DEVELOPMENT OF HIGH PROTEIN BEVERAGE FROM HYDROLYZED EGG WHITE

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วัตถุประสงค์หลักของงานวิจัยนี้คือเพื่อศึกษาผลของการใช้เอนไซม์โปรติเอส (SDAY10, PC10F และ P6SD) ในการปรับปรุงคุณสมบัติเชิงหน้าที่ และเคมีของโปรตีนไข่ขาวพาสเจอร์ไรส์รวมถึงการศึกษาคุณสมบัติในการเป็นสารต้านอนุมูลอิสระ เพื่อใช้ในการพัฒนาเครื่องดื่มโปรตีนเพื่อสุขภาพ จากการศึกษาพบว่าการเพิ่มความเข้มข้นของเอนไซม์ SDAY10 ทำให้ค่าความสว่าง (L*) สีแดง (a*) และค่าสีเหลือง (b*) เพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้ ที่ความเข้มข้นที่สูงขึ้นของเอนไซม์ยังส่งผลให้ค่า pH ความหนืด และการกระจายตัวของอนุภาค ($D_{4,3}$) ของโปรตีนไข่ขาวลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ($P < 0.05$) นอกจากนี้การใช้เอนไซม์ SDAY10 ที่ความเข้มข้น 0.1-0.3% w/v สำหรับการไฮโดรไลซ์ โปรตีนไข่ขาวไฮโดรไลเสตยังช่วยเพิ่มประสิทธิภาพในการเป็นสารต้านอนุมูลอิสระ (วัดโดยการทดสอบ DPPH ABTS และ FRAP) และเมื่อเทียบกับความเข้มข้นของเอนไซม์ที่ต่ำกว่า โดยเฉพาะอย่างยิ่งความเข้มข้น 0.3% ของเอนไซม์ SDAY10 ยังพบว่าการย่อยด้วยเอนไซม์ 0.3% SDAY10 ได้กรดอะมิโนอิสระมากที่สุด 75.64 ± 6.84 mg/mL ที่เวลา 240 นาทีในการจำลองการย่อย และได้คะแนนการประเมินทางประสาทสัมผัสสูงสุดที่ 7.80 ± 1.32 ($P < 0.05$) ดังนั้นการใช้เอนไซม์ SDAY10 ที่ความเข้มข้น 0.3% w/v สำหรับการในการพัฒนาเครื่องดื่มโปรตีนเพื่อสุขภาพ แสดงให้เห็นถึงศักยภาพที่สำคัญในการปรับปรุงคุณสมบัติทางกายภาพและเคมีของโปรตีนไข่ขาว อย่างไรก็ตาม การศึกษาในอนาคตจำเป็นต้องตรวจสอบการประเมินทางประสาทสัมผัสเกี่ยวกับการลดความขมของผลิตภัณฑ์เพื่อพัฒนาให้ผลิตภัณฑ์ได้รับค่าการยอมรับโดยรวมที่มากขึ้น

สาขาวิชา	อาหารและโภชนาการ	ลายมือชื่อนิสิต .....
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KEYWORD: Pasteurized egg white, Hydrolysis, Antioxidant activity

Nuntorn Charoensak : DEVELOPMENT OF HIGH PROTEIN BEVERAGE FROM HYDROLYZED EGG WHITE. Advisor: Asst. Prof. SATHAPORN NGAMUKOTE, Ph.D. Co-advisor: Pornpimol Mahamad, Ph.D.

The primary objective of this study was to examine the impact of enzymatic treatment on pasteurized egg white protein (EWP) and assess its influence on various physicochemical properties and antioxidant activity. Our findings revealed that the addition of SDAY10 enzymes led to a significant increase in lightness (L*), redness (a*), and yellowness (b*) parameters. Furthermore, higher concentrations of the enzyme resulted in a considerable reduction in pH, viscosity, and volume mean diameter (D4,3) of EWP protein compared to the control group ($P < 0.05$). Additionally, the application of SDAY10 enzyme (0.1-0.3% w/v) for EWP hydrolysis exhibited a noteworthy enhancement in antioxidant activity (measured by DPPH, ABTS, and FRAP assays) compared to lower enzyme concentrations. Notably, the 0.3% concentration of SDAY10 exhibited the highest release of amino acids during in vitro digestion and received the highest sensory evaluation score. Consequently, employing SDAY10 enzymes at a concentration of 0.3% w/v for EWP hydrolysis demonstrates significant potential for improving the physical properties of EWP and enhancing antioxidant activity. However, it is imperative to investigate the sensory evaluation with regards to the bitterness of the product developed using such enzymes.

Field of Study:	Food and Nutrition	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....
		Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

1.1 Background of the study

The global population of individuals aged 60 and above has been steadily increasing, with projections indicating a significant rise in the coming years. Developing countries, in particular, are experiencing a rapid growth in their elderly population (Nations, Cite 2020). Thailand, in particular, is facing the challenge of an aging society, with the proportion of elderly individuals reaching 14 percent of the total population by 2023 (Pramote Prasartkul1, 2019). Malnutrition is a significant concern among the elderly, which can lead to various health issues such as high blood pressure, diabetes, and cardiac disease (de Boer *et al*., 2013).

In terms of nutrition, the elderly requires a sufficient intake of protein despite needing fewer calories overall. However, physiological changes like tooth loss and swallowing difficulties can contribute to malnutrition. Therefore, there is a need to develop functional beverages that are safe for swallowing and have desirable textural properties while providing essential nutrients, particularly high - quality protein.

Egg white is a valuable protein source, containing all essential amino acids (Pokora *et al*., 2013). It also contains various proteins and minerals, making it an

attractive option for health - conscious individuals and the elderly (Li-Chan, 1995), (Baron *et al*., 2016). However, egg white proteins are susceptible to denaturation and aggregation during food processing, which presents challenges in product development. Protein hydrolysate, achieved through enzymatic hydrolysis, is a widely - used technology to enhance protein functionality and overcome these challenges.

Protein hydrolysates are derived from the hydrolysis of peptide bonds, resulting in proteins of different sizes and free amino acids. This structural change influences the physical and chemical properties of the proteins, improving their solubility and other characteristics (Severin and Xia, 2006), (Fraenkel-Conrat *et al*., 1945). Enzymatic hydrolysis is preferred in the food industry due to its ability to retain essential amino acids and produce high - quality hydrolysates

Consumer preferences have shifted towards foods that are rich in essential nutrients and beneficial for health (Hernández-Carrión *et al*., 2014). Protein, in particular, has gained popularity for its functional food benefits. Egg white protein hydrolysate has shown promising potential in functional foods, as it contains bioactive peptides that can positively impact health and reduce the risk of certain diseases (Bigliardi and Galati, 2013), (Noh and Suh, 2015), (Kristinsson and Rasco, 2000).

Therefore, the aim of this study was to investigate the effect of enzymatic treatment on developing egg white protein beverage and investigate physicals and chemical characteristic of egg white protein hydrolysate.

The aim of this study is to investigate the effect of enzymatic treatment on the development of an egg white protein beverage and to examine the physical and chemical characteristics of egg white protein hydrolysate. By understanding these factors, we can explore the potential of egg white protein hydrolysate as a valuable ingredient in functional food products.

1.2 Research questions

1. What are the specific characteristics and functional properties of egg white hydrolysates when subjected to enzymatic hydrolysis using three different enzymes?

2. How does the process of hydrolysis impact the antioxidative activities of egg white hydrolysate, and what are the resulting changes in its antioxidative properties?

3. What is the ideal combination or ratio of different egg white hydrolysate solutions and water to optimize the production process of a high protein beverage, considering factors such as protein content, taste, and texture?

4. How do the sensory properties (e.g., taste, aroma, texture) of high protein beverage products derived from egg white hydrolysate?

1.3 Objectives

1. To examine characteristics and functional properties of egg white hydrolysates by using four different enzymes

2. To study the effect of hydrolysis on an antioxidative activities of egg white hydrolysate

3. To investigate optimal ratio of different EW hydrolysates solutions and water on the production process of high protein beverage

4. To test the organoleptic properties of high protein beverage products from egg white hydrolysate

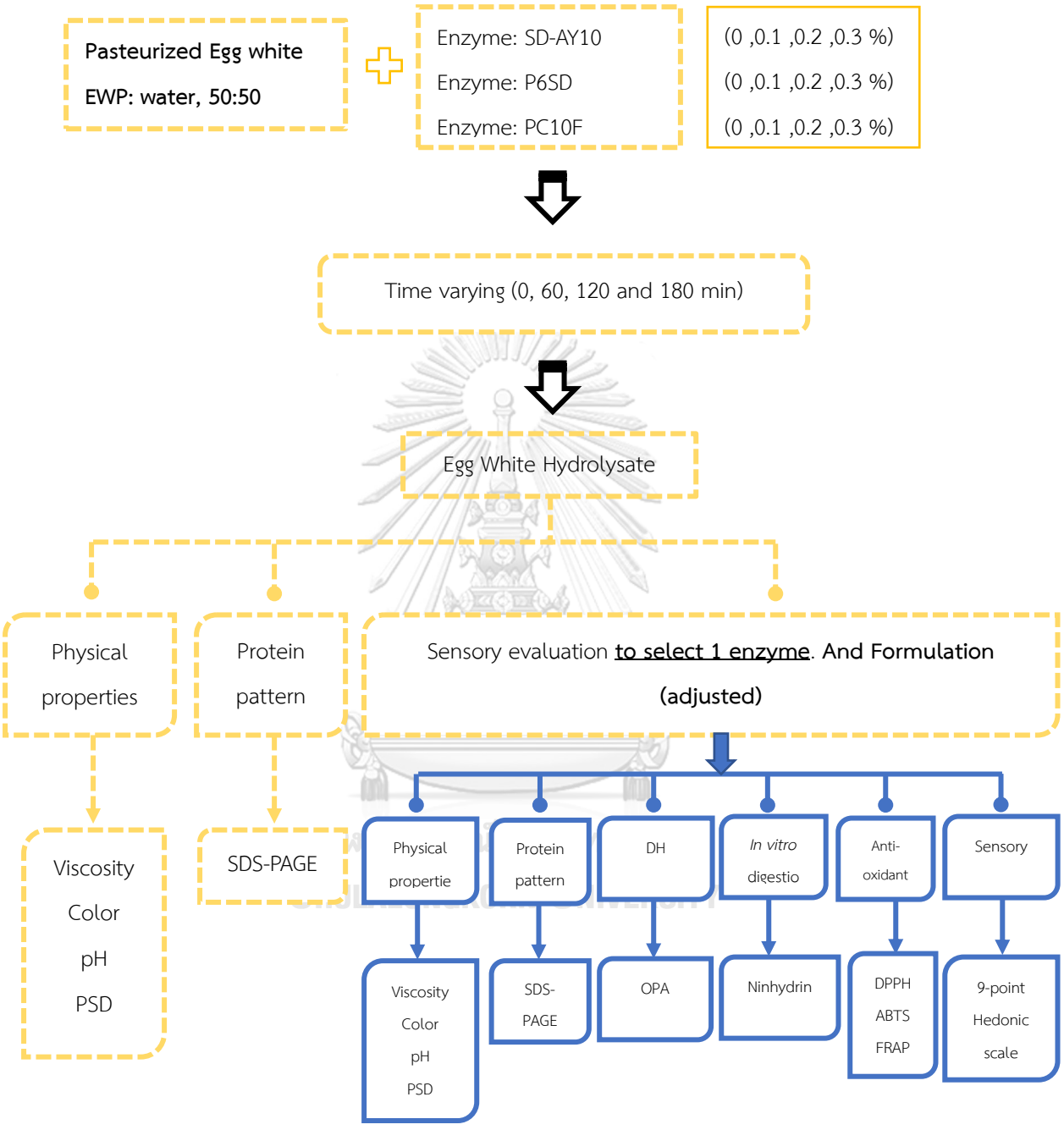
1.4 Hypotheses

1. Different enzymes affect physiochemical properties and acceptability of product differently

2. Egg white hydrolysate may increase antioxidant activity of egg white beverage

3. To deliver the most acceptance formula of the product and be efficient for commercialization

1.5 Conceptual framework



Abbreviation

PSD: Particle Size Distribution

DH: Degree of hydrolysis

CHAPTER II

LITERATURE REVIEW

2.1 Ageing society

Due to the global trend is moving to aging society. The definition of elderly by WHO is a person who age ≥ 60 years but in some developed countries such as France, South Korea and Japan elderly is a person who age ≥ 65 years. Elderly was categorized to three groups, first group is young old age between 65 - 74 years, aged who age between 75 - 84 year and oldest old who has age ≥ 85 years (Saisat S, 2010; "United Nations, Department of Economic and Social Affairs, Population Division (2019)," 2019). The United Nations has assessed the aging situation that between the year 2001 - 2100 will be a century of aging. In year 2005, Thailand aging situation was reported that aging people have more than 7 percent of total population and defined as ageing society. As shown in Figure 1 in year 2020, aging people will reach to 20 percent of total population and continuously increase until year 2031 Thailand will enter to super aged society.

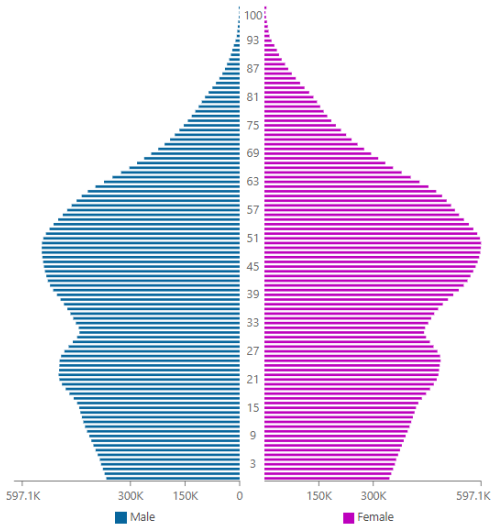


Figure 1 Thailand population pyramid 2020
(World Population Review, 2020)

Nowadays, Thailand has entered the aging society, which has a population of over 60 years, up to 10.78 million people, representing 16.5 percent of the country. Which is conform with the report from United Nations (UN) which the level of progress to enter the aging society, the elderly must have aged 60 years or more, more than 10 percent and have an age of 65 years and over 7 percent Items of the whole country. It is a social and economic issue that Thailand must have a plan to support. The increasing number of elderly populations will affect the public health system in health for the elderly. Age is a major risk factor for non - communicable chronic diseases (NCDs) (Bales and Ritchie, 2002) such as chronic obstructive pulmonary disease, cardiovascular disease (CVD), type 2 diabetes, cognitive decline, dementia, and cancer (Thakur *et al*., 2013). To prevent or delay pathogenesis of all diseases can be done by choosing foods that are useful and suitable for the age.

2.2 Reactive oxygen species (ROS)

Aging stage can affect the human body functions such as changes of metabolic function, gene expression patterns and increasing of high levels of Reactive Oxygen Species (ROS) (Sergiev *et al*., 2015). ROS also called oxygen free radical such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•). All ROS are highly reactive atoms or molecules with one or more unpaired electron in their external shell which become an unstable molecule and can be formed when oxygen interacts with other molecules (Chandrasekaran *et al*., 2017). This unstable can create energy that might damage biological system. ROS are produced by all aerobic cells and play an important role in aging as well as in age - related diseases (Venkataraman *et al*., 2013). There are endogenous and exogenous sources of ROS (Chance et al., 1979). Mitochondria are considered one of the important sources of ROS, cytochrome P450, lipoxygenase, metabolic processes, NADH oxidase and peroxisomes. Moreover, exogenous sources which can also induce the production of ROS, such as pollution exposure; toxin exposure, including exotoxins such as heavy metals like mercury, lead and cadmium. Other exotoxins including anticancer drugs, Ionizing radiation and ultraviolet light (Murphy, 2009) even dietary factors such as excess sugar, saturated fat and fried oils; malnutrition and various disease state.

2.3 Oxidative stress and aging

Under normal circumstances our body has an ability to remove free radicals or to repair the resulting damage from ROS in cells. Oxidative stress causes by overproduction of ROS or decreased level or dysfunction of antioxidants.

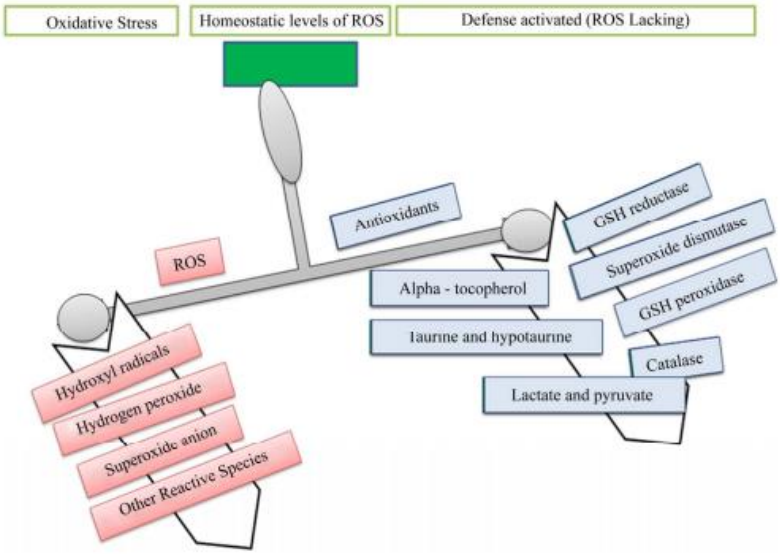


Figure 2 Imbalance between oxidant and antioxidant
(Rahman *et al*., 2012)

The continuous production of ROS by mitochondria throughout cell life produces an age related conversely with the decreasing of antioxidants can lead to damage of cells and biomolecules which impact to the whole organism such as membrane damage, cross linking of proteins or DNA and even lead to cell death and the unbalance of oxidant and antioxidant was shown in Figure 2. All these factors might

lead to the development of acute and chronic diseases in elderly such as CVDs, acute and chronic kidney disease (CKD), neurodegenerative diseases (NDs), macular degeneration (MD), biliary diseases, cancer and cardiovascular (CV). A graphical representation depicting the impact of aging on pathogenesis was shown in Figure 3.

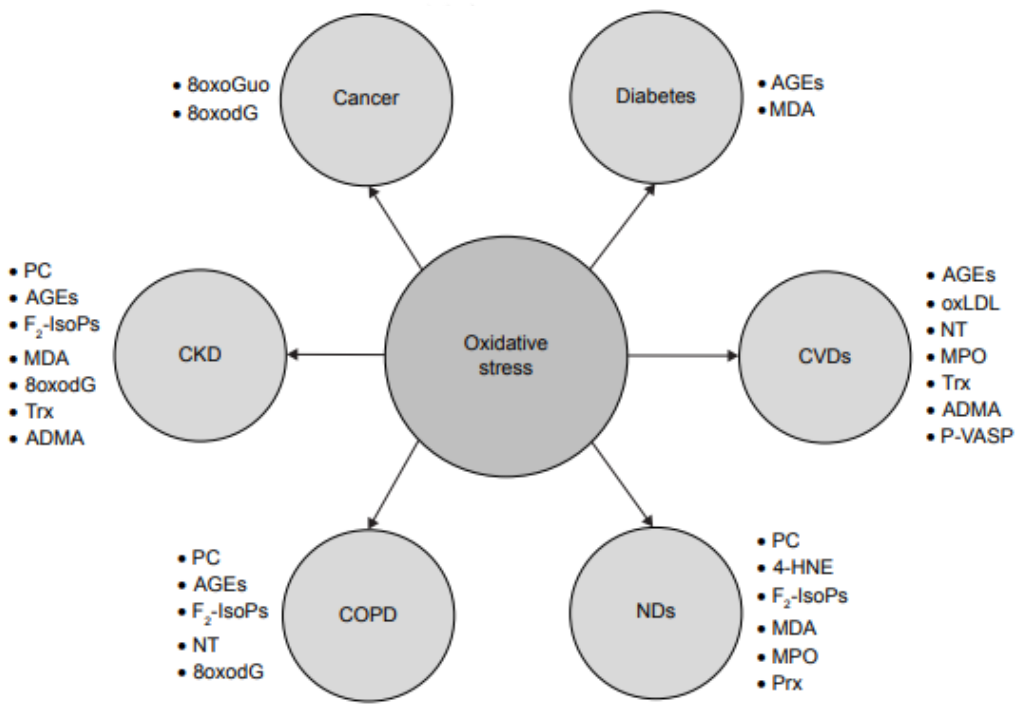


Figure 3 Oxidative stress, age - related diseases, and relative biomarkers

2.4 Dietary recommendations for elderly

Due to there are several factors which affect dietary intake in elderly such as social and economic effect: Poverty, transportation availability, lack of knowledge in nutrition, inadequate in cooking skill, lack of social support (Ramic *et al*., 2011). Psychological effect: depression, loneliness, dementia, food preference (Anyanwu et al., 2011). And most importantly, physical factors: Hormones which altered energy intake and food consumption in older persons changing such as cholecystokinin (CCK), leptin, ghrelin, insulin, peptide YY(PYY) and glucagon - like peptide - 1 (GLP – 1) (Boado et al., 1998). Senses that very important for the enjoyment of food consumption such as decreasing in performance of olfactory processes (Koskinen *et al*., 2003) and visual processes due to age - related (Verhagen and Engelen, 2006). Decreasing in basal metabolic rate due to decreasing in physical activities in elderly (Pannemans and Westerterp, 1995). Decreasing in number of teeth, saliva secretion and tongue pressure also affect food intake in elderly (Razak *et al*., 2014). These factors all contribute to the loss of muscle mass and an increase in oxidative stress which leads to the occurrence of the disease in the elderly (Kalyani *et al*., 2014).

