Assessment of different proteases on degree of hydrolysis, functional properties and radical scavenging activities of salted duck egg white hydrolysate

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Abstract The impact of different proteases (alcalase, ficin, protamex, and neutrase) on the hydrolyzation, foaming and radical scavenging potency of salted duck egg white was studied. Egg white without protease hydrolyzation served as control. Regarding the degree of hydrolysis, neutrase and protamex were more capable of hydrolyzing duck egg white than the others. The α -amino nitrogen content in the egg whites was highly correlated with hydrolysis ability of the enzymes, and it had gradually increased with curing. Foam stability, capacity and emulsion stability were significantly influenced by proteases and curing period. However, the solubility of the egg white hydrolysate was not much affected by curing but showed significant differences between hydrolyzed and control samples. Emulsion stability of hydrolysate decreased with curing times. Neutrase and alcalase hydrolysates gave the best foaming and emulsion properties. Moreover, neutrase and protamex hydrolysates showed good scavenging potency against the DPPH radicals, hydroxyl radicals, hydrogen peroxide, and ferrous ion radicals. Overall, the hydrolysate from neutrase hydrolyzed duck egg white showed the highest foaming and radical scavenging capacities among the other hydrolysis treatments in this study.

Keywords Duck egg white · Salting · Protease · Hydrolysate · Functional properties · Radical scavenging abilities

Introduction

Eggs are inexpensive food materials containing numerous functional and nutritional compounds, especially proteins, vitamins, lipids, minerals among others. The daily consumption of eggs could improve mental and physical health (Ariviani et al. 2018; Fatah et al. 2018). Duck eggs are similarly nutritious as hen eggs and are commonly consumed in many parts of the world, though predominantly in South-East Asia (Venkatachalam 2018). In Thailand, these are usually consumed in cured form. The duck eggs are coated with salted mud paste and are further dipped in charcoal mixed with rice husk, after which they are allowed to cure for a period of 30 days. Normally, salted duck egg yolk is widely used to make various types of snacks, whereas the egg white is usually discarded as it has an extremely salty taste and could adversely impact the consumer's health and may cause the stroke, cardiovascular disease, high blood pressure, stomach cancer, and kidney disease. In addition, the curing also significantly influences the functional properties of egg white. Several studies have attempted to utilize salted duck egg white as a functional ingredient (Chen et al. 2009; Ren et al. 2014; Venkatachalam 2018). Recently, food derived proteins and peptides have spurred high interest as rich sources of bioactive compounds, and they have various therapeutic applications (Jahandideh et al. 2016). Proteins are macromolecules can be broken down into smaller constituents; such processing is done especially with proteases, acids, or heat treatments.

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Proteases commonly hydrolyze peptide bonds in protein molecules, and most proteases cleave the α -peptide bonds between amino acids (Lopez-Otin and Bond 2008). Various protease enzymes can be obtained from numerous sources, and among these, alcalase, neutrase, protamex, flavorzyme, collupulin, and ficin are extensively used to hydrolyze the egg white proteins (Cho et al. 2014; Ren et al. 2014). Moreover, it has been widely reported that protein hydrolysates exhibit stronger antioxidant activities; the protein sources tested include egg white, egg yolk, soybean, canola, whey, fish, and meat (Amarowicz 2008; Cho et al. 2014; Pena-Ramos and Xiong 2003; Raghavan and Kristinsson 2008; Sakanaka and Tachibana 2006). The enzymatic hydrolysis can improve the functional properties (foaming, emulsification and gelling) and biological activities (immunity and antioxidants) of the protein, and furthermore, the hydrolysate can be used as an ingredient in foods and feeds (Pokora et al. 2013). Antioxidants can be of either endogenous and exogenous type and play an important role in protecting against degenerative diseases caused by unstable and highly reactive free radicals. The source of protein, along with the optimum conditions and degree of hydrolysis with protease enzymes, crucially determine the functional properties and the antioxidant activities of the hydrolysate. Numerous studies have addressed egg white protein hydrolysate and its antioxidant properties, but only limited information is available regarding salted duck egg white protein and its hydrolysate. The present study aimed to examine the hydrolysates of salted duck egg white produced with selected protease enzymes (alcalase, ficin, protamex and neutrase), particularly as regards foaming, emulsification, and radical scavenging activities.

