss as well as shrinkage rate decreased when raw meat was immersed in the solution containing hEW (PNY, T, PM and Peptide S) and NEw. The meat treated by sample T possessed an excellent WHC, resulting in the least shrinkage (3.24%).

The ability of an added substance to increase meat batter pH can allow muscle proteins to be much more successful in forming a stable emulsion (Homco-Ryan *et al*., 2003). The water content of cooked meat is related to the juiciness of meat, which is also an important sensory factor. Results showed that there were no significant differences between meat treated by hEWs (PNY, T, PM and Peptide S) and NEw, but a relatively lower water content was observed in the cooked meat treated with only water (control).

4.3.2 Texture.

Consumer satisfaction has been influenced by meat's textural properties (Silva *et al*., 2015). Changes in texture caused by heating can be affected by changes in the soluble proteins, myofibrillar proteins, and connective tissue of meat. Heating produces a softening of connective tissue and a toughening of meat fibers (Bouton and Harris, 1972). In this study, three textural parameters of cooked meat were evaluated (hardness, elasticity and toughness). As shown in Fig.4-1, although relative higher water content was observed in meat immersed in Peptide S and PM solution compared to the control, there was no significant difference observed in the hardness of these three kinds of meat. The greatest elasticity and toughness of cooked meat was observed in the meat soaked in water (control). As myoglobin is water-soluble in meat, when meat was soaked in only water, the loss of myoglobin resulted in the greatest degree of hardness, elasticity and toughness. Conversely, in the meat treated in solution with hEW (PNY, T, PM and Peptide S) and NEw, small-sized proteins with penetrated into the connective tissue matrix of meat muscle tissues. These proteins showed WHC, OBC, and emulsifying capacities, acted as a binder of water and fat components in the meat, and affected textures in the final cooked meat.

4.3.3 Micro structure.

In this study, a scanning electron microscopy (SEM) technique was utilized to investigate the difference between the microstructure of intramuscular connective tissue in pork meat muscles. As shown in Fig.4-2, honeycomb structures of the endomysium were clearly observed within muscle fiber bundles in all the meat. Relatively larger holes and a grainy appearance of endomysium sheaths were distinctive in the meat treated by T, while collagen fibril disruption and the grainy appearance in the

endomysium sheaths were not prevalent in the sample treated by Peptide S. Despite the differences observed among these raw pork meat slices, after cooking, the honeycomb structures in all the meat samples became smaller as shrinkage of their connective tissue networks and muscle fibers occurred.

4.4 Conclusion.

In this study, physical characteristic including solubility, water holding capacity and oil binding capacity were evaluated for different hEWs. Furthermore, shrinkage and cooking loss of pork meat slices soaked in different hEWs were compared. Results showed that, cooking loss and shrinkage rate decreased when pork slices were soaked in solution containing hEWs (PNY, T, PM and Peptide S) and NEw, compared with the slice soaked in only water. The meat treated by sample T possessed an excellent WHC, resulting in the least shrinkage rate (3.2%) compared with the control (shrinkage rate of 22.7%). Treatment with hEWs and NEw contributed to the decrease of elasticity and toughness of cooked meat. Therefore, it is concluded that treatment of pork meat slices in egg white hydrolysates is beneficial for decreasing shrinkage and weight loss during heating.

Table 4-1 Absorption rate (%), cooking loss (%), shrinkage (%) and water content of meat slices soaked in different egg white hydrolysates.

	Absorption rate(%)	Cooking loss(%)	Shrinkage(%)	Water content(%)
PNY	14.88 ± 2.41^{ab}	24.43 ± 0.95^{bc}	6.90 ± 1.38^{c}	68.22 ± 0.48^{a}
T	19.16 ± 2.41^{a}	22.87 ± 1.74^{c}	3.24 ± 0.61^{d}	68.87 ± 1.61^{a}
PM	16.33 ± 1.07^{ab}	24.37 ± 1.95^{bc}	4.67 ± 1.93^{cd}	66.40 ± 0.55^{a}
NEw	11.96 ± 1.38^{b}	27.21 ± 2.29^{b}	9.07 ± 0.18^{b}	67.33 ± 0.96^{a}
Peptide S	14.92 ± 3.38^{ab}	26.75 ± 3.28^{b}	6.14 ± 1.99^{c}	66.42 ± 0.76^{a}
Control	1.47 ± 0.27^{c}	38.02 ± 2.02^{a}	22.74 ± 1.16^{a}	62.40 ± 0.47^{b}