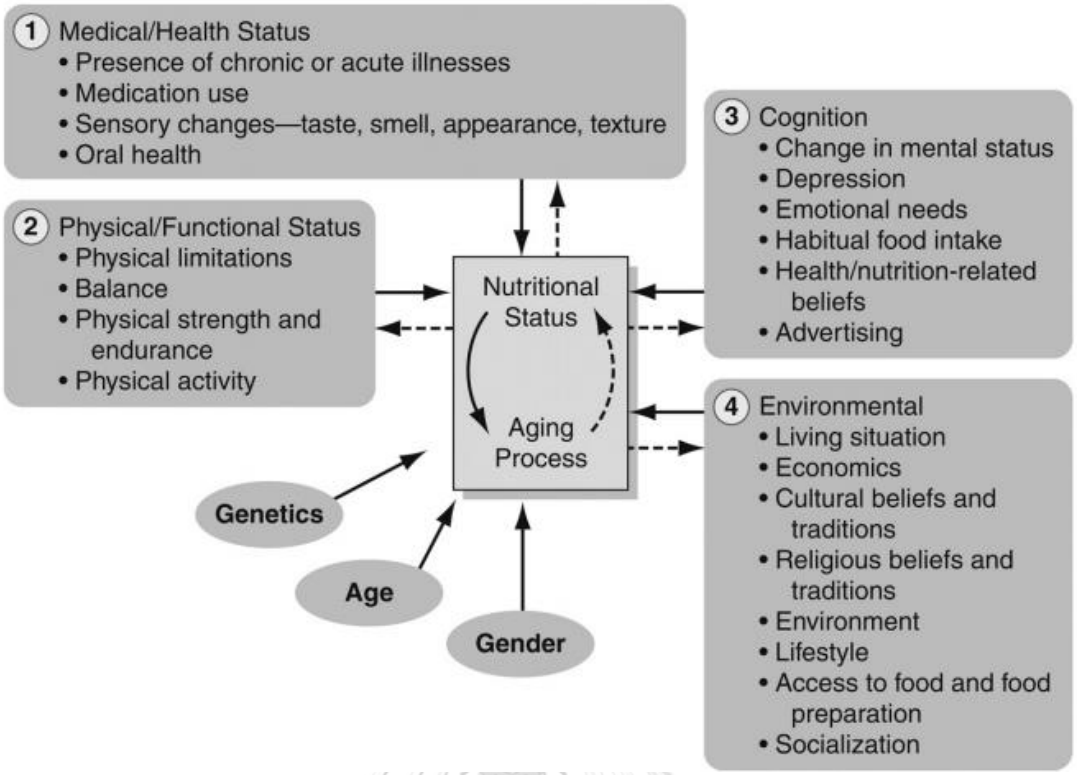


Figure 4 Factors that influence health - related quality of life and the aging process (Richards Adams, 2010)

Energy requirement in elderly decreased due to decrease in physical activities and basal metabolic rate (Keys *et al*., 1973; Roberts and Dallal, 2005). Elderly usually at age 55 year, an increase in food intake is observed which is followed by a reduction in food intake in age over 65 years (Briefel *et al*., 1995). Since both lean body mass and basal metabolic rate decline with age. There is a nutrition guideline for aging to promoting health and wellness to be a healthy aging. The energy that the elderly should receive is divided into three groups, 1,400 kilocalories for the elderly male - female aged 60 - 80 years with light activity, 1,600 kilocalories for the female elderly

aged 60 - 80 years with moderate activity and 1,800 kilocalories for the male elderly aged 60 - 80 years with moderate activity (Kaewanun, May-August 2018).

Carbohydrates are needed for prevent protein from being used as an energy source. Current dietary guidelines recommend that approximately 45 percent to 65 percent of total daily calories, but the carbohydrate should be complex carbohydrate such as legumes, whole grain and vegetables for avoiding overnutrition and the risk of obesity and chronic condition (Shlisky *et al*., 2017) because metabolic system in elderly reduced (Elahi and Muller, 2000).

The dietary guidelines for lipids recommend that no more than 25 percent to 35 percent of total daily caloric intake. Emphasis that fat should be monounsaturated or polyunsaturated fat sources. Conversely, restricting dietary fat to less than 20 percent of the caloric intake may affect the overall quality of the diet and negatively affect taste, satiety and food intake.

As people age experience loss of skeletal tissue mass due to decreased food intake and it is become risk factor for malnutrition (Meydani, 2001). Some studies suggested that an intake of 1 g / kg is needed to maintain skeletal tissue mass in older adult and 1 to 1.25 g / kg was reported that safe for older adults (Stevens, 2001). some evidence - based recommended dietary protein intake for older people shows that older adults need more dietary protein than young adults to support good health, promote recovery from illness, and maintain functionality (Bauer *et al*., 2013). As a result, older people are at higher risk for conditions such as sarcopenia and

osteoporosis than are young people which related to fall and lead to death (de Souza Genaro and Martini, 2010). When considering protein intake, it has been found that older people need greater doses of quality protein which contain essential amino acids (EAA) to increased muscle synthesis (Symons *et al*., 2009).

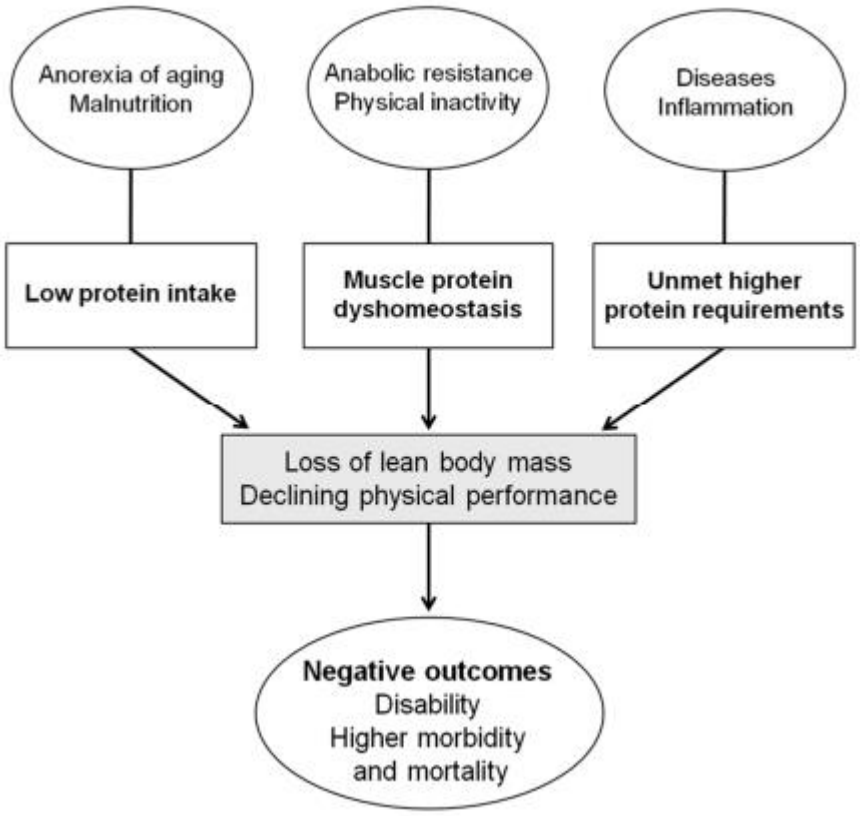


Figure 5 Aging - related causes of protein shortfall.

Such protein deficits have adverse consequences, including impairment of muscular, skeletal, and immune function (Bauer *et al*., 2013)

2.5 Egg white protein

Hen eggs consist of 3 main components: eggshell (9 – 12%), egg white (60%), and yolk (30–33%) as shown in Figure 6. Hen eggs are known as the best source of protein because of its nutritional, egg contains essential lipids, proteins, vitamins, minerals, and trace element while offering a moderate calorie source (about 140 kcal / 100 g), moreover it can offer a great culinary potential, and low economic cost. Eggs have been identified to represent the lowest - cost animal source for proteins, vitamin A, iron, vitamin B12, riboflavin, choline and the second lowest - cost source for zinc and calcium (Drewnowski, 2010).

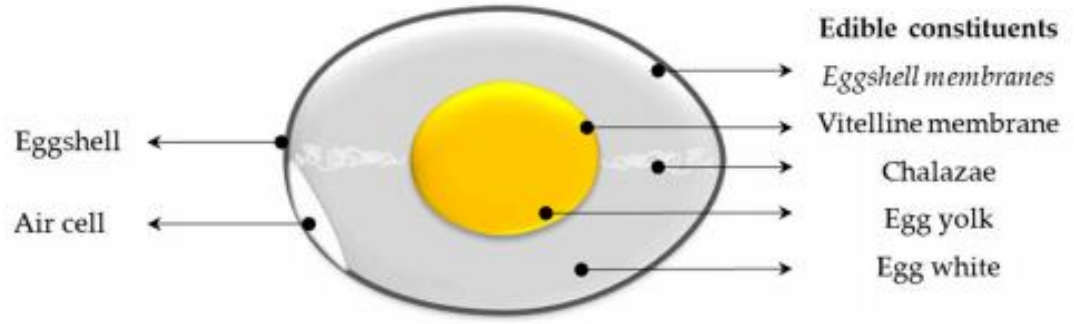


Figure 6 Egg structure

Eggshell membranes are edible but usually not consumed, as they remain tightly associated with the eggshell (Réhault - Godbert *et al*., 2019)

Egg white (EW) represents about 60% of the shell egg by weight, and mainly comprises of water (88%) and protein (11%), with the rest made up of carbohydrates, ash, and trace amounts of lipids (1%) (Li-Chan, 1995). As showed in table 1, egg white is an excellent source of high - quality proteins contain more than 20 different proteins which have been identified and isolated from EW.

Table 1 Shown amount of each proteins found in egg white

Protein	Amount (%)
Ovalbumin	54%
Ovotransferrin	12%
Ovomucoid	11%
Lysozyme	3.5%
Ovomucin	3.5%
Avidin	0.05%
Cystatin	0.05%
Ovomacroglobulin	0.5%
Ovoflavoprotein	0.8%
Ovo - glycoprotein	1.0 %
Ovoinhibitor	1.5 %

Source: Kovacs-Nolan *et al.*, 2005

Table 2 Major egg white proteins and its characteristics

Protein	Molecular Weight (kDa)	PI	Characteristics
Ovalbumin	45	4.5	-
Ovotransferrin	77.7	6	Binds iron and other meal ions
Ovomucoid	28	4.1	Inhibit serine proteinases
Lysozyme	14.3	10.7	Lysis of bacterial cell wall
Ovomucin	220 - 270000	4.5 - 5.0	Interacts with lysozyme

Source: Mine, 1995

As showed in table 2 ovalbumin is the major EW protein with 54% of the EW proteins by weight. It is glycoprotein consisting of 385 amino acids, molecular mass of ovalbumin is about 45 kDa. with an isoelectric point of 4.5 (Geng *et al*., 2012).

Ovalbumin does not have a classical N - terminal ladder sequence (Huntington and Stein, 2001). But it has 3 sites of post synthetic modification in addition to the N - terminal acetyl group (Narita and Ishii, 1962) and the C - terminal proline. Ovalbumin is a highly structured globular protein.

The secondary structure of ovalbumin was showed in Figure7 It is a various motifs including α - helix (41%), β - sheet (34%), β - turns (12%), and random coils (13%) (Stein *et al*., 1990). When ovalbumin passes through heat caused protein structure change from its soluble 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 egg white (Hu and Du, 2000).

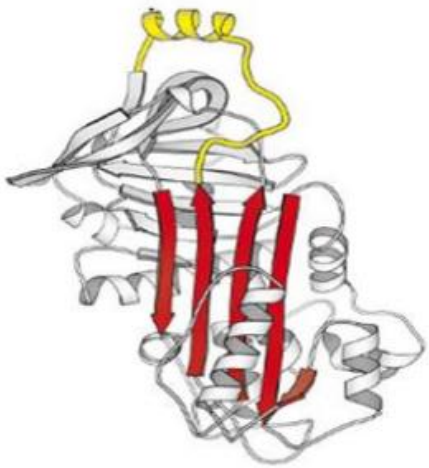


Figure 7 The 3 - D crystal structure of ovalbumin with the α - helix reaction loop in yellow and main β - sheet A in red

(Huntington JA, 2001 May 25)

Ovotransferrin is an iron - binding glycoprotein, found in EW and chicken serum, belonging to the family of transferrin iron - binding glycoproteins (Giansanti *et al*., 2011).

The iron binding ability confers antimicrobial activity by getting iron unavailable for microbial growth. Ovotransferrin consist of 686 amino acids with a molecular weight of 76 kDa (Abeyrathne *et al*., 2013) and isoelectric point of 6.0. It contains 15 disulfide bonds and no free sulfhydryl groups. This single chain is folded into two globular lobes (representing its N - and C - terminal halves) linked by an alpha helix of nine amino acid residues (residues 333–341) that can be released by tryptic digestion (Charter and Lagarde, 1999). Ovotransferrin structure was showed in Figure 8. Ovotransferrin from EW have been demonstrated as biological properties: antimicrobial Activity (Valenti *et al*., 1981), antifungal activity (Valenti *et al*., 1986), antiviral activity (Giansanti *et al*., 2005), immunomodulatory activity (Rath *et al*., 2009), anticancer activity (Ibrahim and Kiyono, 2009) and antioxidative activity (Ibrahim *et al*., 2008). Due to its biological effect, ovotransferrin has already been suggested and used as an infant formula ingredient (Del Giacco *et al*., 1985).

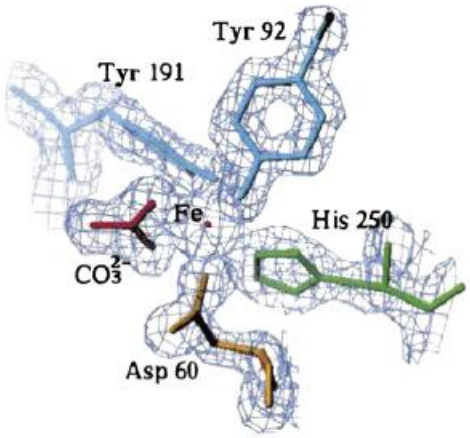


Figure 8 Ovotransferrin with iron - binding site
(Mizutani *et al*., 2001)

Ovomucoid is a protein found in EW with 11% of the egg white proteins by weight. Molecular mass of ovalbumin is about 28 kDa. It is a trypsin inhibitor with three protein domains of the Kazal domain family (Lineweaver and Murray, 1947). Ovomucoid has been studied its biological Activities, the previous study found that ovomucoid can stimulate the immune system (Holen et al., 2001) and drug delivery effect (Agarwal *et al*., 2000).

Lysozyme found in EW with 11 percent. It is a single chain polypeptide containing 129 amino acid residues with molecular mass is about 14.3 kDa. The three - dimensional structure is stabilized by four disulfide bridges which contribute to the exceptional thermostability of the protein. Although thermal stress experiments have shown the formation of catalytically active lysozyme dimers, the reduction of disulfide bridges has generally been associated with the loss of enzymatic activity. Lysozyme has 4 disulfide bridges leading to high thermal stability, and its isoelectric point is 10.7 (Wan *et al*., 2006). Lysozyme's main function is its antimicrobial activity and other physiological functions. It uses to interfere with the growth of bacteria (Silvetti *et al*., 2017). Additionally, antibacterial and antiviral mechanisms of lysozyme have been demonstrated. It has been shown antibacterial activity (Hughey and Johnson, 1987) and antiviral activity (Sava, 1996). Current application of lysozyme in food industry was applied in cheese production to controls the growth of spoilage microorganisms (Doosh and Abdul-Rahman, 2014).

Ovomucin is another major EW protein, which accounts for 3.5% of the total EW protein. It is one of the large molecular weight proteins ranges from 220 to 270 kDa (Omana *et al*., 2010) with carbohydrate attached and it confers gel - like structure of EW (Hiidenhovi *et al*., 1999). Ovomucin mainly composed of two subunits called alpha and beta, α - ovomucin is homogeneous, whereas β - ovomucin is heterogeneous which the beta subunit is much more heavily glycosylated (Offengenden et al., 2011; Robinson and Monsey, 1975). Ovomucin is known to be antimicrobial (Kobayashi *et al*., 2004; Tsuge *et al*., 1996).

Avidin is a minor protein found in EW. Its functions as known as anti - bacterial growth in egg by binding with biotin (Vitamin B7, vitamin H). The binding results in its unavailability for microorganisms and thereby prevents bacterial proliferation (Wellman-Labadie *et al*., 2008). Avidin binds to biotin in the small intestine, preventing its absorption. So, eating uncooked EW may affect the biotin availability resulting as hair loss. The denaturation temperature suggested by Lichan *et al*., avidin can be denatured at 85℃ (Stadelman *et al*., 1996).

2.6 Functional properties of egg white protein

EW proteins are extensively used as a functional ingredient in food industry because of its functional and nutritional properties. The most well - known properties such as: foaming property, emulsifying property, gelling property and bioactive property. These properties have been applied in many food industries for preparation

of meringues, cakes (sponge), ladyfingers (biscuit), soufflés, nougat, bavarois, whipped cream, chocolate, mousse, and bakery product (Raikos *et al*., 2007). There are many researches have been studied on the development of EW protein properties in order to be used in a variety of food industries.

2.6.1 Foaming property

When apply the physical stress of beating EW, it can create a foam. The reason is amino acid in EW contain both hydrophilic and hydrophobic amino acid. The hydrophilic groups (in protein) are arranged towards the water phase and the hydrophobic groups (in protein) towards the air phase. First stress, the beating drags the liquid through itself and creating a force that unfolds the protein molecules. This process is called denaturation. Second stress, the air comes into the solution to form bubbles and the hydrophobic regions facilitate adsorption at the interface. Foam structures from EW was showed in Figure 9 EW protein 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).

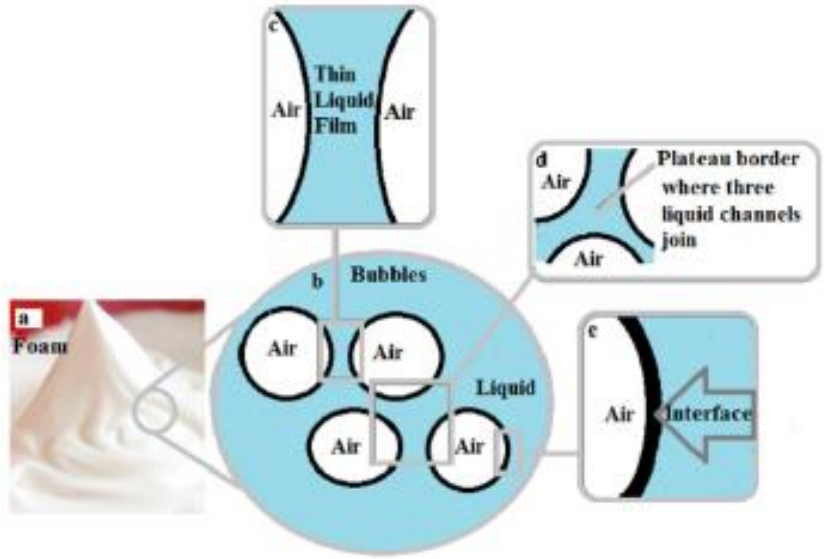


Figure 9 Foam structures from EW

(a) Foam, (b) Air Bubbles, (c) Thin liquid film or Thin film, (d) Plateau border, (e) Air - water interface

(Gharbi and Labbafi., 2019)

There are many studies that improved the foaming property of EW protein by using both thermal and nonthermal treatment. Thermal treatment that use to improve foaming property such as dry heating causing partial hydrolysis of EW protein. They explained that lower molecular weight caused more surface active than unmodified proteins which allowed faster and efficient adsorption to the air - water interface (Lechevalier *et al*., 2017). Nonthermal treatment such as High hydrostatic pressure (HP), HP processing can induce changes in structural and functional properties of egg white proteins. Increasing foaming

properties by HP were dependent on pH and pressure levels. At pH 8.8, pressures above 500 Mpa (9.6 mg / ml, 25 °C) led to the increase in foam volume (Singh *et al*., 2014).

2.6.2 Emulsifying property

Emulsification is the most important process in many food industries. Food emulsion can be categorized as oil in water (O / W) or water in oil (W / O). The difference between O / W and W / O emulsions is that an O / W emulsion commonly exhibits a creamy texture, while a W / O system has greasy textural properties. Emulsifying activity is related to the capacity of surface - active molecules to cover the O / W interface created by mechanical homogenization, so reducing the interfacial tension. Consequently, the more active the emulsifying agent, the more the interfacial tension between hydrophobic and hydrophilic components is lowered. Protein emulsifying activity is the ability of the protein to participate in emulsion formation and to stabilize the newly created emulsion. Protein solubility is an important factor determining emulsifying properties of proteins. The emulsifying property of proteins 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

et al., 1999). Moreover, protein solubility can increase the capacity of a protein to form and stabilize emulsion was improved. Undissolved proteins are poor stabilizers because proteins must dissolve and migrate to the interface (Lin and Zayas, 2006). The recent study to increase EW protein emulsifying activity and stability is partial hydrolysis combined with heat treatment. The result shown that the enzymatic hydrolysate caused higher emulsifying ability and stability than native egg white and close to the properties of egg yolk which was a reference as a food emulsifier (Wang *et al*., 2018).