Materials and methods

Raw materials

The salted duck eggs at various stages of curing under ambient temperature (0, 5, 10, 15, 20, 25 and 30 days) with salted mud coating were purchased from a commercial producer in Chaiya district of Surat Thani province in southern Thailand. The procured eggs were brought to the laboratory on the same day, and the mud coating was removed using tap water. The eggs were then carefully checked for damage or cracks, and defective eggs were discarded. After that, the eggs were cracked open the egg whites were collected without any mix of egg yolks. The egg whites were then analyzed for total protein and salt content, before subjecting to protein hydrolysis. All the enzymes, chemicals and reagents for this study were purchased from Sigma Aldrich, Thailand.

Analysis of egg white

Determination of total protein

The total protein content in the salted duck egg whites from different curing stages was measured by the method of Zaia et al. (2005). The results are expressed as a percentage of total protein.

Determination of salt content

The salt contents of salted duck egg white samples were measured in accordance with the method of Tan et al. (2016). The results are expressed as a percentage of NaCl.

Protein hydrolysis of egg white

The protein hydrolysis of salted duck egg white was conducted in accordance with the method of Cho et al. (2014) with slight modifications. Prior to hydrolysis, the pH of egg white was adjusted to 6.0 using NaOH (0.1 N), and then the egg whites were incubated for 20 min at 45 °C. After incubation, the egg whites were hydrolyzed with alternative protease enzymes with the specific temperature (ST) and pH. The alternative proteases are including alcalase (endopeptidase, ST 55 °C and pH 7.5), ficin (endopeptidase, ST 40 °C and pH 7.0), protamex (complex, ST 60 °C and pH 8.0) and neutrase (neutral peptidase, ST 45 °C and pH 6.5). For hydrolysis, 0.05% (w/w) of a protease enzyme was mixed in the egg white and incubated for 12 h at the specific temperatures and pH as aforementioned. After hydrolyzing the egg white, the enzyme in the samples was completely inactivated in a water bath at 80 °C for 20 min, and then the samples were cooled down to room temperature and centrifuged at 2800g for 20 min at 4 °C. The supernatant was collected and subjected to various analyzes. In the control group, all the above steps were executed similarly, but the protease enzyme was replaced by deionized water and the “hydrolysis conditions” were set to ambient temperature and pH 6.

Analysis of egg white hydrolysate

Quantification of α -amino nitrogen content (α -ANC) and degree of hydrolysis (DH)

The egg white hydrolysate was measured for α -ANC and DH based on the method of Benjakul and Morrissey (1997) with slight modifications. A 125 μ l sample was mixed with 2 ml of 0.2 M phosphate buffer (pH 8.2), and 0.1 ml of 2,4,6-trinitrobenzene 1-sulfonic acid solution (0.01%) was then added. The reaction mixture was mixed well and kept in a water bath (50 °C) under the dark condition for

30 min. Then, 2 ml of sodium sulfite (0.1 M) was added to terminate the reactions, and then the sample was cooled down to room temperature. The absorbance was measured at 420 nm. The α -amino nitrogen content was calculated based on the standard curve for L-leucine, and the results were expressed as mg/ml.

Solubility

The solubility of an egg white hydrolysate was measured using the method of Morr (1985) with slight modifications. The egg white hydrolysate (0.5% w/w) was mixed in 5 mM imidazole buffer containing 0.02% sodium azide (pH 7.6). Then, the mixture was continuously stirred for 1 h at room temperature, followed by centrifugation at 2800g for 30 min. The supernatant was collected and determined for protein content using the Lowry protein assay. The results of protein solubility were expressed as a percentage.

Foaming capacity and stability

The egg white hydrolysates were measured for foaming capacity and stability using the methods of Cho et al. (2014) and Sathe and Salunkhe (2006) with slight modifications. The sample (1% v/v) was dissolved in 50 mM phosphate buffer (pH 7.6) and then whipped for 5 min at 8000 rpm in a food processor. Then, the whipped sample was transferred immediately into a graduated cylinder, and the total volume was measured after 30 s (for foaming capacity). After that, the samples were kept at room temperature for 30 min, and the volume was measured again as an indicator of foam stability. The results of foaming capacity and foam stability were expressed as a percentage.