Means±SD are shown (n=3). In each tested parameter, different superscript letters indicate significant differences between means in the same column **(**$p < 0.05$**).**

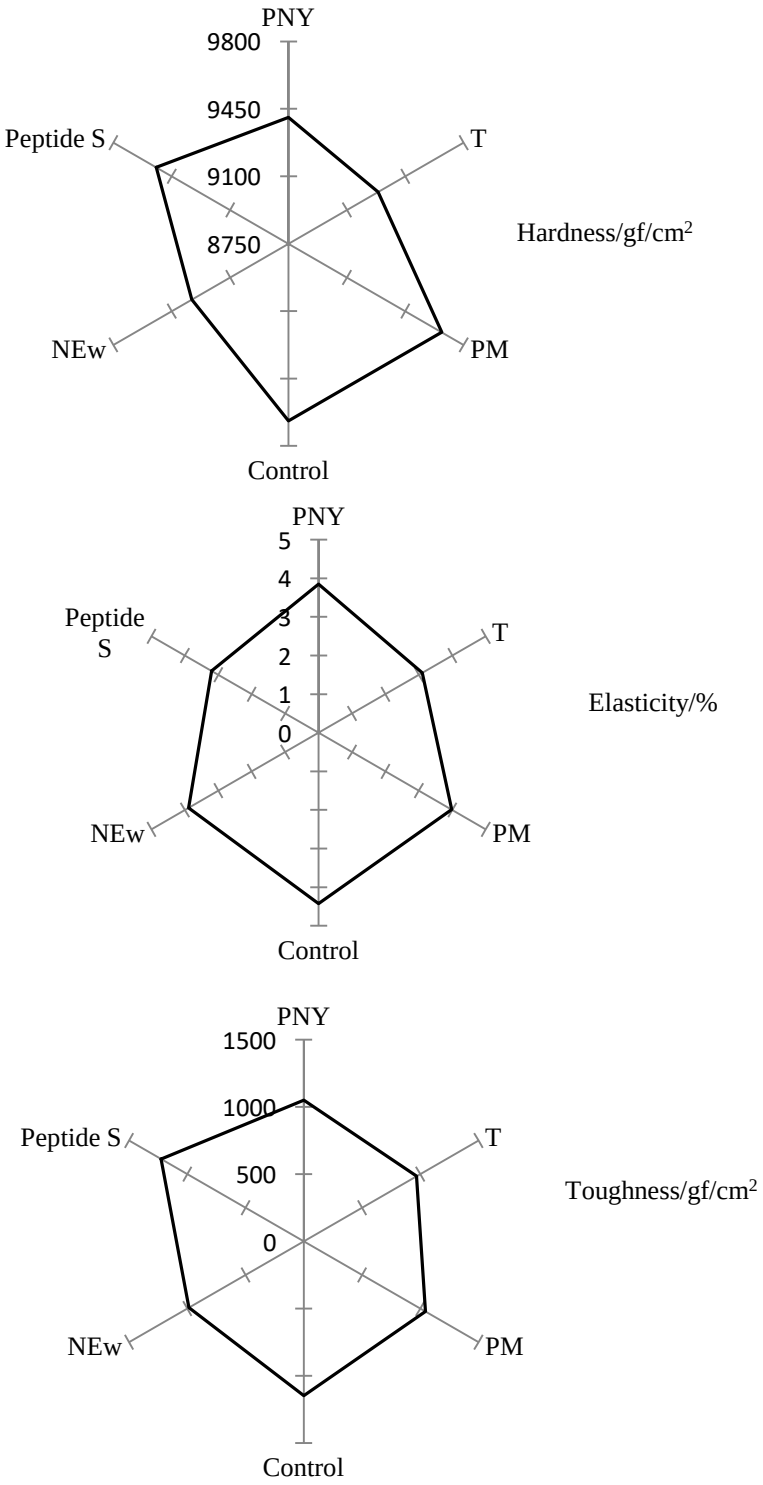

Fig. 4-1 Texture evaluation of meat slices soaked in different egg white hydrolysates.