2.6.3 Gelling property

Gel is an intermediary form between solid and liquid. It is the cross - linking among polymeric molecules which make an intermolecular network within a liquid medium. Gelling formation from egg white was showed in Figure 10. In food processing, gelling property play important role in many food products such as desserts, puddings, reformulated meat products, tofu and surimi (Alleoni, 2006). Ferry (1948) suggested the concept that gelation occurs in two steps. He emphasized the importance of the relation between denaturation and aggregation (FERRY, 1948). The first step involves changes in the conformation (usually induced by heat) or partial denaturation of the protein molecule. In the second step, a gradual association or molecule aggregations of denatured proteins leads to an exponential increase of

viscosity, and to the formation of a continuous network (PHILLIPS, 1994). The nature and properties of gels are influenced by several factors, such as protein concentration, solution pH, nature, and concentration of the electrolyte (Mulvihill and Kinsella, 1988). To improve the gelling properties of EW, Kato et al. (1989) proposed that to improve gelling properties of dried egg white is extended the denaturation of proteins while preventing aggregation. Such conditions can be obtained by heating of egg white powder at high temperatures (80 °C) for a long time (up to 10 days) (Kato *et al*., 1989). Moreover Matsudomi *et al*. (2002) reported that gelling property of dried egg white can be improved by modification with galactomannan through the Maillard reaction (Matsudomi *et al*., 2002).

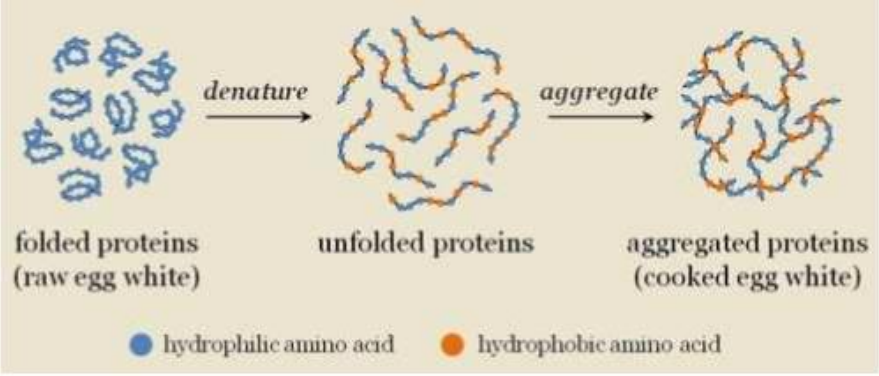


Figure 10 Two - step process of gel formation of proteins

2.6.4 Bioactivity of EW protein

EW 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 is defined as a process to make protein sequences into small peptides with various molecular weights and individual amino acids with the specific of enzymes. Enzymatic hydrolysis of EW proteins can release bioactive peptides and different enzymes have different abilities to release such bioactive fractions (Mine, 2007). The enzymatic hydrolysis products can positively impact on body functions and human health (Eckert *et al*., 2013). Table 3 showed bioactive effect of peptides derived from EWP by using enzymes.

Table 3 Examples of bioactive peptides derived from EW protein by using enzymes with optimal condition

Biological activity	Egg proteins	Enzymatic hydrolysis conditions	Peptides / sequence	Reference
Antioxidant activity	Ovalbumin	Pepsin, pH 7.4, 37°C, 3 h	Tyr - Ala - Glu - Glu - Arg - Tyr - Pro - Ile - Leu - Ser - Ala - Leu - Ala - Met	(Dávalos *et al*., 2004)
	Egg white proteins	Alcalase, pH 10.66, 50°C	Asp - His - Thr - Lys - Glu, Phe -	(Liu *et al*., 2015)

			Phe - Glu - Phe - His, Met - Pro - Asp - Ala - His - Leu	
Antihypertensive activity	Ovalbumin	Pepsin (pH 2.0), trypsin (pH 7.8), and chymotrypsin (pH 8.0), 37°C, 24 h	Arg - Ala - Asp - His - Pro - Phe - Leu, Tyr - Ala - Glu - Glu - Arg - Tyr - Pro - Ile - Leu	(Miguel *et al*., 2004)
	Ovalbumin	Thermolysin (pH 7.5), pepsin (pH 2.0), trypsin (pH 7.5), and chymotrypsin (pH 7.5), 37°C, 2 h	Leu - Trp, Ile - Lys - Trp	(Iroyukifujita *et al*., 2000)
	Ovotransferrin	Thermolysin, pH 8.0, 55°C, 3 h; pepsin, pH 2.0, 37°C, 3 h	Ile - Arg - Try, Leu - Lys - Pro, and Ile - Gln - Try	(Majumder and Wu, 2011)
	Egg white proteins	Alcalase, pH 10.0, 50°C, 180 min	Thr - Asn - Gly - Ile - Ile - Arg	(yu *et al*., 2012)
Antimicrobial activity	Ovalbumin	Trypsin and chymotrypsin, pH 7.8, 37°C, 6 h	Ser - Ala - Leu - Ala - Met, Ser - Ala - Leu - Ala - Met - Val - Tyr, Tyr - Pro - Ile -	(Pellegrini *et al*., 2004)

		Leu - Pro - Glu - Tyr - Leu - Gln, Glu - Leu - Ile - Asn - Ser - Trp, Asn - Val - Leu - Gln - Pro - Ser - Ser, Ala - Glu - Glu - Arg - Tyr - Pro - Ile - Leu - Pro - Glu - Tyr - Leu, Gly - Ile - Ile - Arg - Asn, Thr - Ser - Ser - Asn - Val - Met - Glu - Glu - Arg	
Lysozyme	Pepsin, pH 1.0, 37°C, 1 h, followed by trypsin, pH 8.0, 37°C, overnight	Ile - Val - Ser - Asp - Gly - en (Asp - Gly - Met - Asn - Ala - Trp, His - Gly - Leu - Asp - Asn - Tyr - Arg	(Mine *et al*., 2004)
Lysozyme	Alcalase, pH 7.5, 50°C, 3 h		(Tang et al., 2013)
Ovalbumin	Flavourzyme, pH 7.0, 50°C, 1 h; pepsin, pH 2.0, 37°C, 2 h; trypsin, pH 7.5, 37°C, 3 h; neutrase, pH 7.0,50°C,4	Asx - Ser - Gly - Arg - Ala - Leu Arg - Val - Ala - Ser - Met - Ala - Ser - Glu - Lys - Met - Lys - Ile	(You *et al*., 2010)

		h; papain, pH 6.5, 50°C, 5 h		
Anticancer activity	Ovomucin	Pronase (from Streptomyces griseus), pH 8.4, 37°C, 24 h	Glyceropeptides with 120 000 Da and 220 000 Da	(Watanabe *et al*., 1998)

Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Asx, aspartic acid or asparagine; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

2.6.5 EW protein and heat sensitivity

Due to EW contains several heat sensitive proteins which an important factor when EW proteins were used as protein source in food industry. Especially when going through a process that requires heat such as pasteurization. The previous study of the effect of salt and sugar increased the heat stability of heat sensitive protein. Moreover, several carbohydrates including sucrose, glucose, fructose, arabinose, mannitol and xylose could protect protein from denaturation during pasteurization process (Stadelman *et al*., 1996). As showed in table 4.

Table 4 EW protein and denature temperature

Protein in EW	Denaturation Temperature (°C)
Ovalbumin	84.0
Ovotransferrin	61.0
Ovomucoid	79.0
Ovomucin	-
Lysozyme	75.0
Avidin	85

2.7 Hydrolysis of food proteins

Enzymatic proteolysis is a process by which proteins are degraded into their component polypeptide or amino acid parts. This generally occurs through protease - mediated (enzyme) hydrolysis of peptide bonds, but can also occur through non - enzymatic methods such as by action of mineral acids and heat (Varshavsky, 2001). Protein hydrolysates is a product obtained from hydrolyzed reactions which is the hydrolysis of proteins at the peptide bond area from various protein sources such as fish, milk, eggs, plants etc. From that hydrolysis reaction short chain peptides and free amino acids occurs, Moreover modifying the protein structure will affect the physical and chemical properties of proteins (Severin and Xia, 2006).

Improving the functional properties of proteins will increase the variety of protein utilization in the food industry by changing the protein structure. Protein

hydrolysis is carried out by using thermal, non - thermal, chemical methods. Thermal which is the traditional method to increase food safety from foodborne pathogens, eliminate toxicity and reduce food allergen. Moreover, it can improve texture and taste of the food (Vanga and Raghavan, 2016). Non - thermal methods such as using high - pressure processing (HPP) range 100 to 800 mPa (Farkas and Hoover, 2000). Resulting in changes the bonds within the molecules of the protein. Molecules of protein changing found to be effective in improving the structure of proteins resulting in changes in the properties of proteins, reducing allergies and prolonging shelf life of food product (Rivalain *et al*., 2010). Chemical hydrolysis, which is the most commonly used food products are divided into 3 types, acid hydrolysis, alkali hydrolysis and enzymatic hydrolysis.

Acid hydrolysis by using inorganic acids such as sulfuric acid or hydrochloric acid. Using sulfuric acid to break down peptide bonds will cause unpleasant odor of protein. The hydrolysis of protein with hydrochloric acid causes sodium chloride salt or potassium chloride. Therefore, in the food industry, hydrochloric acid is used to break down peptide bonds because sodium chloride salt or potassium chloride is salt used in general food with has little negative impact on the product. Advantage of this method is easy process with low cost and time saving. But the disadvantage is that the loss of amino acids such as tryptophan and may cause destroyed of some amino acids such as cysteine, serine and methionine (Mustatea *et al*., 2019).

Another way is alkali hydrolysis by using sodium hydroxide, barium hydroxide and potassium hydroxide. It was found that alkali hydrolysis may cause racemization of some types of amino acids which is the conversion of L - amino acids into D - amino acids type that the body cannot utilized. Moreover, causing some amino acid such as arginine, threonine and cysteine destroyed as well bad taste. So, this method is not popular in the food industry (Udenigwe and Aluko, 2012).

Enzymatic hydrolysis is a popular method. Widely used to improve the protein's properties. The most important functional properties of food proteins, in general, are solubility, emulsification, and foaming. In addition to providing nutrition, proteins present in foods. Enzymatic hydrolysis is a method with highly specific which can control the degree of hydrolysis (DH) When compared to acids and alkalis hydrolysis as enzymes are more specific with peptide bonds. Enzymatic hydrolysis should concern in many factors such as proper ratio of enzyme and protein, time, pH and temperature. Normally, the hydrolysis process is performed at the optimum pH and temperature of each enzyme to control the degree of hydrolysis and desired final product which affect the protein properties for being a bioactive ingredient (Vanga *et al*., 2017) and flavor of final products. Most of the enzymes used for protein hydrolysis are from animal sources (such as pancreatin and pepsin), plant sources (such as papain from papaya and bromelain from pineapple), and microbial sources (such as Alcalase). It is important to consider the type of enzyme, hydrolysis conditions, and control of

DH. As Figure 11 showed the mechanism of enzyme action when using different type of enzyme.

Nowadays, food industry often uses protease enzymes that produced from microorganisms such as Alcalase produced from bacteria *Bacillus subtilis* classified as endopeptides, neutrase produced from bacteria *Bacillus amyloliquefaciens* classified as endopeptides and Flavourzyme produced from *Aspergillus sp.* classified as both endopeptides, which break peptide bonds of nonterminal amino acids within the molecule and exopeptidases, which break peptide bonds from terminal amino acids. In which different types of enzymes use different optimal conditions as shown in table 5.

Table 5 Optimum conditions for enzymatic hydrolysis of protease

Enzyme	Optimal pH	Optimal temperature And type of enzyme		Source
Alcalase	8 - 8.5	55 - 60	endopeptidase	*Bacillus subtilis*
Neutrase	5.5 - 7	45 - 55	endopeptidase	*Bacillus amyloliquefaciens*
Flavourzyme	6 - 7	50 - 60	Endo - exopeptidase	*Aspergillus sp.*

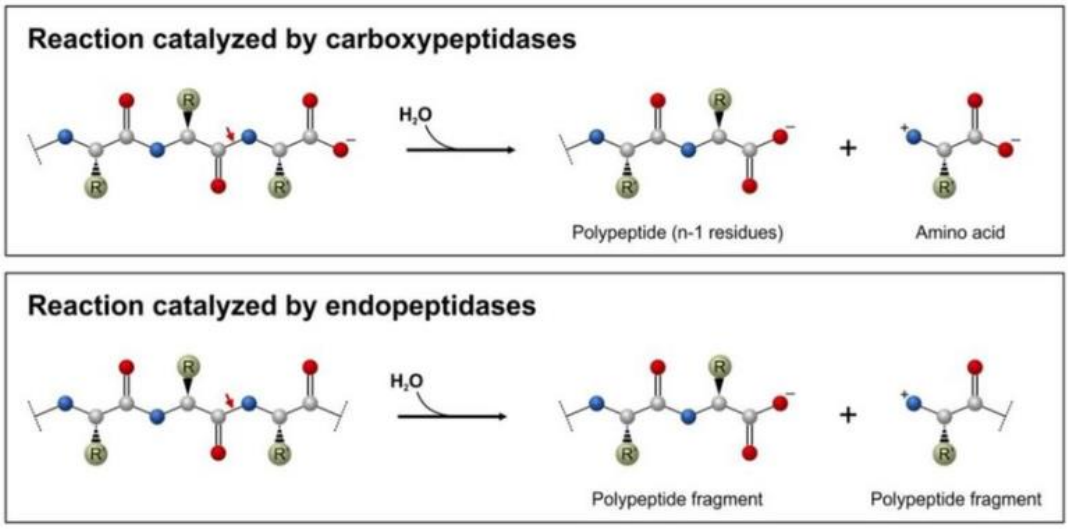


Figure 11 Mechanism of action of endopeptidase and exopeptidase (Noreen *et al*., 2017)

2.8 Egg white protein hydrolysates (EWH)

Nowadays, the importance of proteins in the diet has been increasingly. Food production by using enzymatic hydrolysis has been used for many years to improve and modify protein functions (Liu and Chiang, 2008). As described above that EW contain all essential amino acid (as shown in table 6). Moreover, EW protein hydrolysate contains peptides obtained from the EW protein through the enzyme proteolysis which is released Biological activity which have the antioxidant effect (as shown in table 6) which is an important source and good quality protein for healthy people and those who entering aging society, especially for elderly people.

Table 6 Amino acid composition of EW and egg yolk protein hydrolysates

	Composition (%)		
Amino acid	WP hydrolysate	YP hydrolysate	Reference For human EAA[1]
Hydrophobic amino acid			
Gly	5.83	5.56	-
Ala	8.93	8.18	-
Val[2]	7.94	6.93	1.3
Leu[2]	8.65	8.61	1.9
Lle[2]	5.63	5.29	1.3
Met[2]	3.54	2.29	1.7
Pro	4.25	4.86	-
Hydrophobic, flavor amino acid			
Phe[2]	5.21	3.91	-
Tyr	2.74	3.02	-
Hydrophilic amino acid			
Arg[2]	4.13	5.36	-
His[2]	2.04	2.19	1.6
Ser	7.97	9.21	-
Lys[2]	5.79	6.86	1.6
Asx*	10.08	10.18	-
Glx*	12.51	11.63	-
Thr[2]	4.75	5.92	0.9

Asx*, Asp + Asn, Glx*, Glu + Gln.

WP: Egg white protein, YP: Egg yolk protein

[1] Suggested profile of essential amino acid requirements for adult humans, FAO / WHO (1990). [2] Essential amino acids.

Source: Pokora *et al*, 2013

In addition, EWH is also used as an importance ingredient in the functional food industry because its nutrition. Apart from that it also has a good texture and many other properties, such as the ability to increased solubility from enzymatic hydrolysis. Moreover, improving the emulsifier, foaming, gel formation and antioxidant properties. But nowadays, the use of EW protein added to foods or use protein as a food component. In order to increase nutrition or to improve texture but there are still quite a few limitations as mentioned above. Egg white protein consists of a variety of proteins (as shown in table 1) and each protein can withstand at different temperatures. Currently, there are many studies that focus on the digestion of egg white proteins by enzymes for improving protein properties which can be applied in many food industries. From the previous study shown that the properties of final products are affected by degree of hydrolysis (DH) (Adebiyi *et al*., 2008).

Dong Ouk Noh *et al*. studied on 6 different commercial enzymes with EW liquid protein. They found that enzyme Alcalase gave the highest degree of hydrolysis (DH) value (43.2%) and had the highest radical - scavenging activity (82.5%) at a concentration of 5.0 mg / mL (Noh and Suh, 2015). Castro *et al*. studied the antioxidant activities effect of EW protein by used 2 protease enzymes from *Aspergillus oryzae*

and *Bacillus licheniformis.* They found that antioxidant activity was higher by using protease from *Aspergillus oryzae* than the rest protease. They also found that at 50% of DH gave highest antioxidant activity (ORAC) after hydrolyzed for 120 min (de Castro and Sato, 2015).

The effect of enzymatic hydrolysis on digestion and absorption of EW protein have been studied. Due to, raw EW contains highly abundant protease inhibitors that may delay digestion of egg components in human body, especially when EW is used as a raw ingredient (Saxena and Tayyab, 1997). Evenepoel *et al*. studied digestibility of cooked and raw egg protein in humans. They found that preheat treatment of raw egg protein can enhance digestibility (Evenepoel *et al*., 1998). Moreover, Matsuoka et al. studied the absorption rate of EW protein hydrolysate in rats' model. The result shown that EWH maintain the nutritional value and rapidly absorbed by the body (Matsuoka *et al*., 2019). So it could be conclude that enzymatic modification is another effective way to improve the functional properties of proteins, such as enhance thermal stability, protein solubility (Pacheco-Aguilar *et al*., 2008). These properties are useful for many food applications. Moreover, enzymatic modification may suitable for people who have digestion and absorption problems such as elderly.

2.9 Egg white protein hydrolysis application in food products

Nowadays, consumers prefer to consume foods that contain essential nutrients and more beneficial to their health. Enzymatic hydrolysis is a well - known method for

increasing the added value of food proteins by modifying their physicochemical and nutritional properties. Enzymatic hydrolysis provided better solubility of food proteins and changed their surface properties which essential for the stabilization of emulsions and foams.

The pervious of Rimon *et al*. had studied the application of EWH to be a new texture for novel food. The enzyme used in this study was food grade aminopeptidase from *Aspergillus oryzae*. First product was cream as showed in 12. It was obtained by egg white hydrolysis and centrifuge. Flavor and texture like lemon cream. Second product was obtained by egg white hydrolysate with the addition of salt before enzyme inactivation, giving an egg - based product with a taste and mouthfeel similar to fresh or cottage cheese, but fat, milk protein and lactose free (Garcés-Rimón *et al*., 2016). Product number 3, sugar and liquid honey extract were added before enzyme inactivation and the gel was not centrifuged, so the resulting product had a flavor and texture comparable to those of junket, a dairy dessert made by enzymatic milk coagulation, but somehow lighter and smoother.

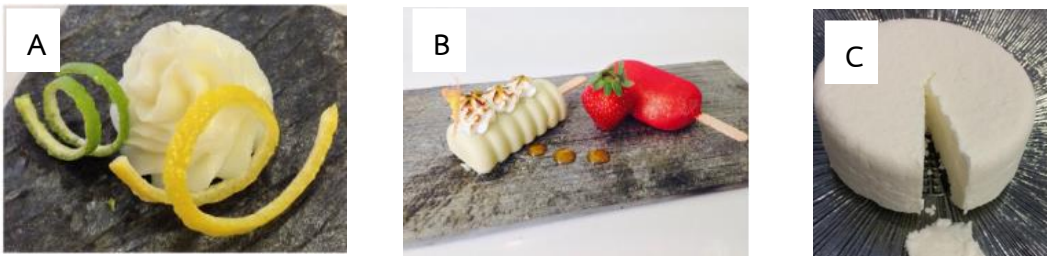


Figure 12 Product produced from EWH

(A) Cream obtained by hydrolysis and centrifugation of egg white. (B) Ice - creams produced by freezing this product. (C) Fresh cheese - like product obtained by hydrolysis and centrifugation of egg white (Garcés-Rimón *et al*., 2016)

Moreover, the previous research from Kaewmanee *et al*. had studied the effect of salted EW protein hydrolysate on the quality of pacific white shrimp. The result shown that salted EW protein hydrolysate can improve the cooking yield and reduced the cooling loss (Kaewmanee *et al*., 2009). Wang et al. studied the effect of partially hydrolyzed EW and its application on pork meat after soaked of slide meat in EWH solution. They found that EWH solution can be a natural antioxidant for the preservation of pork meat by improving color stability during storage time (Yu. Wang, 2019). In addition, Han et al. studied the use of EWH powder to develop gluten free bread. instead of gluten. The recipes were substituted with 0% – 15% of two type of egg white solids (M200 and P110). The result suggested that EWH powder 15% yielded

high foam formation and stability which improved the texture and properties of bread during storage as showed in Figure 13.

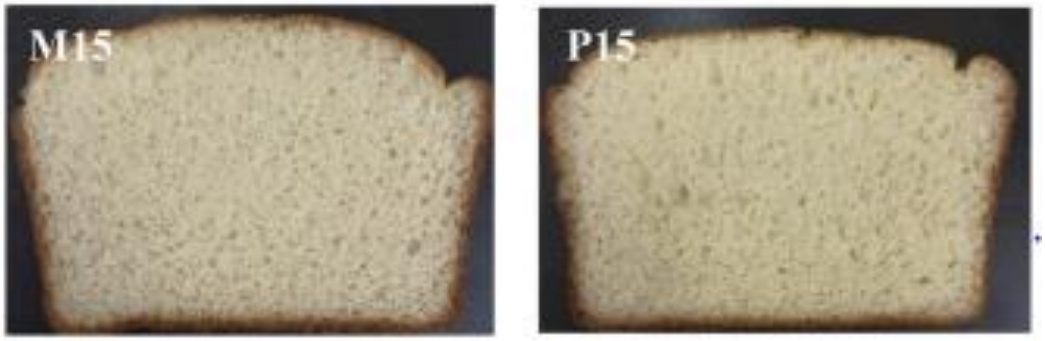


Figure 13 Appearance of crumb structure of gluten - free bread used 15% of Egg white solids (Han *et al*., 2018)

2.10 Nutrition claim (Thai FDA)

According to the nutrient content claims set by the Thai FDA for the development of high protein beverages, the protein content claim as good source, contains or provides for solid food groups should meet the following criteria: The product must contain no less than 5 grams of protein per 100 grams of food or not less than 2.5 grams per 100 kcal of energy. For liquid foods, the protein content claim should be no less than 2.5 grams of protein per 100 milliliters or per 100 kcal of energy.