Emulsification stability

The emulsion stability of the egg white hydrolysates was measured in accordance with the method of Thamnarathip et al. (2016) with slight modifications. The egg white hydrolysate (0.5% w/w) was mixed with vegetable oil (2.5%) and 50 mM phosphate buffer (pH 7.6). The mixture was converted to emulsion by homogenizing at 16,000 rpm for 2 min in a food processor and then sonicated using an ultrasonic processor in an ice bath for 10 min at 40% of maximum amplitude. Then, the emulsion (10 g) was poured into a graduated cylinder and stored at ambient temperature for 24 h. The results of emulsion stability were expressed as a percentage.

Radical scavenging abilities

The radical scavenging abilities of egg white hydrolysates were assessed against the DPPH radical (Lu and Yeap

2000), hydroxyl radical (Chung et al. 1997), hydrogen peroxide radical (Müller 1985), and ferrous ion chelating activity (Singh and Rajini 2004). The results are expressed as percentages.

Statistical analysis

All the experiments were done in six replicates, and the data are shown as a mean $\pm$ standard deviation. One-way ANOVA was applied to determine significant ($P < 0.05$) differences between the treatments. The SPSS software v6 for Windows was used for statistical analyzes.

Results and discussion

Salt content and Protein loss

Egg white with its combination of functional properties plays a vital role in the food processing industries. However, these properties are strongly influenced by salt concentration, ionic strength, type of salts, and pH (Croguennec et al. 2002). Figure 1. shows the salt and protein contents during prolonged salt curing of duck egg white. The salt content in the duck egg white gradually increased from 0.63 to 3.51%. Xu et al. (2017) reported that during salting the salt gradually diffuses into the egg white via the pores in the egg white membrane and in the eggshell. Kaewmanee et al. (2009) also observed increasing salt content in the duck egg white during prolonged salting. On the other hand, the protein content tends to decrease gradually as the salt content increases steadily in the samples. The initial protein level in the duck egg white was 11.12%, and by the end of the salting period, it had decreased to 8.01%. Ganesan et al. (2014) reported that prolonged salting increases protein loss, and also that the

Fig. 1 Influences of prolonged salting on the salt accumulation and the protein loss in duck egg white. Data shown are mean $\pm$ SD

egg white becomes watery and less viscous due to the migration of moisture from the egg yolk to the egg white by diffusion. Ünlüsayın et al. (2011) observed decreasing protein content in cuttlefish during prolonged salting. Schäfer et al. (1999) observed a decline of protein content in the egg white during prolonged storage at ambient conditions. Furthermore, the egg white naturally has a rich source antiproteases that contribute to embryogenesis and antimicrobial defense. Prolonged salting could affect the antiproteases and leads to natural protein hydrolysis (Réhault 2007). This is in agreement with the present study, as DH and α -ANC increased in the control samples without any protease treatment (Fig. 2).

Degree of hydrolysis (DH) and α -amino nitrogen content (α -ANC)

Enzymatic hydrolysis of food proteins modifies their physical, functional and nutritional properties (Garcés-Rimón et al. 2016). Figure 2 shows DH of salted duck egg white using various proteases during at the initial curing period (0 days). Among these enzymes, neutrase gave the highest DH (36.67%), followed by protamex (27.67%), alcalase (18.33%), ficin (15.33%) and the control (0.50%). DH is an indicator of proteolytic processes, and a high DH means that the protease effectively hydrolyzed the proteins. The degree of egg white protein degradation can be assessed from α -amino nitrogen content (Niewiarowicz et al. 1986; Noh and Suh 2015). α -ANC in the salted duck egg white, after hydrolysis with the various proteases, is shown in Fig. 3. Prolonged salting significantly affected the amount of hydrolysis generated α -ANC in the duck egg white. Among the protease enzymes, neutrase gave the highest α -ANC, followed by protamex, alcalase, ficin, and the control. All the samples showed linear trends for the

Fig. 2 The degree of hydrolysis, obtained with the tested hydrolase enzymes, in hydrolysates of duck egg white on the initial day of salt curing (0 days). Data shown are mean $\pm$ SD

Fig. 3 The dependence of α -amino nitrogen content in egg white hydrolysates on the duration of salt curing of duck eggs. Data shown are mean $\pm$ SD

salted duck egg white's α -ANC that gradually increased during salting. The control samples did not receive any hydrolase treatment but still showed a slight increment in α -ANC. This indicates that during prolonged salting protein degrades and generates peptide bonds. Cho et al. (2014) reported that the degree of hydrolysis is highly correlated with the α -ANC in the egg white protein. Pliansrithong et al. (2013) also observed that the application of neutrase enzyme greatly increased the α -ANC in beer wort. Godbert et al. (2010) observed increased proteolytic fragments in the egg white during the prolonged storage.