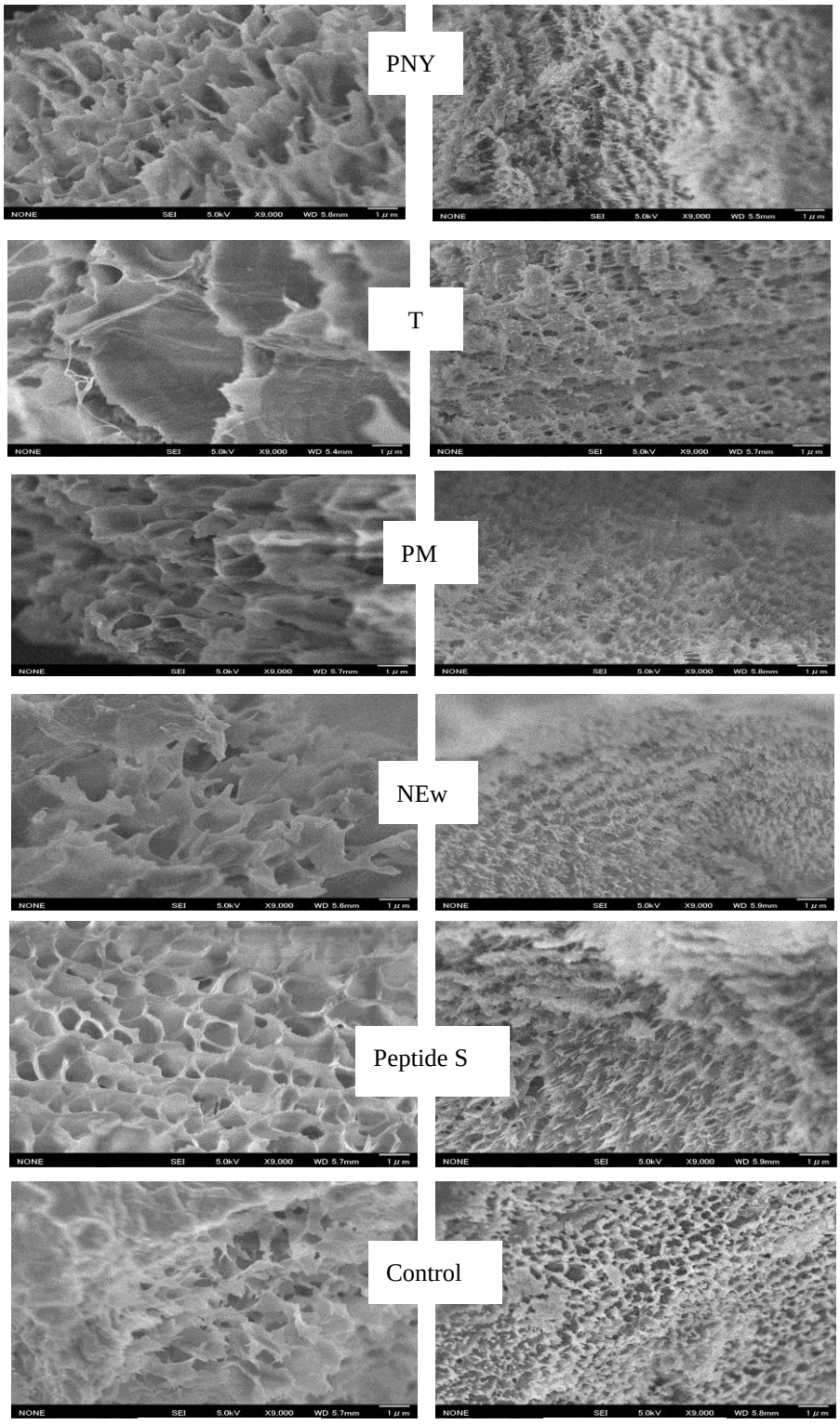

Fig.4-2 Microstructure of meat slices soaked in different egg white hydrolysates.

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Chapter V

Summary and conclusion

In recent years, various bioactivities have been unlocked in low molecular weight peptides obtained by hydrolysis of protein. Moreover, it is known that hydrolysis of a protein also results in a change its physical function due to change in its three-dimensional structure. These functional changes can include solubility, gelling property, foaming property, and emulsify properties. Hence, the functional modification of food proteins using proteolytic enzymes has drawn attention as a way to increase the added value of foods. Native egg white (NEw) has heat gelling property and foaming property. However, egg white (EW) protein loses its heat gelling property by partial hydrolysis. The protein is denatured by heating and an amphiphilic structure is formed, so that emulsification characteristics are obtained.

Protein-type water retention agents such as milk protein, EW, soybean protein, and polymerized phosphate have been widely used for maintaining juicy taste and quality in processed meat. When a protein type water retention agent is applied, gelation during heating causes meat to have a hard texture. When a polymerized phosphate salt is applied, melting of myofibrillar protein causes the meat to lose its original texture. So, it is difficult to keep a good mouthfeel while avoiding the loss of juiciness. In this study, the effect of partially hEW on meat quality was examined.