The protein content claim as high, rich, rich in or excellent source of for solid food groups should meet the following criteria: The product must contain no less than 10 grams of protein per 100 grams of food or not less than 5 grams per 100 kcal of

energy. For liquid foods, the protein content claim should be no less than 5 grams of protein per 100 milliliters or per 100 kcal of energy.

Please note that this information is based on the guidelines provided by the Thai FDA regarding nutrient content claims for high protein beverages. It's always recommended to refer to the latest regulations and guidelines issued by the relevant authorities for accurate and up-to-date information.

2.11 Food and nutrition trend

The data from Euromonitor International shown that the oldest and youngest consumers are two groups that have been focal points in the drive for more segmented product portfolios. As the global population ages as described, food manufacturers are innovating with new ways to meet the nutritional needs of older consumers. According to lifestyles survey more than half of consumer willing to spend their money on new food experience such as texture, mouthfeel and flavor. Moreover, older people want to feel, behave and be treated as younger

Protein as one of the most important nutrients for aging people. Recently, protein has become a mega trend globally. According to Euromonitor International's Health and Nutrition Survey in Thailand, the belief that protein is "better for me" is said as the top reason why consumers seek for protein on food and drink. Followed by, protein "makes me feel better".

Food Ingredients Asia 2019 reported the value of healthy food and beverages market in year 2018 approximately around 86,648 million baht and it is predicted that the value in 2019 will be approximately 88,731 million baht or expansion growth rate 2.4 percent. Especially the protein group is worth around 6,725 million baht and is likely to grow by 6.4 percent according to the increasing popularity of protein consumption for strengthening and maintaining muscle mass. In this regard, the health food and drink mentioned above most of the demand comes from the 3 main groups of consumers. Including health - conscious people who want to have a good body and the elderly. Kasikorn Thai Research Center views that healthy food and drink will play an important role in the prevention and treatment of diseases which mostly occur in elderly. Currently, Thailand has an aging population of more than 10 million or 15 percent of the total population and is expected to increase to 20 percent of the total population by 2021, which will be an aging society. Completely (Aged Society) will inevitably result in the demand for healthy products.

When looking at the mega trend of increasing demand for healthy products. It is an opportunity for manufacturers to find new ways to differentiate their products. Moreover, Kasikorn SME Analysis also suggested that the new technology and innovation such as biotechnology could help to increase the beneficial of product to be more value added.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Chemicals	Company
Protease - SDAY10 (≥ 80,000 u / g)	Amano Enzyme Inc.
Protease - PC10F (≥ 90,000 u / g)	Amano Enzyme Inc.
Protease - P6SD (≥ 600,000 u / g)	Amano Enzyme Inc.
O - phthalaldehyde (OPA)	Sigma - Aldrich
Sodium borate	LOBA CHEMIE PVT. LTD.
Sodium dodecyl sulfate (SDS)	Bio - Red Laboratories
Dithiothreitol (DTT)	Calbiochem
Tris - HCl buffer	Vivantis Biochemical
Coomassie Brilliant Blue R - 250	Applichem
Acetic acid	Merck
Precision protein standard	Bio - Rad
Porcine pepsin	Sigma - Aldrich
Porcine pancreatin	Sigma - Aldrich
Calcium chloride ($CaCl_2$)	Merck
Bile Acid Assay Kit	Sigma - Aldrich
Sodium hydroxide (NaOH)	Merck
Hydrochloric (HCl)	Merck

Potassium chloride (KCl)	Merck
Potassium phosphate (KH_2PO_4)	J. T. Baker
Sodium bicarbonate ($NaHCO_3$)	Merck
Sodium chloride (NaCl)	Merck
Magnesium chloride hexahydrate $MgCl_2(H2O)_6$	Merck
Ammonium carbonate $(NH4)_2CO_3$	Sigma - Aldrich
3,5 - Dinitrosalicyclic acid	Sigma - Aldrich
Sodium potassium tartrate	Sigma - Aldrich
Sodium acetate trihydrate	Qrec
Tin (II) choloride	KEMAUS
Ethylene glycol	KEMAUS
Ninhydrin	KEMAUS
Ascorbic acid	Sigma - Aldrich
2,2'- azinobis(3-ethylbenzothiazoline-6-sulfonate)	Sigma - Aldrich
Potassium persulfate ($K_2S_2O_{4)}$	Sigma - Aldrich
2,4,6 - Tris(2 - pyridyl) - s - triazine (TPTZ)	Sigma - Aldrich
Iron (II) sulfate ($FeSO_4$)	Ajax Finchem
Iron (III) Ferric chloride anhydrous ($FeCl_3$)	Ajax Finchem

Lab devices	Company
Microcentrifuge tube (1.5 mL)	Hettich Zentrifugen

Lab instruments	Company
Autopipette (20, 200, 1,000 µL)	Labnet International, Inc
Freezer (- 20 °C)	Sanyo Cool
Gel documentation system	Biorad
Hot plate	Labnet International, Inc
Spectrophotometer	Biotek Instrument
Vortex mixer	Becthai, Bangkok
Water bath	Boss scientific, Thailand
pH - meter	Mettler - Toledo
Viscometer	BROOKFIELD ENGINEERING LABORATORIES, INC.
Hunter Lab colorimeter	Hunter Associates Laboratory, Inc.
Mastersizer 3000 laser diffraction particle size analyzer	Malvern Panalytical Ltd
Model GS - 700 Imaging Densitometer	Bio - Red Laboratories
ROTINA 380 and 380 R centrifuge	Andreas Hettich GmbH and Co. KG

Gel Doc™ EZ Imager	BIO - RAD
Gel Doc™ EZ Imager (White tray)	BIO - RAD

3.2 Methods

3.2.1 Preparation of egg white protein hydrolysate (EWH)

Pasteurized egg white liquid (EWP) were purchased from a nearby grocery store. Then EWP were directly homogenized using a magnetic stirrer after being directly diluted with distilled water to a 50% concentration of egg white (EW). The homogenized EW was then combined with a specific quantity of different enzyme concentration from 0%, 0.1%, 0.2%, 0.3% W/V. The safety use of enzyme as recommended by Thai FDA (Administration, 2562). The hydrolysis conditions are reported in Table 7.

Table 7 Protease enzyme characteristics

Enzyme	Source	Optimum condition		Type
		Temp (°C)	pH	
SDAY10	*B. licheniformis*	70	10.0	Endopeptidase
PC10F	*B. stearothermophilus*	70	10.0	Endopeptidase
P6SD	*A. melleus*	50	7.0	Endo - exopeptidase

Next, EWP were hydrolyzed in water bath at optimal temperature of each enzyme for 3 hours and then heat - treated for 15 minutes at temperature of 95 °C to stop the hydrolysis reaction. EWH were immediately cooled in ice - chilled water and sample were collected and stored at - 20 °C for further analysis.

3.2.2 Evaluation of the physicochemical and functional characteristics of EWH

3.2.2.1 Evaluating the viscosity properties of EWH

The viscosity analysis of EWH was measured by viscometer (The Brookfield DV - II + Pro) to investigate the shear rates of EWH product. EWH was prepared in 250 mL beaker at chilling temperature. Each sample was determined in triplicate for viscosity for about 1 min by using RV 02 Spindle of Brookfield DV – II + Pro. The units of measurement of viscosity was expressed as mPa·s or cP.

3.2.2.2 Determination of color characteristics in EWH

Color measurements of EWH were performed by using Hunter Lab colorimeter (Model : ColorFlex 4.5 / 0, USA). First, the instrument was calibrated with black and white plate standards before color measurement. Color parameters that are observed on EWH formula including lightness (L*), redness (a*), and yellowness (b*). Value L represents the brightness (lightness) of the sample. While the value of + a represents the red color of the sample, conversely the value of - a represents the green color. The value + b represents the yellow of the sample, while The value - b represents the blue

of the sample. The color measurement of each EWH was calculated to compare with control egg white protein.

3.2.2.3 Investigation of pH during hydrolysis process of EWH

The hydrolysis process was performed under controlled experimental conditions, including temperature, pH, enzyme concentration, and reaction time. These parameters were optimized based on company suggestion. The pH of the hydrolysis reaction mixture was monitored at regular intervals using a calibrated pH electrode and a precision pH meter. The electrode was calibrated with standard buffer solutions prior to each measurement to ensure accuracy.

3.2.2.4 Determination of particle size distribution in EWH

Particle size distribution (PSD) of EWH was analyzed with a laser diffraction particle analyzer (Mastersizer 3000 Ver 3.40, Malvern, Scientific and technological Research Equipment Centre, Chulalongkorn University, TH). The stirrer speed achieved is 1500 rpm with 100 mL MilliQ® water in the sampling chamber. The test tubes were tilted three times to get a homogenous sampling. Each emulsion replicate measured three times and the average was reported. The dispersant refractive index (RI) is 1.33 for the water. The laser obscuration was between 10% to 20%. From the particle size distribution, the volume weighted mean ($D_{4,3}$), mode (top of the peak), and volume (%) was calculated.

3.2.2.5 Determination of degree of hydrolysis (DH) in EWH

The O - phthalaldehyde (OPA) assay was used to determine DH of EWH by investigated the concentration of available amino groups during the hydrolysis of EWP as an accurate measurement of DH. The OPA assay solution contained 0.1 M sodium borate, 0.1% SDS (w / v), 0.3 mM OPA, 2% ethanol (v / v), and 5.7 mM dithiothreitol. Sample (50 µL) was incubated with 1 mL of OPA assay solution for exactly 2 min before reading the absorbance at 340 nm. The measurements were carried out in triplicate and samples and standards were blanked against 50 µL of H_2O with 1 mL of OPA assay solution. To calculate the DH from the absorbance readings, the method of Nielsen (Nielsen et al., 2001), which calculated by following equation.

$$DH = \frac{h}{h_{tot}} \times 100\%$$

with DH being the percentage degree of hydrolysis, h the hydrolysis equivalents formed during proteolysis in mmol / g protein and h_{tot} is the hydrolysis equivalents at complete hydrolysis to amino acids in mmol / g protein. The value of h_{tot} was set to 8 mmol / g, assuming an average weight of 125 g / mol of amino acids within proteins based on literature precedent. (Nielsen et al., 2001).

3.2.2.6 Protein pattern determination in EWH by SDS - PAGE

Two SDS - PAGE gels were prepared, with a separating gel concentration of 10% and a stacking gel concentration of 4%. The composition of the 10% separating gel consisted of a mixture of 10% acrylamide, which included 30% acrylamide / bis - acrylamide, 37.5 : 1 (2.7% crosslinker) solution (Bio - Rad), 0.375 M Tris - HCl (pH 8.8), 0.1% (w / v) ammonium persulfate, and 4 µl Tetramethylethylenediarnine (TEMED). Following the solidification of the separating gel, the stacking gel was applied atop it, and a gel comb was introduced into the stacking gel. The 4% staking gel was formulated using a mixture of 10% acrylamide, 0.1 M Tris - HCl (pH 6.8), 0.1% (w / v) ammonium persulfate, and 4 µl TEMED. The inner chamber was filled with a prepared SDS running buffer consisting of 0.2501 M Tris base, 1.924 M glycine, and 0.03467 M sodium dodecyl sulfate (SDS). The samples were mixed with 6X Laemmli SDS sample buffer and 9% beta - mercaptoethanol. The samples were heated by boiling them for 5 - 10 minutes and loaded into wells without overflowing. The chamber was covered to the top, the appropriate voltage (approximately 100 volts) was set, and electrophoresis was run for 1 hour. The protein patterns were visualized using Bio - Safe Coomassie stain, a product manufactured by Bio - rad. ImageJ densitometry software (Version 1.50b, National Institutes of Health, Bethesda, MD, USA) was used to analyze the negative picture in order to calculate the percentage of protein degradation.

3.2.2.7 Evaluation of EWH digestion profile using *In Vitro* digestion model

To mimic *In Vivo* digestion process, an *In Vitro* digestion model system using enzymes like those in the upper gastrointestinal digestive tract of humans was used. EWH of each enzyme were prepared before the investigation of in vitro digestion. The invitro digestion method was previously described by Minekus *et al*. (Minekus *et al*., 2014) Briefly, 5 ml of samples were mixed with simulated saliva fluids (SSF) without amylase to the final volume of 10 mL, and was pre - incubated at 37°C for 2 min. After the samples were mixed with simulated gastric fluid (SGF) containing pepsin (2000 U / mL of digest), the pH was adjusted to 3 with 5 mol / L of HCl. The final digest volume was adjusted to 20 mL. The mixtures were placed into a temperature - controlled water bath with continuous shaking at 100 rpm / s. After 2 h of incubation, the final intestinal step was carried out by adding simulated intestinal fluid (SIF), contained with pancreatin (100 U / mL of digest) and bile salts (10 mM of digest). The pH was adjusted to 7 by adding 5 mol / L of NaOH. The final volume of the samples were 40 mL. For each sampling time point 5, 8, 10, 15, 30, 45, 60, 90 and 120 min of gastric (G) and intestinal (I) phase), separate tubes were used to analyze amino acid release by ninhydrin method. The reaction was terminated by heating to 95 °C for 10 min in a heat - box followed by cooling to room temperature.

3.2.2.8 Analysis of protein degradation into intermediate peptides using Ninhydrin assay

To investigate the breakdown of protein into intermediate peptide in gastric (G) and intestinal (I) phase of *in vitro* digestion by ninhydrin assay. The ninhydrin assay was previously described by Bryan *et al*., (Bryan *et al*., 2018) Briefly, each sample was mixed with DW and ninhydrin reagent in micro test - tube. The test - tube was placed in heat - box at 100 °C for 10 min. The test - tube was cooling for 5 min after that 200 µl of sample were pipetted into 96 well - plate. The samples were read at 568 nm using a microplate reader. Lysine was used as a standard curve.

3.2.3 Evaluation of EWH's free radical scavenging activity

3.2.3.1 DPPH assay

The radical scavenging of EWH was determined by using DPPH method which was previously described by Cheung et al., (Cheung *et al*., 2003) with slight protocol modification. DPPH is electron transfer assay, based on the capacity of antioxidants to reduce an oxidant by measuring the decrease of its absorbance. Briefly, 20 µl aliquot of each obtained hydrolysate was mixed with 180 µl of 0.2 mM DPPH solution in methanol and the samples were incubated for 30 min at room temperature in the dark. After 30 min, the decrease in the solution absorbance was measured at 517 nm by using UV–Vis spectrophotometer. The DPPH radical scavenging activity was calculated from a standard curve using ascorbic acid. The DPPH radical scavenging activity was expressed as an equivalent of ascorbic acid (mg ascorbic acid). Ascorbic acid was used as a positive control for this study.

3.2.3.2 ABTS assay

ABTS assay also known as Trolox equivalent antioxidant capacity (TEAC) assay. The ABTS radical scavenging activity of EWH was measured using the decolorization assay (Re *et al*., 1999). In this study, ABTS method was previously described by (Mäkynen *et al*., 2013) (Mäkynen *et al*., 2013). Briefly protocol, a

2,2’ - azinobis (3 - ethylbenzothiazoline - 6 - sulfonate) free radical ($ABTS^{\cdot+}$) solution was prepared by mixing 7 mM $ABTS^{\cdot+}$ in 0.1 M phosphate buffer saline (pH 7.4) with 2.45 mM $K_2S_2O_4$ in distilled water. The solution was kept for 16 h at room temperature in the dark. The $ABTS^{\cdot+}$ was diluted with 0.1 M phosphate buffer saline (pH 7.4) until the initial absorbance value reached between 0.900 and 1.000 at 734 nm. 10 µL of EWH was mixed with 90 µL the diluted $ABTS^{\cdot+}$ solution. After 6 min, the absorbance was measured at 734 nm. The TEAC value was calculated from the calibration curve of Trolox. The TEAC value was expressed as mg Trolox equivalents / mL of EWH. All experiments were carried out in triplicate.

3.2.3.3 FRAP assay

The reducing ability of EWH was determined by FRAP (ferric reducing antioxidant power) assay. The FRAP assay is based on the ability of PH to reduce Fe^{3+} to Fe^{2+}. The working FRAP reagent was previously described by (Mäkynen *et al*., 2013) (Mäkynen *et al*., 2013). Briefly, The FRAP reagent was mixed with 10 ml of 0.3 M sodium acetate buffer solution (pH 3.6), 1 ml of 10 mM 2,4,6 - tripyridyl - S - triazine (TPTZ) in 40 mM HCl and 1 ml of 20 mM ferric chloride ($FeCl_3$). A 20 µl of EWH was added to 180 µl of FRAP reagent and well mixed. After that incubated for 30 min at room temperature before reading absorbance at 593 nm. The standard curve was prepared by using $FeSO_4$ for

evaluating FRAP value. All solutions were used on the day of preparation. In the FRAP assay, the values was expressed as mM $FeSO_4$ equivalents / mL of EWH. All measurements were carried out in triplicate.

3.2.4 Sensory analysis

To determine the acceptability of four different enzymes. Three formulations of the beverage were prepared and evaluated its acceptability using 9 - point hedonic scale. Samples were evaluated for acceptance by 30 panelists. A 9 - point hedonic scale, in which a score of 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely, was used for evaluation. The one who response for preparing EWH was the primary researcher (Egg white protein hydrolysate). Whole process was control under Thai FDA regulations for made pasteurized beverages, the hygiene and safety controls for EWH were regulated follow by, all sample were cooked in the food processing laboratory at Chulalongkorn University, Bangkok. Hygiene and quality were maintained by using stainless steel cookware to prepare all EWH beverage sample.

3.2.5 Statistical analysis

Data was reported as mean of values ± SEM. Statistical analysis was performed by one - way analysis of variance (ANOVA) followed by Duncan's multiple range test and using SPSS statistical. The significance different test used for mean comparison

and P - value < 0.05 was considered as statistically significant. Calculations was performed with Microsoft Excel 2013. The graphs were generated with Sigma Plot, version 28 software.

CHAPTER IV
RESULTS

4.1 Physicochemical and functional characteristics of egg white hydrolysate (EWH)

4.1.1 Viscosity properties of EWH

The pasteurized egg white (EWP) was blended with an equal volume of water, resulting in a ratio of 50 : 50 (v / v). The resulting mixture was further diluted with water containing commercially available protease enzymes at different concentrations (0.1%, 0.2%, and 0.3% w / v), employing optimal conditions for enzymatic activity. The hydrolysis treatment was conducted for a duration of 3 hours, maintaining a constant temperature using a water bath (Boss Scientific, Thailand). Following the hydrolysis process, the solutions were carefully transferred into sealed containers and stored under refrigeration conditions at 2 - 6 °C to ensure sample preservation for subsequent analysis.

Table 8 Viscosity properties of EWH

Sample	Viscosity (cP)
Initial	17.60 ± 0.00
Control SDAY10	UD
0.1% SDAY10	UD
0.2% SDAY10	167.27 ± 1.22^{aA}
0.3% SDAY10	102.83 ± 1.29^{bA}
Control PC10F	UD
0.1% PC10F	108.13 ± 0.07^{a}
0.2% PC10F	46.33 ± 0.24^{bB}
0.3% PC10F	38.67 ± 0.47^{cB}
Control P6SD	UD
0.1% P6SD	156.33 ± 0.33^{aB}
0.2% P6SD	22.00 ± 0.00^{bC}
0.3% P6SD	12.00 ± 0.00^{cC}

Different lowercase letters (a - c) indicate significant difference ($P < 0.05$) within the same enzyme group but with different enzyme concentrations.

Different uppercase letters (A - C) indicate significant difference ($P < 0.05$) different enzymes at the same concentration.

Initial = 50 EWP : 50 Water (v/v)

UD = Undetectable values (due to exceeding the measurement range) using spindle No. 02 at 100 rpm speed, cP = A unit of measurement for viscosity equivalent to one - hundredth of a poise.

The result shown in Table 8 Demonstrated the viscosity properties of EWH, the enzyme concentration has a significant impact on the viscosity of the EWH samples. For each enzyme group, as the enzyme concentration increased (from 0.1% to 0.3%), the viscosity generally decreases from UD to 102.83 ± 1.29 cP, 108.13 ± 0.07 to 38.67 ± 0.47 cP and 156.33 ± 0.33 to 12.00 ± 0.00 cP in EW group treated with enzyme SDAY10, PC10F and P6SD respectively. UD or undetectable values, can occur when the measured parameter exceeds the measurement range of a particular instrument or technique. In the case of viscosity measurements using a viscometer with spindle No. 02 at a speed of 100 rpm, UD values may arise when attempting to measure the viscosity of an egg white solution untreated with an enzyme and low enzyme concentration. Moreover, when examining the different enzymes at the same concentration could affect statistically significant differences in viscosity for the EWH samples. This might indicate that the choice of enzyme could have a notable impact

on the viscosity properties of the material, even when the enzyme concentrations are identical.