First, Chapter 1 describes the change in EW properties caused by partial hydrolysis. Three kinds of proteases (Protin NY 100®, Thermoase PC 10F®, Protease M®) were used at a concentration of 0.4% for partial hydrolysis of EW. The egg white hydrolysates (hEW) obtained using these three enzymes were named PNY, T, and PM,

respectively. Examination of the molecular weight distribution of hEWs by SDS-PAGE showed that ovotransferrin (78 KDa) disappeared completely, but remarkably remained in a protein gel having a molecular weight of ovalbumin (45 KDa) or less, namely a decomposition product. Based on the results of measuring the emulsifying property of hEWs by the turbidity method and measurement of the emulsified particle size, it was shown that hEW-T has an emulsifying activity comparable to that of egg yolk. All hEWs showed higher emulsifiability than NEw and low molecular weight EW peptide - Peptide S. In addition, it was shown by the ANS method that the surface hydrophobicity of hEW - T was the highest. Furthermore, the obtained hEW exhibited a higher ORAC value than that of NEw.

Next, in Chapter 2, the effect of hEWs on the stability of meat color was examined. When fresh meat is exposed to air, it produces bright red oxymyoglobin (oxygenation), and if it is exposed to air for a long time, the bivalent iron of heme is oxidized to trivalent iron, resulting in brown metmyoglobin. In this study, sliced fillets of pork (4 to 8 mm of thinkness) were soaked in each hEW (PNY, T and PM) and a peptide S solution with a protein concentration of 10% (w / w). Meat soaked in the same manner using NEw was as a positive control and water alone was used as a negative control. After storage in a refrigerator for 24 hours, the color of each meat slice before immersion, after dipping and after heating was measured with a colorimeter, and the effect of hEW on the change in coloration was analyzed.

Results showed that, when immersed for 24 hours at 4 °C, the meat slice treated with peptide S with the highest ORAC value (389 μmol TE / g) showed the least color change ($\Delta E=2.8$). Furthermore, it was shown that each meat slice soaked in hEW solution was superior to the negative control (water only) considering the effect of

suppressing change in color (ΔE). According to measurement of metmyoglobin, meat treated with Peptide S showed the lowest value. On the other hand, there was no significant difference between the metmyoglobin volume in meat treated with PM and NEw.

Finally, in Chapter 3, the effect of hEW on grilled meat was investigated. In the grilled meat experiment, each immersed meat was baked uniformly on both sides on a hotplate, and the amount of expressible moisture and the area of the meat were measured. As a result, it was shown that cooking loss (%) and shrinkage rate (%) decreased when the meat was dipped in a solution containing hEWs or NEw. Meat subjected to hEW-T treatment resulted in the minimum shrinkage (3.2%) compared with control meat (shrinkage rate of 22.7%). Furthermore, using a scanning electron microscope, large pores and granular sheath were observed in meat treated with hEW-T compared to control meat. In addition, immersion treatment with hEW and NEw contributed to a decrease in elasticity and toughness of the cooked meat. Therefore, treatment with hEW was found to be helpful in decreasing the cooking loss and shrinkage of grilled pork slices.

From the above, it can be concluded that when EW protein is partially hydrolyzed with a proteolytic enzyme, the gelation property is lost and; an emulsifiability comparable to egg yolk can be obtained. The obtained hEW also possessed excellent water holding capacity and antioxidative capacity (ORAC value). hEW is a promising natural additive for suppressing color change in meat during storage period, and also for decreasing shrinkage and juice loss during cooking.

Appendix I

Summary in Japanese

卵白部分加水分解物の特徴とその肉への応用 – 博士論文和文要旨

第一章　「緒論」：　近年、蛋白質加水分解物である低分子ペプチドに様々な生理活性が見いだされている。また、蛋白質の加水分解は、その立体構造の変化によって、溶解性やゲル化性、 起泡性、乳化性などの物性機能が変化することも知られている。このように、蛋白質分解酵素を用いた食品蛋白質の機能性改変は、食品蛋白質の用途拡大に役立つ手段の一つとして注目されている。卵白(NEw)は加熱ゲル化性と起泡性を有するが、卵白蛋白質を部分加水分解し、低分化することで加熱ゲル化性を失い、酵素失活時の加熱によって蛋白質が変性し、両親媒性構造になるため乳化特性を獲得する。従来、加工用の食肉の保水や品質保持には、乳蛋白、卵白蛋白、大豆蛋白といった蛋白質系の保水剤や重合リン酸塩が用いられてきた。蛋白系の保水剤では、加熱時のゲル化によりカマボコのような食感が生じ、また重合リン酸塩では筋原繊維蛋白質の溶融化により、肉本来の繊維感がなくなってしまうなど、保水と食感を両立させにくいという問題があった。そこで、本研究では、加熱ゲル化性を無くした卵白部分加水分解物の肉質改質効果を検討した。