4.1.2 Color characteristics in EWH

Color is an important parameter in the development of any beverage, as the base color affects the final product's color. L* represents the lightness of the sample, with higher values indicating a brighter or lighter color. In the table, the result could explain that as the concentration of the enzyme increases, the L* values generally decrease for two enzyme groups (PC10F, and P6SD) from 79.20 ± 0.16 to 69.63 ± 0.08 and 78.92 ± 0.16 to 57.10 ± 0.44 respectively. Conversely, the L* values generally increase form 79.28 ± 0.08 to 84.66 ± 0.03 in EWP treated with enzyme SDAY10.

The a* parameter represents the red - green axis, with positive values indicating a more reddish color, and negative values indicating a more greenish color. Looking at the table, we can see that the a* values vary depending on the enzyme group and concentration, in the SDAY10, PC10F and P6SD group, as the enzyme concentration increases from 0.1% to 0.3%, the a* values shift from - 3.92 ± 0.01 to - 2.77 ± 0.02, - 3.91 ± 0.03 to - 3.48 ± 0.01 and - 3.99 ± 0.03 to 3.13 ± 0.01 respectively. This indicates a shift towards a less greenish color as the concentration increases.

The b* parameter represents the yellow - blue axis, with positive values indicating a more yellow color, and negative values indicating a more blue color. The

result indicated that the b* values increased from 2.11 ± 0.01 to 6.62 ± 0.04, 2.16 ± 0.01 to 4.44 ± 0.10 and 2.13 ± 0.04 to 7.58 ± 0.06 in the SDAY10, PC10F and P6SD group respectively. This implies a shift towards a more yellow color with increasing enzyme concentration.

Table 9 Color characteristics in EWH

Sample	L*	a*	b*
SDAY10 control	79.28 ± 0.08[a]	- 3.92 ± 0.01[a]	2.11 ± 0.01[a]
SDAY10 0.1%	83.61 ± 0.01[b]	- 3.13 ± 0.00[b]	5.07 ± 0.01[b]
SDAY10 0.2%	83.95 ± 0.03[c]	- 2.88 ± 0.02[c]	5.57 ± 0.02[c]
SDAY10 0.3%	84.66 ± 0.03[d]	- 2.77 ± 0.02[d]	6.62 ± 0.04[d]
PC10F control	79.20 ± 0.16[a]	- 3.91 ± 0.03[a]	2.16 ± 0.01[a]
PC10F 0.1%	78.77 ± 0.04[b]	- 2.94 ± 0.02[b]	6.19 ± 0.06[b]
PC10F 0.2%	73.35 ± 0.03[c]	- 3.61 ± 0.03[c]	6.26 ± 0.04[b]
PC10F 0.3%	69.63 ± 0.08[d]	- 3.48 ± 0.01[d]	4.44 ± 0.10[c]
P6SD control	78.92 ± 0.16[a]	- 3.99 ± 0.03[a]	2.13 ± 0.04[a]
P6SD 0.1%	74.80 ± 2.01[b]	- 3.01 ± 0.00[b]	6.13 ± 0.04[b]
P6SD 0.2%	68.44 ± 0.13[c]	- 2.85 ± 0.03[c]	9.05 ± 0.03[c]

P6SD 0.3%	57.10 ± 0.44^{d}	- 3.13 ± 0.01^{c}	7.58 ± 0.06^{d}

Different lowercase letters (a - d) indicate significant difference ($P < 0.05$) within the same enzyme group but with different concentrations.

4.1.3 pH

The pH results presented in Table 10 demonstrate the pH changes observed during the hydrolysis process of EW at various time points and enzyme concentrations. The samples were treated with different enzymes, namely SDAY10, PC10F, and P6SD, along with corresponding control samples. The initial pH values of the control samples ranged from 9.00 ± 0.00, 8.90 ± 0.03 and 7.06 ± 0.01 respectively. From this result indicated to the specific optimal pH conditions required for each enzyme used in hydrolysis.

When comparing the same enzyme group at each time point but with different concentrations, the pH results showed significant differences in acidity levels. Within the SDAY10, PC10F and P6SD enzyme group at the 1 - hour time point, the pH values for the 0.1% to 0.3% concentrations were 8.46 ± 0.03 to 6.70 6.70 ± 0.03, 8.25 ± 0.01 to 6.35 ± 0.11 and 7.05 ± 0.00 to 6.27 ± 0.01 respectively. This could indicate that the enzyme concentration increased, the pH decreased, reflecting a higher level of acidity. Within the PC10F enzyme group at the 1 - hour time point,

Observing the enzyme group with a fixed concentration across different time from 1 hour to 3 hour intervals, the result could determine that the pH values decrease

as the hydrolysis progresses within all enzymes group. these results demonstrate that as the hydrolysis process extends over time, the pH of the reaction mixture tends to decrease. This decline in pH could explain the accumulation of acidic products resulting from the breakdown of proteins into smaller peptides and amino acids.

Table 10 pH determination during hydrolysis at optimal temperature and pH

Sample	Initial pH of EWP solution	1 Hour	2 Hour	3 Hour	Inactivated at 3 hours
Control SDAY10	9.00 ± 0.00^{A}	8.46 ± 0.03^{aBC}	8.48 ± 0.02^{aB}	8.32 ± 0.02^{aC}	8.50 ± 0.10^{aB}
0.1% SDAY10	8.98 ± 0.03^{A}	7.54 ± 0.08^{bB}	7.32 ± 0.10^{bBC}	7.15 ± 0.16^{bC}	7.36 ± 0.12^{bBC}
0.2% SDAY10	8.97 ± 0.04^{A}	6.77 ± 0.05^{cC}	6.62 ± 0.03^{cDC}	6.57 ± 0.07^{cD}	6.93 ± 0.02^{cB}
0.3% SDAY10	9.00 ± 0.02^{A}	6.70 ± 0.03^{cC}	6.58 ± 0.03^{cD}	6.69 ± 0.04^{cC}	6.93 ± 0.02^{cB}
Control PC10F	8.90 ± 0.03^{A}	8.25 ± 0.01^{aC}	8.12 ± 0.07^{aD}	8.11 ± 0.01^{aD}	8.70 ± 0.00^{aB}
0.1% PC10F	8.89 ± 0.02^{A}	6.72 ± 0.33^{bB}	6.58 ± 0.27^{bB}	6.49 ± 0.17^{bB}	6.96 ± 0.32^{bB}
0.2% PC10F	8.91 ± 0.01^{A}	6.39 ± 0.14^{bC}	6.33 ± 0.12^{bC}	6.35 ± 0.04^{bC}	6.80 ± 0.21^{bB}
0.3% PC10F	8.90 ± 0.02^{A}	6.35 ± 0.11^{bC}	6.24 ± 0.11^{bC}	6.26 ± 0.12^{bC}	6.79 ± 0.17^{bB}
Control P6SD	7.06 ± 0.01^{D}	7.05 ± 0.00^{aD}	7.25 ± 0.01^{aC}	7.37 ± 0.00^{aB}	7.41 ± 0.00^{aA}
0.1% P6SD	7.06 ± 0.00^{A}	6.56 ± 0.00^{bCD}	6.57 ± 0.00^{bC}	6.56 ± 0.00^{bD}	6.73 ± 0.00^{bB}

0.2% P6SD	7.07 ± 0.00^{A}	6.36 ± 0.00^{cB}	6.37 ± 0.00^{cB}	6.37 ± 0.01^{cB}	6.32 ± 0.01^{cC}
0.3% P6SD	7.07 ± 0.01^{A}	6.27 ± 0.01^{dC}	6.32 ± 0.00^{dB}	6.31 ± 0.01^{dBC}	6.18 ± 0.02^{dD}

Mean with different lowercase (a - d) are significant different ($P < 0.05$) within the same enzyme group at each time point.

Mean with different uppercase (A - D) are significant different ($P < 0.05$) during hydrolysis time.

4.1.4 Particle size distribution (PSD) in EWH

The PSD analysis in this study employed one parameters: $D_{4,3}$ (Volume Weighted Mean Diameter) data were express micrometers (μm). The experimental results presented in Table 11. demonstrate the impact of varying enzyme concentrations (specifically SDAY10, P6SD) ranging from 0.1% to 0.3% on the PSD of the EWH samples.

Upon increasing the enzyme concentration from 0.1% to 0.3% for SDAY10 and P6SD treatments, a notable and statistically significant reduction in the Volume Weighted Mean Diameter ($D_{4,3}$) was observed. Specifically, the $D_{4,3}$ values decreased significantly from 27.47 ± 0.80 μm to 19.27 ± 0.15 μm for SDAY10, and from 11.73 ± 0.15 μm to 8.75 ± 0.02 μm for P6SD. The corresponding PSD graphs for SDAY10 and P6SD can be found in Figure 14. and Figure 16. respectively. Conversely, no significant

differences in the PC10F group were observed as the enzyme concentration increased. The PSD graph for PC10F is provided in Figure 15.

Table 11 The distribution of EWH particle sizes after being treated with various enzyme concentrations

PSD (μm)	SDAY10			PC10F			P6SD		
	0.1%	0.2%	0.3%	0.1%	0.2%	0.3%	0.1%	0.2%	0.3%
$D_{4,3}$	27.47 ± 0.80^{a}	9.28 ± 0.04^{b}	19.27 ± 0.15^{c}	7.31 ± 0.42^{ab}	6.95 ± 0.36^{b}	7.98 ± 0.29^{a}	11.73 ± 0.15^{a}	8.80 ± 0.03^{b}	8.75 ± 0.02^{b}

Mean with different lowercase (a - c) are significant different ($P < 0.05$) within the same group at different concentration.

$D_{4,3}$: Volume Weighted Mean Diameter.

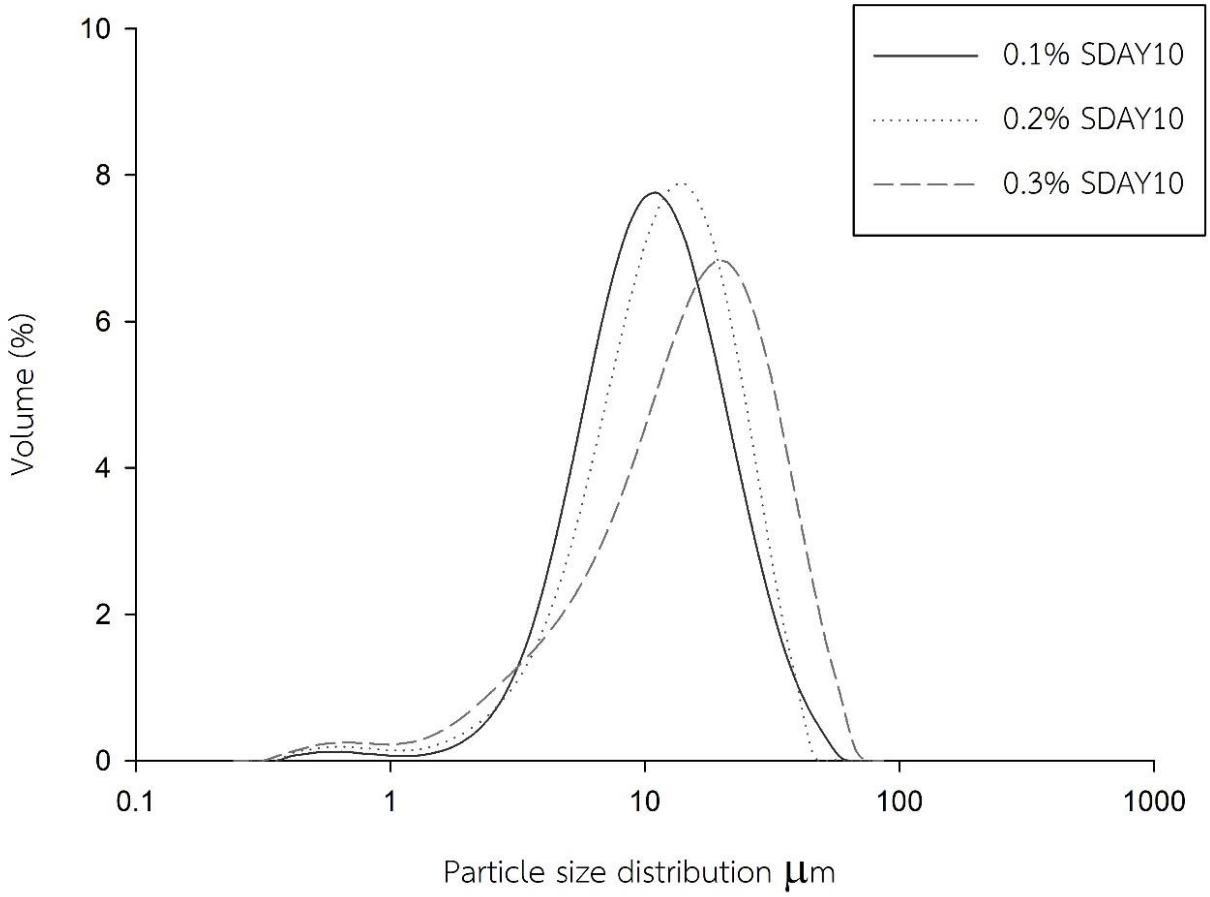


Figure 14 The particle size distribution by volume of EW treated with protease enzymes SDAY10

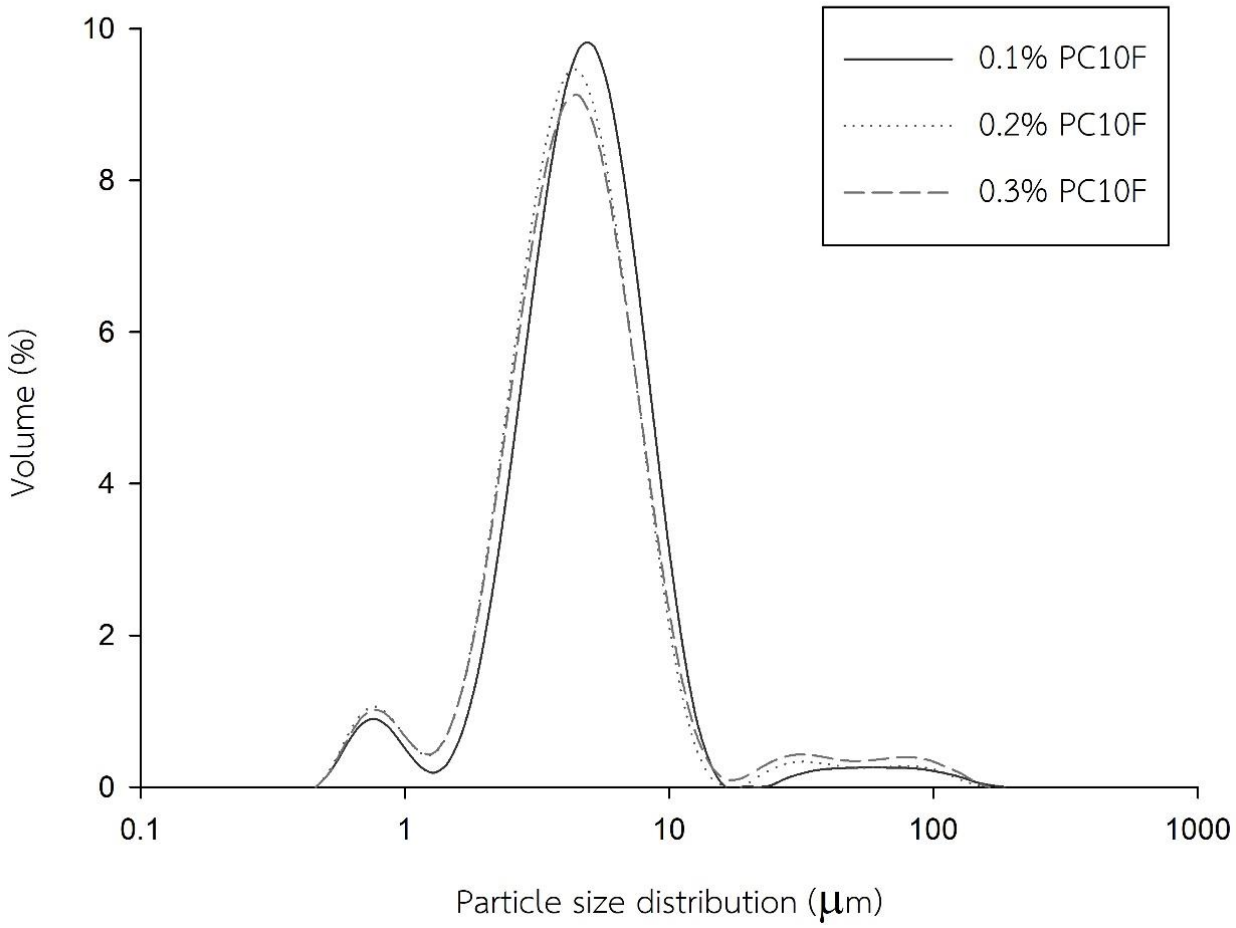


Figure 15 The particle size distribution by volume of EW treated with protease enzymes PC10F

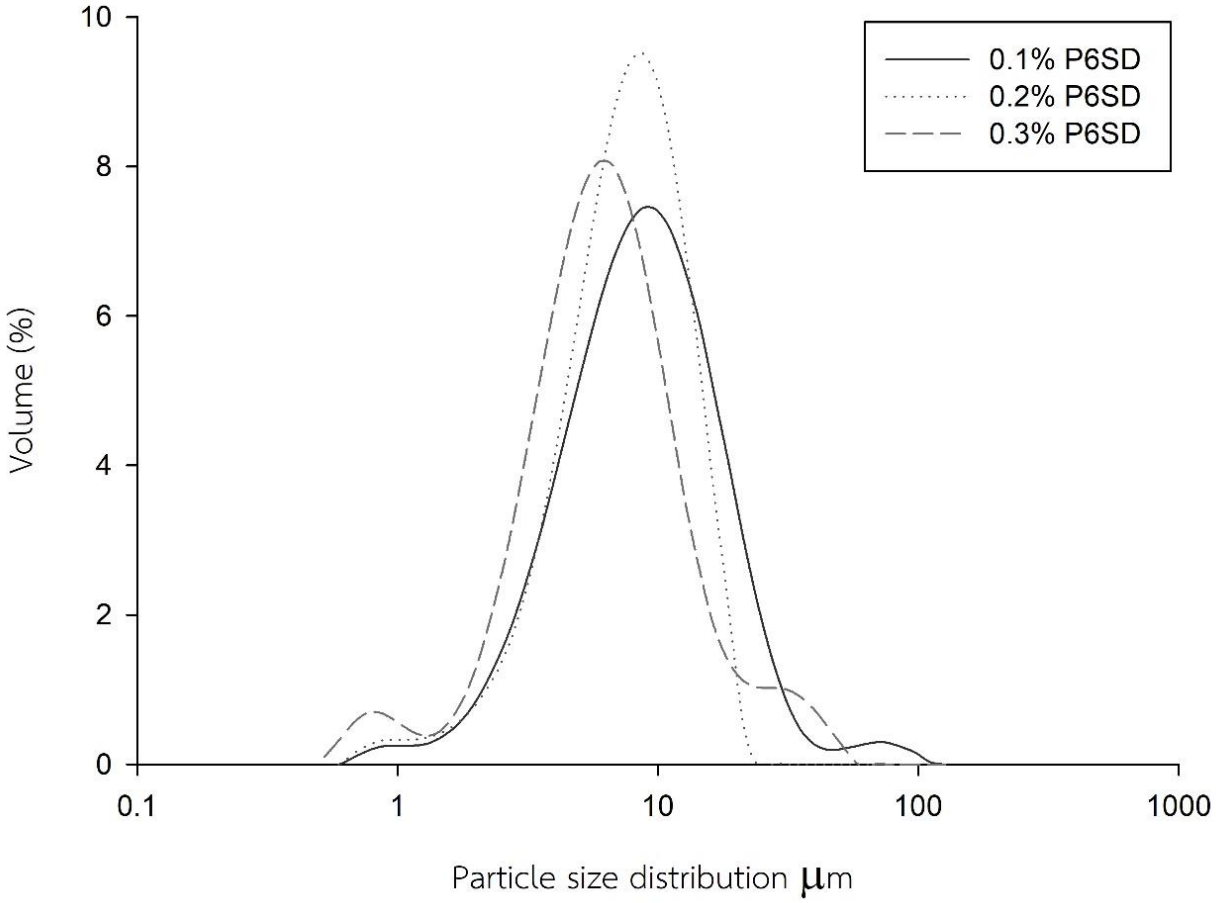


Figure 16 The particle size distribution by volume of EW treated with protease enzymes P6SD

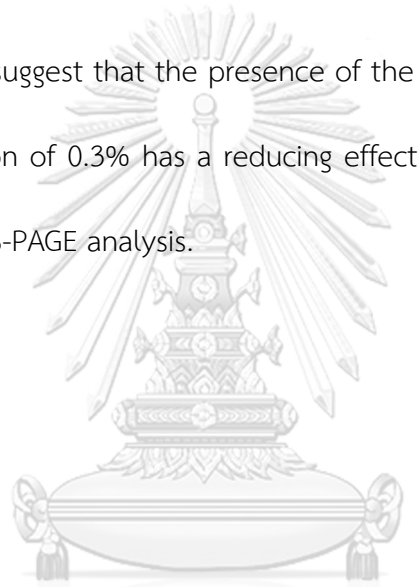
4.1.5 Protein pattern in EWH by SDS - PAGE

Gel filtration is an efficient protocol widely used for separating protein macromolecules, including peptides, based on their molecular weight. The results shown in Figure 17 demonstrate the SDS - PAGE profiles of the control (non - hydrolyzed EWP) in lane B, while lane C, D, and E show the profiles of WEH. Comparing them to the control, the number of protein or peptide bands increased. This observation could be explained by the smaller molecular weight of proteins, indicating a higher success rate of protein cleavage during the hydrolysis process. The results from the SDS - PAGE gel indicate noticeable alterations in the macrostructure of EW protein after treatment. The protein pattern of EWP (non - hydrolysate) revealed molecular weights ranging from 75.37 to 14 kDa, as compared to the marker. Major protein bands were observed with molecular weights of 75 - 37 kDa.

In SDS-PAGE, proteins are separated based on their molecular weight using a polyacrylamide gel matrix. The intensity of a protein band refers to the darkness or brightness of the band, which can be quantified using densitometry. Densitometry measures the intensity of bands on a gel by quantifying the amount of light absorbed or emitted by the dye-stained protein bands. In this study, the band intensity of EWH protein was analyzed using SDS-PAGE. The results, as shown in Table 12, indicate a decrease in band intensity when comparing the control group with three different treatments using different enzyme concentrations.

In the control group, the band intensity of the EWH protein was measured at 25,810.437. However, when treated with enzyme SDAY10 at a concentration of 0.3%, the band intensity decreased to 8,922.007. Similarly, when treated with enzyme PC10F at a concentration of 0.3%, the band intensity decreased from 25,709.960 to 7,467.279. Lastly, treatment with enzyme P6SD at a concentration of 0.3% resulted in a decrease in band intensity from 28,674.002 to 12,135.019.

These findings suggest that the presence of the enzymes SDAY10, PC10F, and P6SD at a concentration of 0.3% has a reducing effect on the band intensity of the EWH protein in the SDS-PAGE analysis.



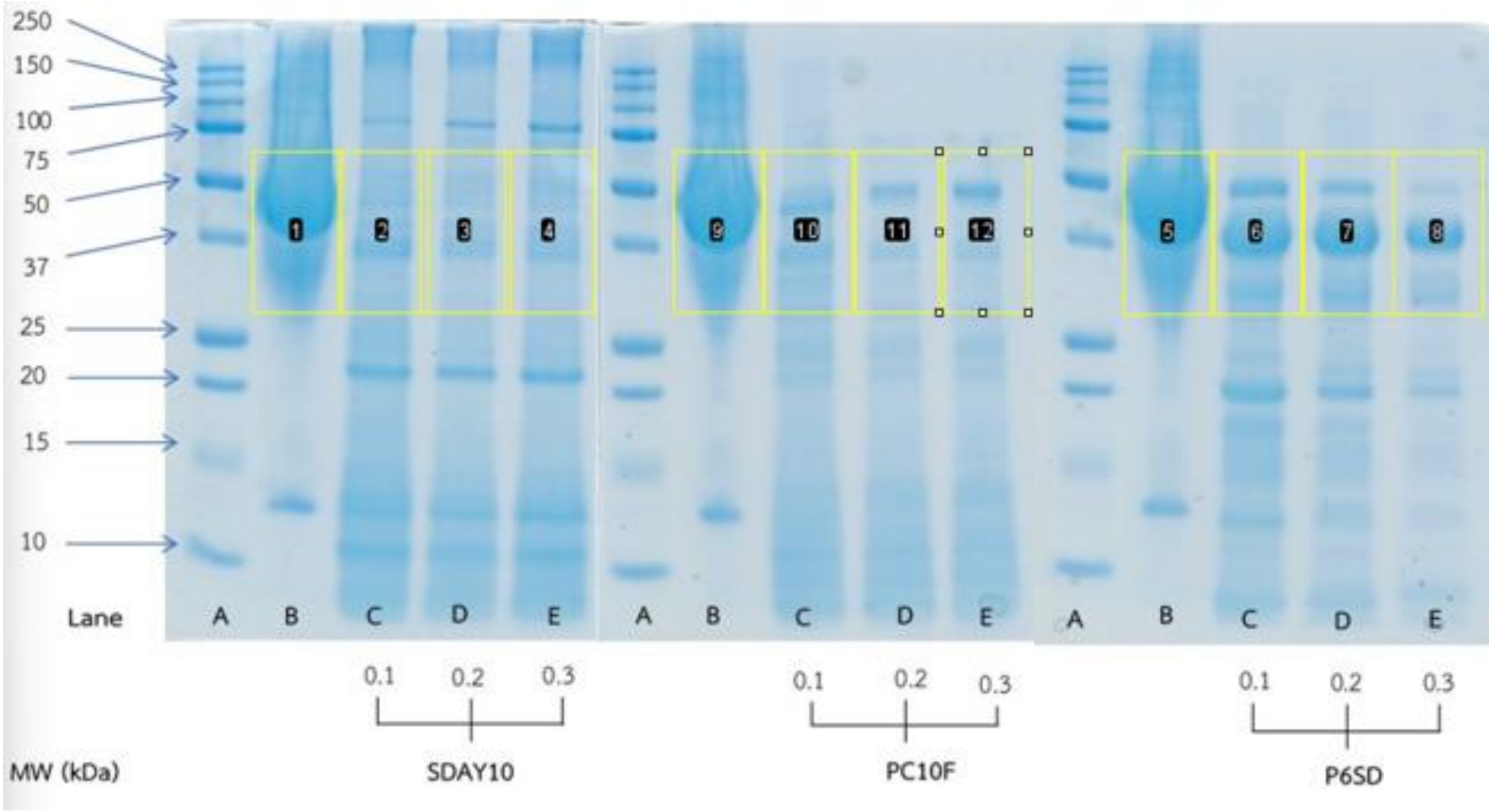


Figure 17 SDS - PAGE gel of EWH

with and without protease enzyme and protein marker Description: A = Protein marker, B = Control (without protease enzyme), C = Substitution 0.1% enzyme, D = Substitution 0.2% enzyme, E = Substitution 0.3% enzyme

Table 12 Peak area of several protein bands EWH with and without the addition of protease enzyme SDAY10 (Protein band density)

MW 50 - 37	Formula (% of protease enzyme in EWH) Area			
kDa.	Control	0.1%	0.2%	0.3%
SDAY10	25,810.437	7,792.907	8,635.806	8,922.007
PC10F	25,709.960	7,962.685	6,833.744	7,467.279
P6SD	28,674.002	18,649.810	16,431.253	12,135.019

kDa = kilodalton, MW = Molecular Weight

4.1.6 Sensory analysis

To evaluate the acceptability of a high protein beverage (EWH) treated with different enzymes, a sensory evaluation was conducted at Chulalongkorn University in sensory evaluation rooms (room 211) on the 2nd floor of the Chulapat 14 building. The evaluation involved panelists who received three EWH samples: one treated with the SDAY10 enzyme, another treated with the PC10F enzyme, and the third treated with the P6SD enzyme. The sensory evaluation took place in a controlled environment consisting of eight individual sensory booths. Panelists were instructed to rate the samples on a scale of 1 to 9 for various attributes, including appearance, color, flavor, taste, sweetness, bitterness, viscosity, aftertaste, and overall acceptability.

The sensory evaluation results demonstrated that the group treated with the SDAY10 enzyme consistently received significantly higher scores in terms of

appearance, color, taste, and sweetness compared to the other groups ($P < 0.05$). These findings indicate that the SDAY10 enzyme treatment positively influenced these attributes, making the beverage more visually appealing, flavorful, and sweet.

However, there were no significant differences in overall acceptability among all the groups ($P < 0.05$). Despite the score in specific sensory aspects, the participant's overall perception and acceptance of the beverage did not vary significantly between the groups. This suggests that factors beyond appearance, color, taste, and sweetness contribute to the overall acceptability of the product.

Based on the valuable feedback received during the sensory evaluation, the panelists recommended reducing the concentration of an ingredient referred to as "EW" to improve the acceptability of the final product and make it easier to consume. Taking this into consideration, the researchers decided to decrease the concentration of EW from 50% to 30% for further analysis and enhancement of the beverage.

Furthermore, when focusing specifically on the taste preference scores, the group treated with the SDAY10 enzyme significantly received a higher average score (6.35 ± 1.95) compared to the PC10F group (4.90 ± 2.38). However, there were no significant differences in viscosity between the two groups. This indicates that the SDAY10 enzyme treatment specifically improved the taste preference of the beverage, while viscosity remained unaffected.

Overall, the findings suggest that the SDAY10 enzyme treatment positively influenced the sensory attributes of appearance, color, taste, and sweetness, leading

to improved taste preference. However, for the overall acceptability of the beverage, factors other than these specific sensory characteristics played a significant role. By considering the panelists' recommendations and reducing the concentration of the EW ingredient, the researchers aim to further enhance the product's acceptability and overall consumer satisfaction.

Table 13 9 - point hedonic and scales as a ready to drink high protein beverage from EWH

Attributes	SDAY - 10	PC10F	P6SD
Appearance	8.45 ± 0.60[a]	7.60 ± 1.40[b]	-
Color	8.45 ± 0.60[a]	7.45 ± 1.53[b]	-
Flavor	7.40 ± 1.90	7.10 ± 1.97	-
Taste	6.35 ± 1.95[a]	4.90 ± 2.38[b]	-
Sweetness	7.85 ± 1.42[a]	6.40 ± 2.30[b]	-
Bitterness	6.65 ± 2.48	5.55 ± 2.84	-
Viscosity	7.75 ± 1.55	6.90 ± 1.55	-
After taste	6.45 ± 2.19	5.35 ± 2.48	-
Overall	5.85 ± 2.32	5.35 ± 2.73	-

Mean with different lowercase (a - b) are significant different ($P < 0.05$).

4.2 Physicochemical and functional characteristics of Egg White Hydrolysate (EWH)

Based on the previous findings, the enzyme SDAY10 was chosen for further evaluation of its physicochemical and functional characteristics. In order to conduct a comprehensive analysis, the pasteurized egg white (EWP) was assessed at an adjusted concentration 30% : water 70%.

4.2.1 Viscosity determination

The data presented in Table 13. includes two sets of samples with different ratios of EWH to water (50 : 50) and (30 : 70) ratio. Each set consists of control samples without any added enzyme and three samples with varying concentrations (0.1%, 0.2%, and 0.3%) of an enzyme SDAY10. In the 30 : 70 ratio set, the control sample and the samples with 0.1%, 0.2%, and 0.3% SDAY10 exhibited viscosities of 70.27 ± 0.22 cP, 22.00 ± 0.00 cP, 10.00 ± 0.00 cP, and 9.20 ± 0.00 cP, respectively. These results indicate that as the concentration of the SDAY10 enzyme increased, there was a significant decrease in viscosity ($P < 0.05$). Furthermore, when comparing the 30 : 70 ratio to the 50 : 50 ratio of EWH to water at the same enzyme concentration, the results also showed a significant decrease in viscosity ($P < 0.05$).

Table 14 Viscosity of EWH

Sample	Viscosity (cP)
EW : WATER (50 : 50)	
Control SDAY10	UD
0.1% SDAY10	UD
0.2% SDAY10	167.27 ± 1.22^{aA}
0.3% SDAY10	102.83 ± 1.29^{bA}
EW : WATER (30 : 70)	
ControlSDAY10	70.27 ± 0.22^{a}
0.1% SDAY10	22.00 ± 0.00^{b}
0.2% SDAY10	10.00 ± 0.00^{cB}
0.3% SDAY10	9.20 ± 0.00^{dB}

Mean with different lowercase (a - d) are significant different ($P < 0.05$) within the same group at different enzyme concentration.

Mean with different uppercase (A - B) are significant different ($P < 0.05$) among group at same enzyme concentration.

UD : means the unit is over-range (greater than 100%) using spindle no.02, speed 100 rpm

4.2.2 Color determination

The result indicated that higher concentrations of SDAY10 lead to higher L*, a* and b* values, indicating a lighter color, more red more yellow in both group. Furthermore, when we focused on decreased on EW ration. The result showed that the formula contains EW 30% gave the lower L* and b* (light color, less yellow), higher in a* (more red).

Table 15 Color determination

Sample	L*	a*	b*
	EW : WATER (50 : 50)		
SDAY10 control	79.28 ± 0.08^{aA}	$- 3.92 \pm 0.01^{aA}$	2.11 ± 0.01^{aA}
SDAY10 0.1%	83.61 ± 0.01^{bA}	$- 3.13 \pm 0.00^{bA}$	5.07 ± 0.01^{bA}
SDAY10 0.2%	83.95 ± 0.03^{cA}	$- 2.88 \pm 0.02^{cA}$	5.57 ± 0.02^{cA}
SDAY10 0.3%	84.66 ± 0.03^{dA}	$- 2.77 \pm 0.02^{dA}$	6.62 ± 0.04^{dA}
	EW : WATER (30 : 70)		
SDAY10 control	71.66 ± 0.01^{aB}	$- 3.57 \pm 0.02^{aB}$	$- 4.99 \pm 0.13^{aB}$
SDAY10 0.1%	75.89 ± 0.10^{bB}	$- 2.98 \pm 0.01^{bB}$	$- 0.68 \pm 0.02^{bB}$
SDAY10 0.2%	76.83 ± 0.02^{cB}	$- 2.76 \pm 0.02^{cB}$	0.37 ± 0.02^{cB}
SDAY10 0.3%	76.54 ± 0.02^{dB}	$- 2.62 \pm 0.02^{dB}$	0.71 ± 0.03^{dB}

Different lowercase letters (a - d) indicate significant difference ($P < 0.05$) within the same group but with different concentrations.

Different uppercase letters (A - B) indicate significant difference ($P < 0.05$) among group but with same concentrations.

4.2.3 pH determination during hydrolysis at optimal temperature and pH

Table 15. presents pH measurements taken at different time points during hydrolysis experiments conducted at the optimal temperature and pH. The objective was to investigate how hydrolysis time and pH changes affect the concentration of the enzyme SDAY10. The experiments utilized three different enzyme concentrations (0.1%, 0.2%, and 0.3%) and were divided into two sections based on the composition of the solvent EW : WATER (50:50) and EW : WATER (30 : 70).

In both solvent compositions, it was observed that as the hydrolysis time increased, there was a significant decrease in the pH of the samples ($P < 0.05$). This indicates that the hydrolysis process caused acidification of the solutions.

When comparing the pH values at the 3 - hour mark for the different concentrations (Control, 0.1%, 0.2%, and 0.3%), a notable difference was observed between the two solvent compositions. In the EW : WATER (30 : 70) composition, the pH values were 7.94 ± 0.00, 6.60 ± 0.00, 6.50 ± 0.00, and 6.46 ± 0.00 for the respective concentrations. On the other hand, in the EW : WATER (50 : 50) composition, the pH values were 8.50 ± 0.10, 7.36 ± 0.12, 6.93 ± 0.02, and 6.93 ± 0.02.

Therefore, the results indicate that at the 3 - hour mark, the EW : WATER (30 : 70) composition exhibited lower pH values compared to the EW : WATER (50 : 50)

composition. This suggests that the hydrolysis process in the EW: WATER (30 : 70) composition resulted in a more pronounced acidification effect.

Table 16 pH determination during hydrolysis at optimal temperature and pH

Sample	Initial	1 Hour	2 Hour	3 Hour	Inactivated at 3 hours
			EW : WATER (50 : 50)		
ControlSDAY10	9.00 ± 0.00^{A}	8.46 ± 0.03^{aBC}	8.48 ± 0.02^{aB}	8.32 ± 0.02^{aC}	8.50 ± 0.10^{aB}
0.1% SDAY10	8.98 ± 0.03^{A}	7.54 ± 0.08^{bB}	7.32 ± 0.10^{bBC}	7.15 ± 0.16^{bC}	7.36 ± 0.12^{bBC}
0.2% SDAY10	8.97 ± 0.04^{A}	6.77 ± 0.05^{cC}	6.62 ± 0.03^{cDC}	6.57 ± 0.07^{cD}	6.93 ± 0.02^{cB}
0.3% SDAY10	9.00 ± 0.02^{A}	6.70 ± 0.03^{cC}	6.58 ± 0.03^{cD}	6.69 ± 0.04^{cC}	6.93 ± 0.02^{cB}
			EW : WATER (30 : 70)		
ControlSDAY10	8.95 ± 0.00^{A}	8.30 ± 0.00^{aB}	8.28 ± 0.00^{aC}	8.25 ± 0.00^{aD}	7.94 ± 0.00^{E}
0.1% SDAY10	8.95 ± 0.00^{A}	6.91 ± 0.00^{bB}	6.77 ± 0.00^{bC}	6.67 ± 0.00^{bD}	6.60 ± 0.00^{E}
0.2% SDAY10	8.95 ± 0.00^{A}	6.71 ± 0.00^{cB}	6.65 ± 0.00^{cCD}	6.45 ± 0.11^{cD}	6.50 ± 0.00^{CD}
0.3% SDAY10	8.95 ± 0.00^{A}	6.63 ± 0.00^{dB}	6.59 ± 0.00^{cC}	6.52 ± 0.00^{bcD}	6.46 ± 0.00^{E}

Values with the different superscript letters (uppercase) within each row are significant different ($P < 0.05$) ($n = 3$) (To investigated the effect of hydrolysis time with pH changing in each enzyme concentration) To examine how hydrolysis time and pH changes affect the concentration of each enzyme.

Values with the different superscript letters (Lowercase) within each column are significant different ($P < 0.05$) ($n = 3$) investigate pH changing with different enzyme concentration at the same time.

4.2.4 Particle size distribution (PSD)

Surprisingly, when we focus on the results of particle size distribution (μm) and compare the two formulas of EW 50% and EW 30%, we found that in the EW 30% group treated with ENZ SDAY10, the particle size distribution (μm) significantly increased from 13.77 ± 0.06 to 19.40 ± 0.00 ($P < 0.05$) when treated with ENZ concentrations ranging from 0.1% to 0.3%. The particle size distribution (μm) of EW 30% treated with ENZ SDAY10 was shown in Figure 18.

Table 17 The distribution of egg white hydrolysate's particle sizes after being treated and left untreated with various enzymes

Particle size distribution	SDAY10 (50 : 50)			
(μm)	Control	0.1%	0.2%	0.3%
[$D_{4,3}$]	ND	27.47 ± 0.80^{a}	9.28 ± 0.04^{b}	19.27 ± 0.15^{c}
Particle size distribution	SDAY10 (30 : 70)			
(μm)	Control	0.1%	0.2%	0.3%
[$D_{4,3}$]	43.07 ± 0.31^{a}	13.77 ± 0.06^{b}	14.77 ± 0.0^{c}	19.40 ± 0.00^{d}

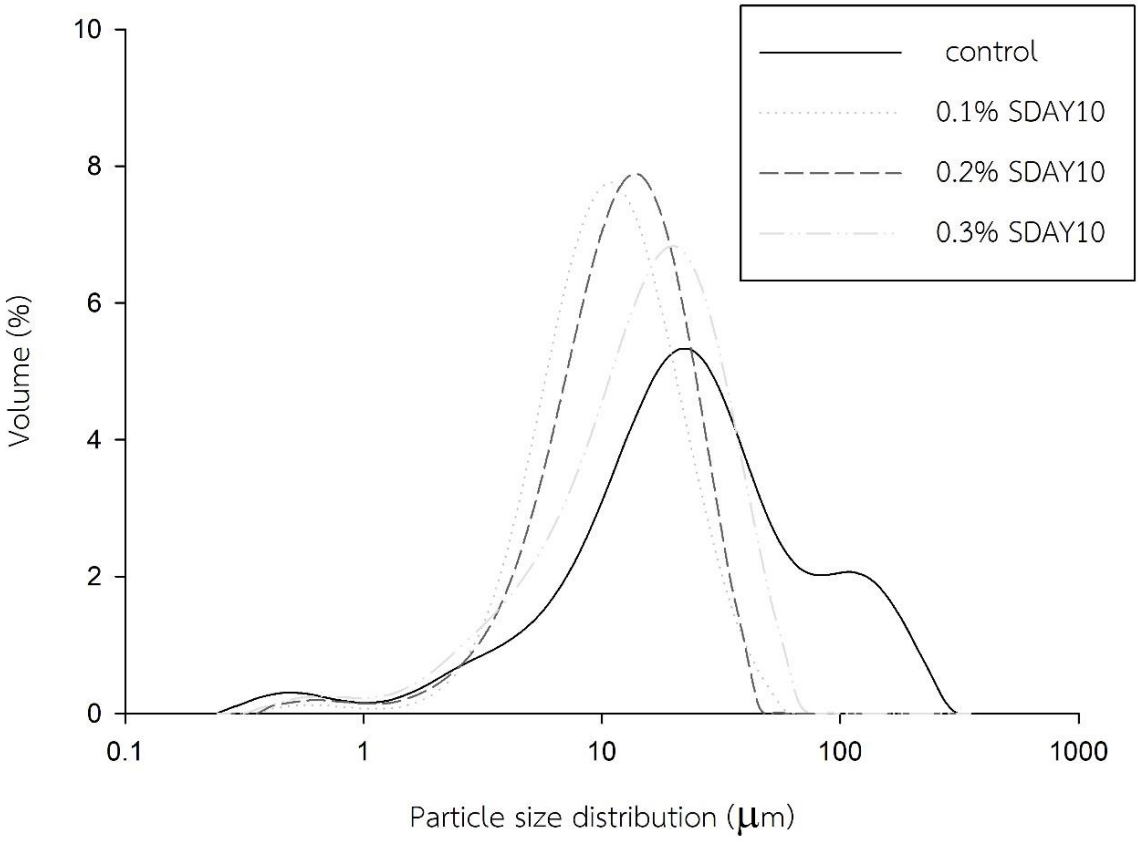


Figure 18 Effect of different type of protease enzymes SDAY10 on particle size distribution curves of EWH beverage

4.2.5 Protein pattern by SDS - PAGE

Figure 19 demonstrates the SDS - PAGE profiles of the control (non - hydrolyzed EWP) in lane B, with the protein marker presented in lane A. In addition, lane C1, D1, and E1 show the profiles of WEH for the formulation of EW 50%, while lane C2, D2, and E2 show the profiles of WEH for the formulation of EW 30%. Comparing them to the control, an increase in the number of protein or peptide bands is observed, along with smaller molecular weights.