第二章　「卵白部分加水分解物の性質」: 卵白液の部分加水分解には、三種類のプロテアーゼ(Protin NY100®、Thermoase PC10F®、Protease M®: 天野エンザイム社製品)を用いた。卵白液量に対する酵素濃度を 0.4%として、酵素を 50°C で添加し、その後、各酵素の至適温度と至適 pH で 30 分間加水分解を行い、90°C でそれぞれ 8 分間の加熱により失活させた。これら 3 つの酵素を用いて得られた卵白加水分解物を、それぞれ PNY、T および PM とした。これら加水分解物の分子量分布を SDS-PAGE で調べた結

果、オボトランスフェリン(78KDa)は完全に消失しているが、オボアルブミン(45KDa)とリゾチーム（14KDa）の間に加水分解物がゲル内に顕著に残り、いわば部分加水分解物であった。卵白部分加水分解物の乳化活性と乳化安定性を濁度法で測定した結果と乳化粒径の測定結果により、部分加水分解物 T は卵黄の乳化性に匹敵する乳化力を有することを見出した。いずれの部分加水分解物も、生卵白および低分子卵白ペプチド-S (ランペップ®:ファーマフーズ社製品)よりも高い乳化性が示された。また、疎水性プローブ法(ANS 法)で、卵白加水分解物 T の蛋白質の表面疎水性は最高レベルであることを示した。さらに、得られた加水分解物は良好な保水性と保油性を有し、また活性酸素の消去活性 ORAC (Oxygen Radical Absorption Capacity)値も強く発現した。

第三章 「卵白部分加水分解物の肉色への効果」: 新鮮な生肉が空気に触れると(酸素化)明るい赤色のオキシミオグロビンを生じる。 さらに、長時間、空気にさらしておくと、ヘムの 2 価鉄が 3 価鉄に酸化され、褐色のメトミオグロビンを生じる。本研究では、豚のヒレ肉を薄切り(4~8 mm)に切り、蛋白質濃度として 10%(w / w)に揃えた、各部分卵白加水分解物(PNY、T および PM)やペプチド S 溶液に個別に 4℃ で 24 時間浸漬した。なお、陽性対照には NEw を、陰性対照には水のみを用いて、同様にヒレ肉を浸漬した。色彩色差計で浸漬前、浸漬後、それぞれの肉色を測定し、卵白部分加水分解物の肉色変化抑制効果の有無を解析した。結果として、4℃ で 24 時間浸漬した場合、抗酸化力を示す ORAC 値 (389 μ mol TE / g) が最も高いペプチド S で処理したヒレ肉が最も良好な肉色の変化(ΔE= 2.8)抑制効果を示した。さらに、各卵白部分加水分解物の浸漬肉は、肉色の変化(ΔE)抑制効果では、陰性対照(水のみ)より優れていることが示された。なお、メトミオグロビン量の測定では、低分子の卵白ペプチド S 処理肉が最も低値を示した。一方、PM および NEw で 処理された肉のメトミオグロビンの量は大差なかった。

第四章 「卵白部分加水分解物の焼肉への改質効果」: 焼肉実験では、薄切り(4~8

mm)した豚ヒレ肉を、蛋白質濃度として 10%(w/w)に揃えた各部分卵白加水分解物(PNY、T および PM)やペプチド S 溶液に個別に 4°C で 24 時間浸漬した。なお、陽性対照には NEw を、陰性対照には水のみを用いて、同様にヒレ肉を浸漬した。その後、各浸漬肉をホットプレートで、両面を同一条件で均一に焼成し、焼肉からの遊離水分量や肉の面積を測定した。その結果、肉を卵白部分加水分解物または NEw を含む溶液に浸漬すると、 調理損失(%)および収縮率(%)が有意に減少することが示された。特に卵白部分加水分解物 T で処理した肉は、同じ水分に浸漬したコントロールの肉(収縮率 22.7%)と比較して、最小の収縮率(3.2%) を示した。さらに、走査型電子顕微鏡を用いて、卵白部分加水分解物 T で処理した肉ではコントロール肉と比較して、顕著に大きな孔および顆粒状の鞘状鞘が観察された。また、卵白部分加水分解物と NEw による浸漬処理は、調理された肉の弾力性と靭性の低下に寄与した。したがって、卵白部分加水分解物による処理は、豚肉スライスの調理損失 および収縮率の減少を改善するのに役立つことが認められた。