This observation can be attributed to the smaller molecular weight of proteins, indicating a higher success rate of protein cleavage during the hydrolysis process. The results from the SDS - PAGE gel reveal noticeable alterations in the macrostructure of EW protein after treatment. Continuing with further analysis of protein band density in the formulation of EW 30%, the results are presented in Table 17. Protein band density, as observed in SDS - PAGE, At molecular weight between 75 - 100 kDa protein band density was significantly decreases from 5,034.610 to 2885.054 and At molecular weight between 20-25 kDa protein band density was significantly decreases from 7,231.004 to 3,159.770 with increasing enzyme concentration 0.1% to 0.3% respectively .

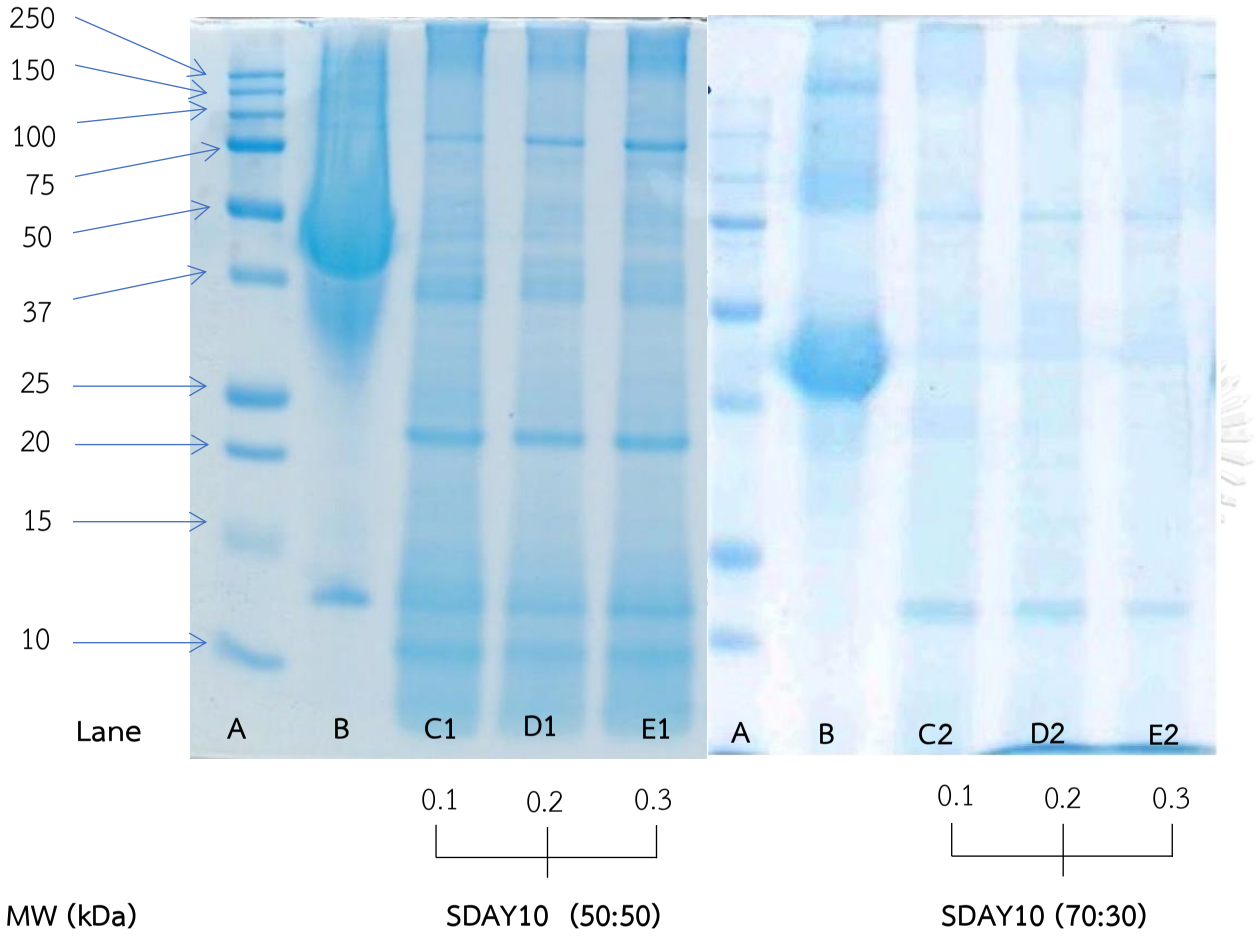


Figure 19 SDS-PAGE gel of EWH with and without protease enzyme (SDAY10) and protein marker

Description: A (Lane 1) = Protein marker, B (Lane 2) = Control (without protease enzyme), C (Lane 3) = Substitution 0.1% protease enzyme, D (Lane 4) = Substitution 0.2% protease enzyme, E (Lane 5) = Substitution 0.3% protease enzyme

Table 18 Peak area of several protein bands EWH with and without the addition of protease enzyme SDAY10 (Protein band density)

Molecular Weight	Formula (% of protease enzyme in EWH)		
	0.1%	0.2%	0.3%
75 - 100 kDa	5,034.610	4,439.891	2,885.054
20 - 25 kDa	7,231.004	6,047.861	3,159.770

4.2.6 Degree of Hydrolysis (DH) in Egg white hydrolysate (EWH)

The experimental findings revealed the impact of varying concentrations of Protease SDAY10 and different time durations on the degree of hydrolysis (DH) in Egg White Hydrolysate (EWH) was showed in table 19. The DH, expressed as a percentage, was assessed to determine the extent of protein breakdown.

At a concentration of 0.1%, the DH values observed were 3.20 ± 0.01% and 3.54 ± 0.00% at 60 and 180 minutes, respectively. These results exhibited a statistically significant increase in DH with an extended reaction time, indicating a progressive protein hydrolysis process. Similarly, at a concentration of 0.2%, the DH values were 3.47 ± 0.02% and 3.56 ± 0.07% at 60 and 180 minutes, respectively. The significant difference observed between these values signifies a notable rise in DH over time.

At the highest concentration tested, 0.3%, the DH values were 3.51 ± 0.02% and 3.59 ± 0.04% at 60 and 180 minutes, respectively. These outcomes demonstrate a statistically significant enhancement in DH as the reaction time increased.

Table 19 Degree of hydrolysis (DH) of EWH

Enzyme	Concentration	DH (%)
Protease SDAY10	0.1%	$3.20 \pm 0.01^{a,A}$
Hydrolysis	0.2%	3.47 ± 0.02^{b}
Time 60 min	0.3%	3.51 ± 0.02^{b}
Protease SDAY10	0.1%	3.54 ± 0.00^{B}
Hydrolysis	0.2%	3.56 ± 0.07
Time 180 min	0.3%	3.59 ± 0.04

Different lowercase letters (a - b) indicate significant difference ($P < 0.05$) within group at different concentrations.

Different lowercase letters (A - B) indicate significant difference ($P < 0.05$) among group at different time point.

4.2.7 EWH Digestion Profile using In Vitro Digestion Model

Ninhydrin Assay

Following an in vitro digestion technique, samples from the gastric and intestinal phases of both EW treated and non - treated with protease enzyme. The invitro protein digestibility was determine using the ninhydrin assay. The ninhydrin - reactive amino nitrogen produced at a certain digestion time point, the in vitro protein digestibility of untreated (control) and treated (test) EW protein was calculated (Figure. 20). The amount of free amino-NH_2 (%) detected in samples. Protein degradation during egg white gastrointestinal digestion was followed by ninhydrin assay (Figure. 20).

The investigation aimed to mimic gastrointestinal digestion and observe the differences in amino acid release in EWH samples treated with different concentrations of enzyme SDAY10 compared to the control (non - treated with enzyme). The results are shown in Figure 20. Based on ninhydrin - reactive free amino - NH_2 acids released during gastric - small intestinal digestion (n = 3), significant differences were observed ($P < 0.05$). The results depicted in Figure 20 show the impact of increasing time points on amino acid release at the same enzyme concentration (Control, 0.1%, 0.2%, and 0.3%). Initially, at 0 minutes, the amino acid release was quantified as 9.80 ± 0.36 mg lysine equivalent for the control, 18.98 ± 2.20 mg for 0.1% SDAY10, 36.60 ± 2.46 mg for 0.2% SDAY10, and 34.27 ± 0.93 mg for 0.3% SDAY10. As the experiment progressed through the gastric and small intestinal phases, there was a notable increase in amino

acid release until the 240 - minute mark, where the values reached 49.58 ± 1.36 mg, 60.35 ± 3.68 mg, 67.34 ± 2.65 mg, and 75.64 ± 6.84 mg lysine equivalent for the respective concentrations.

These findings indicate that, regardless of the enzyme concentration, amino acid release gradually increased over time. Furthermore, focusing on each time point's impact on amino acid release concerning enzyme concentration, it was observed that at the final time point (240 min), the release significantly rose from 49.58 ± 1.36 mg to 75.64 ± 6.84 mg lysine equivalent as the enzyme concentration increased from 0% to 0.3%. This observation suggests that the enzyme concentration of SDAY10 influences amino acid release at each time point, with higher concentrations resulting in a more substantial release compared to lower concentrations (0% and 0.1%). As shown in Figure 21. 0.3% and 0.2% of SDAY10 treated in EWH demonstrated the increasing of AUC for amino acid release (mg lysine equivalent / mL sample).

To summary, this study could provide evidence for the time - dependent increase in amino acid release at a constant enzyme concentration. Additionally, the results demonstrate that higher enzyme concentrations significantly enhance amino acid release compared to lower concentrations. These findings contribute to our understanding of the enzymatic processes involved in amino acid release and their modulation by enzyme concentration and time.

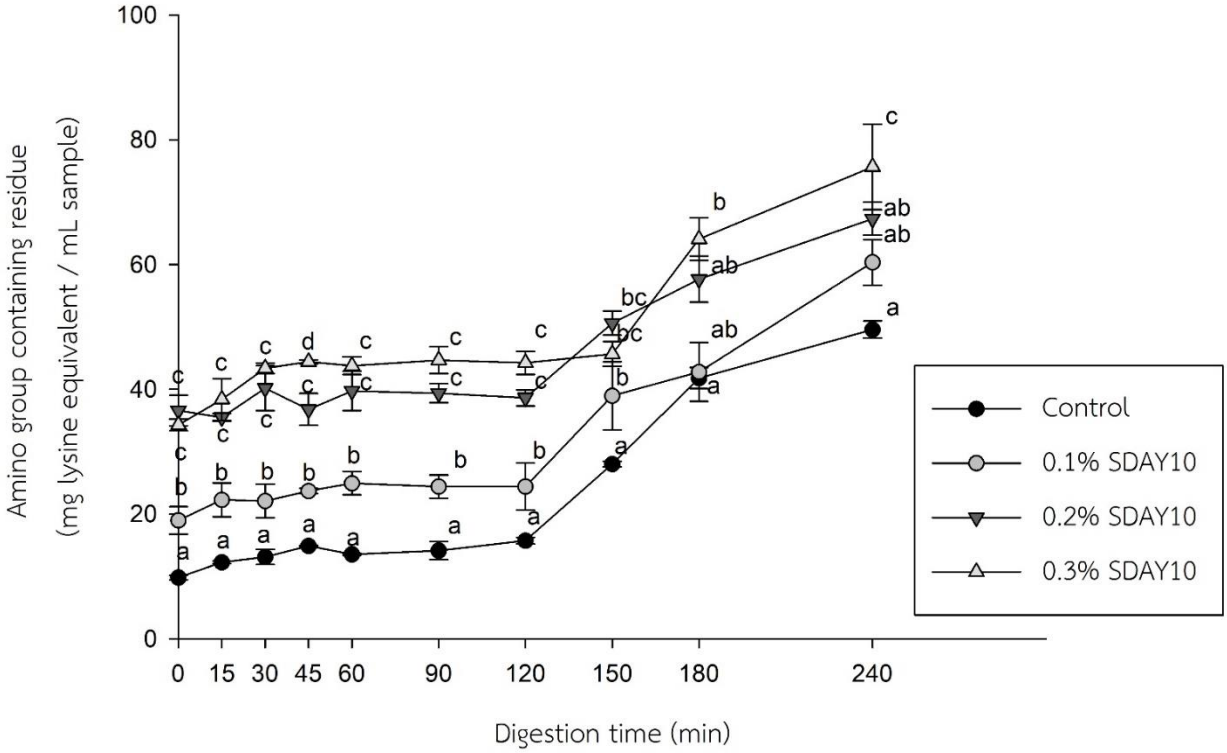


Figure 20 In vitro digestion of Non - EWH (untreated) compared with EWH (treated) determined based on ninhydrin - reactive free amino - NH2 acid released during gastro - small intestinal digestion (n = 3) Different ($P < 0.05$)

Table 20 Amino group containing residues release at different time point

	Egg white hydrolysate sample			
Time (min)	Control	0.1%	0.2%	0.3%
0	9.80 ± 0.36^{a}	18.98 ± 2.20^{b}	36.60 ± 2.46^{c}	34.27 ± 0.93^{c}
15	12.26 ± 0.22^{a}	22.29 ± 2.71^{b}	35.50 ± 0.61^{c}	38.37 ± 3.35^{c}
30	13.12 ± 1.21^{a}	22.09 ± 2.70^{b}	40.17 ± 3.60^{c}	43.36 ± 0.83^{c}
45	14.88 ± 0.10^{a}	23.71 ± 0.42^{b}	36.76 ± 2.58^{c}	44.41 ± 0.30^{d}
60	13.54 ± 0.13^{a}	24.95 ± 1.88^{b}	39.73 ± 3.14^{c}	43.77 ± 1.43^{c}
90	14.14 ± 1.45^{a}	24.39 ± 1.86^{b}	39.36 ± 1.54^{c}	44.68 ± 2.16^{c}
120	15.75 ± 0.54^{a}	24.39 ± 3.78^{b}	38.62 ± 1.28^{c}	44.24 ± 1.85^{c}
150	28.03 ± 0.44^{a}	38.96 ± 5.46^{b}	50.60 ± 1.93^{bc}	45.67 ± 1.97^{bc}
180	41.80 ± 1.69^{a}	42.78 ± 4.71^{ab}	57.66 ± 3.71^{ab}	64.08 ± 3.41^{b}
240	49.58 ± 1.36^{a}	60.35 ± 3.68^{ab}	67.34 ± 2.65^{ab}	75.64 ± 6.84^{c}

Each value is the mean ± SEM (n = 3). Different ($P < 0.05$)

Different lowercase letters (a - d) indicate significant difference ($P < 0.05$) within row

Data were express as mg lysine equivalent

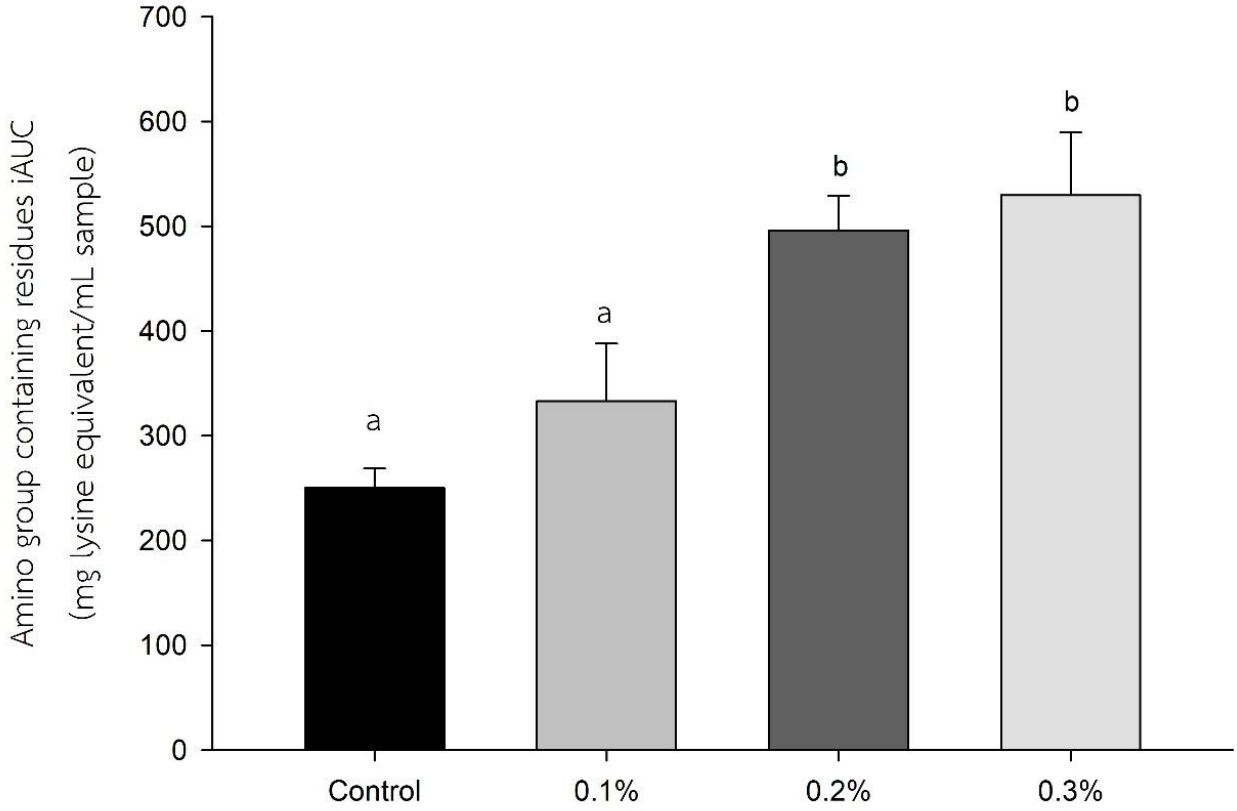


Figure 21 Area under the curve (AUC) for Amino group containing residues Means with different superscripts are significantly different ($P < 0.05$). Control : Non enzyme treated), 0.1% : EW treated with 0.1% SDAY10, 0.2% : EW treated with 0.2% SDAY10, 0.3% EW treated with 0.3% SDAY10

4.2.7 Antioxidant activity of EWH by protease SDAY10

In order to assess the antioxidant potential of EWH samples, the researchers conducted three different assays : ABTS (Trolox Equivalent), DPPH (Ascorbic acid Equivalent), and FRAP (Ferric reducing antioxidant power). These assays are commonly used to measure the radical scavenging capability of compounds.

Figure 22. depicts the results of the DPPH assay, which assesses radical scavenging activity. The findings indicate a significant ($P < 0.05$) increase in radical scavenging activity from 8.07 ± 0.04 to 8.63 ± 0.04 (mg Ascorbic Acid Equivalent / mL sample) when the ENZ concentration is elevated from 0.1% to 0.3%. Therefore, similar to the ABTS assay, higher concentrations of ENZ SDAY10 correlate with enhanced radical scavenging activity as measured by the DPPH assay.

Figure 23. illustrates the results of the ABTS radical scavenging activity. The data revealed a significant ($P < 0.05$) effect with increasing concentrations of ENZ SDAY10. Specifically, when the ENZ concentration was raised from 0.1% to 0.3%, the ABTS radical scavenging activity also increased from 1,752.68 ± 15.20 to 2,046.458 ± 31.60 (mg Trolox Equivalent / mL sample), respectively. This suggests that higher concentrations of ENZ SDAY10 are associated with greater ABTS radical scavenging activity.

Additionally, Figure 24 presents the antioxidant activity of EWH samples treated with 0.1 - 0.3% of SDAY10 enzymes, as determined by the FRAP assay. The data demonstrate that the FRAP values of 0.3% SDAY10 (216.91 ± 0.68) mg / mL were

significantly higher than those of 0.1% SDAY10 (167.22 ± 1.17) mg / mL ($P < 0.05$). This indicates that increasing the concentration of SDAY10 enzymes from 0.1% to 0.3% leads to a significant improvement in the FRAP antioxidant power of the EWH samples.

In summary, the results of these experiments consistently show that higher concentrations of ENZ SDAY10 are associated with increased antioxidant activity in EWH samples, as assessed by the ABTS, DPPH, and FRAP assays. These findings suggest that the ENZ SDAY10 enzymes have a positive impact on the radical scavenging capability and overall antioxidant potential of the EWH samples.

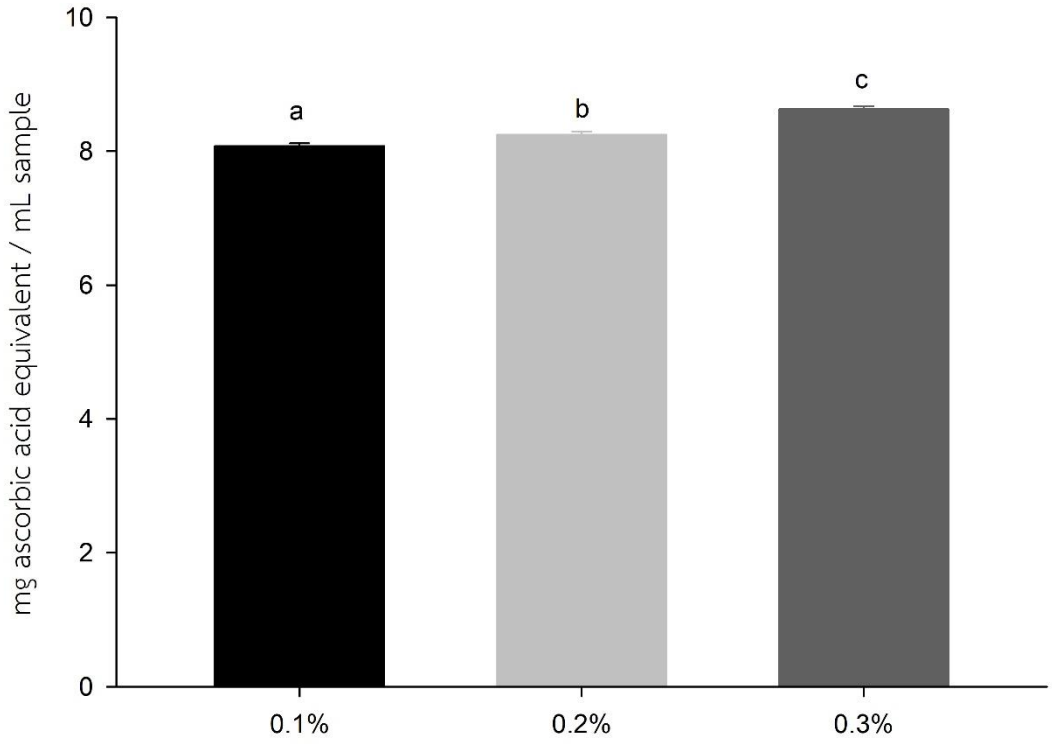


Figure 22 Antioxidant activities of egg white protein hydrolysate (EWPH) with various enzyme (Protease SDAY 10) concentration for DPPH radical.
Different letters (a - c) indicate that the samples were considered significantly different at $P < 0.05$ ($n = 3$)

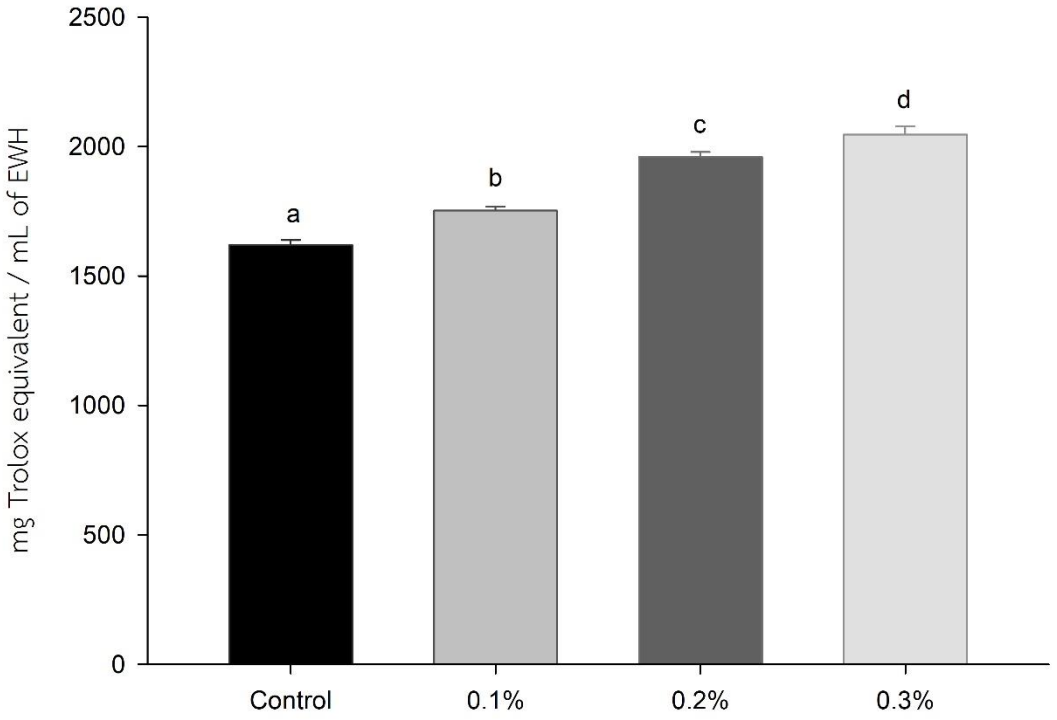


Figure 23 Antioxidant activities of egg white protein hydrolysate (EWPH) with various enzyme (Protease SDAY10) concentration for the ABTS radical.

Different letters (a - d) indicate that the samples were considered significantly different at $P < 0.05$ ($n = 3$)

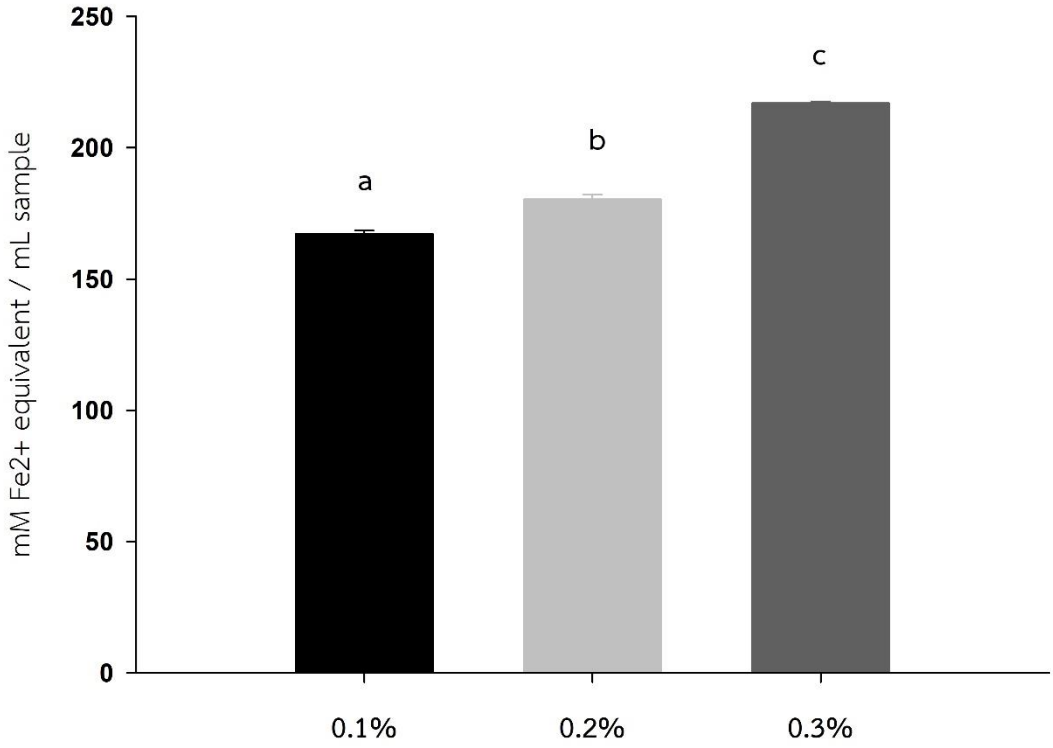


Figure 24 Antioxidant activities of egg white protein hydrolysate (EWPH) with various enzyme (Protease SDAY10) concentration for reducing power activity
Different letters (a - c) indicate that the samples were considered significantly different at $P < 0.05$ ($n = 3$)

In order to determine the optimal formulation for the high - protein beverage made from hydrolyzed egg white (EWH), the panelists were provided with two samples of EWH treated with SDAY10 using different formulations. The sensory evaluation took place in sensory evaluation rooms located in Room 211 of the Chulapat 14 building, 2nd floor, at Chulalongkorn University. The evaluation was conducted in a sensory testing room equipped with eight individual sensory booths. During the evaluation, the panelists rated various attributes of the samples using a 9 - point hedonic scale. The attributes included appearance, color, flavor, taste, sweetness, bitterness, viscosity, aftertaste, and overall acceptability. Each attribute was scored on a scale ranging from 1 to 9.

The choice of the protease ENZ SDAY10 at a concentration of 0.3% was based on the physicochemical results, specifically the highest antioxidant activity observed. This choice of enzyme was presumably made because higher antioxidant activity is generally desirable due to its potential health benefits.

The results of the sensory evaluation, as shown in Table 19, indicated that there were no significant differences ($P < 0.05$) in the attributes of appearance, color, flavor, taste, sweetness, bitterness, viscosity, and aftertaste between the two groups of samples. This suggests that the different formulations did not have a significant impact on these sensory attributes.

However, when considering the overall acceptability of the samples, the group with a formulation ratio of 70 : 30 (presumably referring to the proportions of EWH

treated with SDAY10) received the highest score of 7.80 ± 1.32. This score was significantly different ($P < 0.05$) compared to the group with a formulation ratio of 50 : 50. This indicates that the formulation ratio had a significant influence on the overall acceptability of the high - protein beverage.

Table 21 9 - point hedonic and scales as a ready to drink high protein beverage from hydrolyzed egg white.

Attributes	50:50	70:30	P value
Appearance	8.45 ± 0.60	8.20 ± 0.95	<0.05
Color	8.45 ± 0.60	8.30 ± 0.98	<0.05
Flavor	7.40 ± 1.90	7.80 ± 1.28	<0.05
Taste	6.35 ± 1.95	7.90 ± 1.25	<0.05
Sweetness	7.85 ± 1.42	8.10 ± 1.17	<0.05
Bitterness	6.65 ± 2.48	7.75 ± 1.16	<0.05
Viscosity	7.75 ± 1.55	7.25 ± 1.89	<0.05
After taste	6.45 ± 2.19	7.35 ± 1.60	<0.05
Overall	5.85 ± 2.32[a]	7.80 ± 1.32[b]	<0.05

Different lowercase letters (a - b) indicate significant difference ($P < 0.05$)

CHAPTER V

DISCUSSION

The physical characteristics (viscosity, color properties, pH and volume mean diameter ($D_{4,3}$) of pasteurized egg white (EWP) after being treated with enzymes were investigated. It was found that the viscosity of untreated EWP (control) showed an excessively high value when investigated with the same spindle and speed. However, an increase in enzyme concentration from 0.1% to 0.3% significantly decreased the viscosity in all sample groups treated with ENZ SDAY10, PC10F, and P6SD. Furthermore, when we adjusted the ratio of EWP and water from 50 : 50 to 30 : 70, the results also correlated with the previous investigation regarding the reduction in viscosity. The reduction in viscosity can be attributed to the change in EW protein structure caused by enzymatic hydrolysis, leading to a decrease in their molecular mass and an increase in protein solubility (Jung *et al*., 2006). This result is consistent with a previous study on soy protein hydrolysates, which demonstrated that a higher degree of hydrolysis leads to a decrease in viscosity (Lamsal *et al*., 2007).

The results of the Hunter L*, a*, and b* values, corresponding to lightness, redness, and yellowness, respectively, showed variations depending on the addition of different enzymes. The optimum temperature during the hydrolysis process affected the color of EW. The EWP treated with P6SD and PC10F enzymes significantly decreased L*, while SDAY10 significantly increased L*. However, when focusing on a*

and b*, all three enzyme groups showed a significant increase in a* and b* compared to untreated EW (control) ($P < 0.05$). These finding could explain by the heat effect on protein which is ovotransferrin, ovotransferrin is one of the most heat - sensitive egg white proteins, undergoes structural changes during the cooking process. Initially, the proteins are folded into compact globular structures held together by chemical bonds. However, during cooking, these proteins unfold, and water is dispersed within the protein matrix. As a result, the egg white protein transforms into a semi - solid state, which directly affects the color of the EW (Payawal *et al*., 1946). This can also explain how the choice of protease enzyme may influence the final color of EWH.

The pH investigation of EWH treated with enzyme SDAY10, PC10F and P6SD both water to EW ratio (50 : 50), (70 : 30). The different of initial pH between group because of its different optimal pH condition for specific enzymes used for hydrolysis. After 3 hours of hydrolysis with optimal condition, the result demonstrated that pH values of EW treated group were significantly decreased when compared to initial point in both enzymes. Furthermore, the reduction of pH was increased when the concentration of the enzyme was increased ($P < 0.05$). The previous study showed that the pH of dietary protein drop during in vitro digestibility correlated with pH reduction and protein hydrolysis (Butts et al., 2012). The responsible for pH reduction is the peptide bonds cleavage which lead to the reduction in pH value. Overall, the results demonstrate that the duration of the hydrolysis process has a notable effect on the pH drop. Prolonged hydrolysis could lead to a more substantial decrease in pH,

reflecting the accumulation of acidic products as the proteins are further broken down. This information is crucial for optimizing the hydrolysis process and understanding the kinetics of egg white hydrolysis.

The quality and safety of products in the food and beverage industry have a direct impact on their performance. Additional concerns include organoleptic quality, shelf life, stability, process stability, and quality control. When developing functional food products from EW protein, particle size plays an important role in the emulsifying properties of the protein, affecting stability, texture, and appearance (Fernandez et al., 2004). The previous study found that the particle size in beverage samples had increased after pass through the high thermal process because the denaturation and coagulation of protein in beverage (Liu et al., 2016). To address this issue, a hydrolysis process was employed to examine the impact of enzymes on the particle size distribution (PSD) of egg white protein (EWP). The study investigated the PSD curve of EWP treated with different types of enzymes, namely SDAY10, PC10F, and P6SD, at various concentrations. The results, depicted in Figures 14 - 16, indicated that the particle size of EWP decreased as the enzyme concentration increased (with a significance level of ($P < 0.05$). This finding is in line with previous research that demonstrated the influence of enzyme concentration on particle size distribution. Specifically, higher concentrations of enzymes led to smaller particle sizes. This observation can be associated with the emulsifying property of the treated EWP. Smaller particle sizes in the PSD generally indicate a higher emulsifying capacity,

implying that the enzymatic treatment enhanced the emulsifying properties of the egg white protein.

By effectively reducing the particle size of the treated EWP through higher enzyme concentrations, the enzymatic hydrolysis process likely disrupted the protein structure, resulting in the formation of smaller peptides or protein fragments. These smaller components are more conducive to emulsification, as they possess increased surface area and improved solubility. (Wang *et al*., 2018). As showed in table 11, the higher of enzyme concentration from 0.1 to 0.3% significantly decreased the volume diameter ($D_{4,3}$) from 11.73 ± 0.15 μm to 8.80 ± 0.03 μm in P6SD enzyme treated group ($P < 0.05$) whereas the higher concentration from 0.1% to 0.2% significantly reduced the volume diameter ($D_{4,3}$) from 7.31 ± 0.42 μm to 6.95 ± 0.36 in PC10F treated group and also significantly reduced the volume diameter ($D_{4,3}$) from 27.47 ± 0.82 μm to 9.28 ± 0.04 in SDAY10 treated group. These results suggested that enzymatic hydrolysis can improve emulsifying activity by lower volume mean diameter ($D_{4,3}$) (Chen *et al*., 2011). The data from previous study found that egg white hydrolysate can lead to the higher emulsifying ability and stability when compared to native egg white (Wang *et al*., 2018).

To investigate the changes in the protein molecular profile, the SDS - PAGE method was employed. Based on the previous findings regarding the main proteins present in egg white, it was determined that ovalbumin constitutes approximately 54% of the total protein content. This is followed by ovotransferrin, ovomucoid, lysozyme, which make up approximately 12%, 11%, and 3.5% of the total protein content,

respectively (E. D. Abeyrathne *et al*., 2013). After subjecting the samples to SDS - PAGE and comparing the protein patterns, it was observed that the results were consistent with the previous findings related to protein hydrolysate. Specifically, it was noted that certain main protein bands, such as ovotransferrin and ovomucoid, exhibited a slight decrease or disappearance following the treatment of EWP with enzymes (Yu Wang, 2019). Mover from the previous study, the result also showed the effect of increasing concentration of PC10F enzyme effect on EWP protein band pattern by reducing the protein band area among 29.0 - 44.3 kDa became abundant with some light and thin band (Chang *et al*., 2017).

In this study, the degree of hydrolysis (DH), which is a measure of protein degradation, was examined. A higher DH value indicates a higher level of hydrolysis of egg white protein (EWP). The results revealed that an increase in enzyme concentration had an impact on achieving a higher DH. Specifically, when using 0.3% SDAY10 enzyme, a hydrolysis time of 3 hours resulted in the highest DH compared to lower enzyme concentrations. These findings align with previous research on the hydrolysis of EWP using the enzymes Alcalase and Protamex. The previous studies on Alcalase and Protamex, which are extracted from *Bacillus sp*., also demonstrated comparable results. The highest DH achieved with Alcalase and Protamex was approximately 6%. It is worth noting that SDAY10, Alcalase, and Protamex share the same origin, being derived from Bacillus sp. This similarity in source suggests that SDAY10 may exhibit comparable hydrolytic properties to Alcalase and Protamex (Cho *et al*., 2014). In

conclusion, the present study might indicate that increasing the enzyme concentration can lead to higher DH values. Moreover, using 0.3% SDAY10 enzyme and a hydrolysis time of 3 hours resulted in the highest DH. These findings align with previous research using Alcalase and Protamex, which demonstrated similar DH values around 6%. This study contributes to the existing scientific knowledge and reinforces the coherence between the hydrolysis of EWP with different enzymes of *Bacillus sp*.

To determine the protein digestibility, the release of ninhydrin - reactive amino nitrogen at specific time points was measured (as shown in Figure 20). The ninhydrin assay is commonly used to quantify the amount of free amino acids or peptides produced during protein digestion. These experimental results provide insights into the effect of SDAY10 enzyme treatment on the in vitro protein digestibility of the egg white samples. By comparing the digestibility profiles of the control group and the samples treated with different concentrations of SDAY10, it could assess that the higher enzyme concentration impact on the protein digestion process and release which consist with the previous studied that demonstrated that concentration of amino release during digestion time indicates the amount of peptide bond cleaved (Aspmo *et al*., 2005).

As shown in Figure 22 - 24. EW treated with 0.1 - 0.3% of SDAY10, PC10F and P6SD enzymes demonstrated antioxidant activity. The results showed that DPPH, ABTS and FRAP radical scavenging activity of higher enzyme concentration resulted in higher antioxidant activity. Because of enzymatic hydrolysis of animal or plant proteins is a wildly used method for produced bioactive peptides (Tadesse and Emire, 2020). $ABTS^{+}$

radical scavenging activity is a well - known assay of antioxidant activity and can be applied to both lipophilic and hydrophilic compounds (Miliauskas *et al*., 2004). The increasing in the radical - scavenging activity of EW with various enzymes types and concentrations were found when enzyme concentration increased. When EW protein hydrolyzed with protease enzyme, the generation of lower molecular weight with shorter peptide (tri and dipeptides) as known as bioactive peptide and single amino acid are occurred. The result is related to the previous study of egg white liquid hydrolysate that found an EW treated with protease enzyme yielded bioactive compounds with antioxidant activities (Noh and Suh, 2015). Moreover, the other previous studies have shown that peptide and protein hydrolysate of soy protein, whey protein (Peña-Ramos and Xiong), and egg albumin (Dávalos *et al*., 2004) also increase antioxidant activities. These bioactive peptides from EW protein can positively impact on body functions and human health (Eckert *et al*., 2013) by preventing or scavenging the excessive oxidative stress in human body. However, the yielding of antioxidant activities from protein hydrolysates also depend on many factors such as type of protease enzymes, ratio of enzymes to substrate and optimal hydrolysis condition (Klompong *et al*., 2007).

One significant issue encountered in sensory evaluation when producing egg white hydrolysate is the presence of an undesirable bitter taste, which has a detrimental effect on the overall product score. This bitterness arises due to the generation of specific amino acids and peptides during the hydrolysis process, which

can be quantified using the degree of hydrolysis (DH). The intensity of bitterness experienced is contingent upon both the concentration of enzymes employed and the extent of hydrolysis achieved (Liu *et al*., 2022). Typically, an increase in DH results in an elevation of bitter taste perception. The bitter sensation is often ascribed to the formation of low molecular weight peptides composed predominantly of hydrophobic amino acids. Moreover, for the protein content in final product couldn't claim as rich of protein content due to the regulation of Thai FDA but could claim as good source of protein due to the protein content in final product has approximately 4 grams protein in 100 milliliters.

CHAPTER V
CONCLUSION

The present study examined the impact of enzymatic hydrolysates SDAY10, PC10F, and P6SD on pasteurized egg white (EWP), with a focus on their potential applications in the egg white industry. Our findings demonstrated that varying concentrations (0.1%, 0.2%, and 0.3%) of SDAY10, PC10F, and P6SD enzymes had distinct effects on the physicochemical properties of EWP after treatment.

One notable outcome of this study was the observed reduction in viscosity with increasing enzyme concentration, indicating improved fluidity of the egg white hydrolysate (EWH). Additionally, the application of the SDAY10 enzyme led to a lighter color in the EWH, suggesting potential aesthetic enhancements. Furthermore, the particle size distribution (PSD) was positively influenced by the enzymatic treatment, which could have implications for the properties of the final beverage product during storage.

In addition to the improvements in physical properties, our study also revealed enhanced levels of bioactive ingredients in the treated samples. Specifically, higher levels of released amino acids compared to the control group were observed, indicating increased availability of these essential nutrients. Moreover, the enzymatically treated EWP exhibited antioxidant properties, further highlighting the potential health benefits of the hydrolysates.

Based on these findings, it can be concluded that the SDAY10 enzyme holds promise for improving the physicochemical properties of EWP as a raw material. However, for the development of ready to drink beverages utilizing egg white hydrolysates, further research is necessary to ensure consumer acceptability and optimize product formulations.

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