以上のことから、卵白蛋白質を蛋白質分解酵素で部分的に加水分解し、ゲル化性を消失させて加熱変性させると、分子表面の疎水性が上昇し、卵黄に匹敵する乳化性が得られることがわかった。このようにして得られた卵白部分加水分解物は優れた保水性と保油性および抗酸化力(ORAC 値)も有することが確認された。卵白部分加水分解物は、蛋白質分解酵素処理と加熱変性処理により、その本来の機能性が改変（ゲル化性消失、乳化性獲得）され、新たな機能性として、食肉保存中の肉色退色抑制効果や食肉加熱中の収縮および調理中の肉汁損失抑制効果を獲得することを見出した。

Appendix II

Publications

1) **Wang Y,** Horimoto Y, Nau F, Hatta H. Improving Emulsifying Properties of Egg White Protein by Partial Hydrolysis Combined with Heat Treatment. Advance Journal of Food Science and Technology. 2018, 14(2):50-55.

2) **Wang Y,** Kimura T, Nohara T, Shen Jf, Hatta H. Proposal of a Micro Analysis for Singlet Oxygen Absorption Capacity using a Disposable 96-Well Microplate, Advance Journal of Food Science and Technology. 2018,14(4):126-130.

3) **Wang Y,** Shen Jf, Hatta H. Evaluation of Antioxidant Activity of Egg White Hydrolysates and Their Application on Color Stability of Pork Meat Slices. Advance Journal of Food Science and Technology. 2018,14(5):148-154.

4) **Wang Y,** Enomoto H, Shen Jf, Hatta H. Effect of egg white hydrolysates on the shrinkage, cooking loss and texture of pork meat slices. Advance Journal of Food Science and Technology. 2018,14(6): 186-193.

5) 八田 一，王 玉。食品加工における酵素利用の最前線。 冷凍。2019 年 1 月号第 94 巻第 1095 号：19-24。

Acknowledgements

It is with great pleasure that I write this section of my thesis. In my heart and mind, it is the most important part of this thesis. I get to say “thank you” to all the wonderful people who have made it possible for me to go through this program.

I am deeply indebted to my supervisor, Professor Hajime Hatta. The first time we met in INRA-France, he explained to me about egg knowledge on five pages fully covered writing, which I still have now in my apartment in Shanghai. He opened a door for me to a new world that I would like to discover. I am particularly grateful for the advice, guidance and inspiration he offered throughout the course of this work.

I am also very grateful to Dr. Nagayama Reiko, Dr. Narita Hiroshi, Dr. Matsumoto Shinya, Dr. Kawamura Yukio, and all the teachers in the faculty of Food and Nutrition. They never made me feel nervous for expressing myself in my poor Japanese at the beginning; you always encouraged and pushed me throughout. Special thanks to Dr.Yasumi Horimoto from Guelph University in Canada, who offered valued suggestions on my manuscript of thesis.

I also would like to thank everyone studying in the laboratory of Professor Hajime Hatta, for their patience with my poor Japanese. Special thanks to Mayuko Yamashita, Nanase Kubo, and Reina Fujishin who always acted as my assistant and interpreter, for sharing ideas and best practice as well as keeping me motivated throughout my research.

I would like to thank Professor Yasuki Matsumura of Kyoto University for his instructions in the determination of particle size distributions of egg white hydrolysates. I also would like to thank Dr. Yosie Ueno of Kyoto Prefectural Technology Center for

Small and Medium Enterprises for his help in the observation of microstructures in meat using scanning electron microscopy.

Lastly, I would like to thank China Jilin Jinyi company, who gave me the opportunity to complete this degree under its financial support. Especial thanks to Mr. Shen for his valuable comments throughout these years.

I would like to thank my husband for his endless encouragement and support in keeping me focused on my research and helping me manage my time, and especially, for bearing with my bad temper when I was frustrated and for always waiting for me in Shanghai. My deepest gratitude is to my parents, for their encouragement in obtaining my PhD degree. In my most difficult moments, the words from them always served to uplift my spirit, and gave me a sense of purpose.