Solubility, foaming, and emulsification

The solubility of protein hydrolysates of salted duck egg white, hydrolyzed by the various protease enzymes, as shown in Fig. 4a. The solubility of the samples was significantly affected by the choice of the protease enzyme. Thamnarathip et al. (2016) reported that enzymatic hydrolysis improves solubility and heat stability from the native state of a protein. The control samples were the least soluble ($< 20\%$), indicating a lack of ionic strength and/or having hydrophobic amino acids on the protein surfaces. This is in concordance with Trevino et al. (2008). On the other hand, the different proteases increased the solubility by hydrolysis of the egg white protein. Among the proteases, ficin gave the least solubility while neutrase gave the highest. Overall, the egg white hydrolysates had above 70% solubility. Pokora et al. (2013) reported that solubility is in direct correlation with the DH of hydrolysis by protease enzymes. However, prolonged curing slightly decreased the solubility of the protein hydrolysate. The foaming capacity and foam stability of duck egg white hydrolysates are shown in Fig. 4b, c. The foaming capacity of protein hydrolysates gradually decreased with salt

Fig. 4 Solubility, foaming capacity, foaming stability and emulsion stability of the hydrolysates from egg white of salted duck egg. Data shown are mean $\pm$ SD

curing, while the salt content of the duck egg white increased ($P < 0.05$). Hydrolysis with ficin gave the lowest foaming capacity, whereas neutrase gave the highest foaming capacity. The control samples without enzymatic hydrolysis were the poorest in developing foam. On the other hand, the foam stability did not significantly differ between the control and the ficin hydrolyzed egg white ($< 40\%$). However, the other proteases gave hydrolysates with significantly improved foam stability between 75 and 95%. Cho et al. (2014) observed that neutrase hydrolysis of hen egg white protein improved foaming capacity and stability. The emulsification stability of the duck egg white

hydrolysates is shown in Fig. 4d. Initially, the alcalase gave best emulsion stability, but during prolonged curing and increased salt concentration in the egg white, the emulsion stability became worse. Neutrase and the other proteases gave hydrolyzed samples with better emulsion stability. Overall, the control samples had poor solubility and foaming properties compared to the hydrolysates. The egg white functional properties in foaming and emulsification mainly depend on the amount and solubility of the protein. Pokora et al. (2013) reported that a protein hydrolysate's functional properties are significantly

associated with its solubility. This is in accordance with the present study.

Radical scavenging abilities

Proteins after enzymatic modification may function as natural antioxidants and possess potential health benefits (Amarowicz 2008). The radical scavenging abilities of the salted duck egg white protein hydrolysates, produced by the various proteases, are shown in Fig. 5a–d. This study found that duck egg white hydrolysates, prepared at various stages of salt curing, exhibited stronger scavenging activities against free radicals than the control samples. Among

the various hydrolysates, those hydrolyzed with neutrase, protamex and alcalase had the best scavenging performance against DPPH, hydroxyl, hydrogen peroxide, and ferrous ion radicals. Neutrase hydrolysates showed the highest scavenging activities while nicin hydrolysates exhibited the lowest activities. Normally, the radical scavenging abilities of egg white hydrolysates produced by proteases mainly depend on the specificity of the enzyme and on the hydrolysis conditions (Je et al. 2009). Ren et al. (2014) stated that peptides in the duck egg white hydrolysate, hydrolyzed using protease enzymes, scavenge highly reactive oxygen species. Several studies have reported that those protein hydrolysates that have free

Fig. 5 Antioxidant capacities of the various hydrolysates from egg white of salted duck egg. Data shown are mean $\pm$ SD

amino acids and small peptides also have stronger scavenging activities (Amarowicz 2008; Pokora et al. 2013). Furthermore, α -ANC is considered to be the main contributor of antioxidant activities in the hydrolyzed duck egg whites. The α -ANC (Fig. 3) in egg white hydrolysates was highly correlated with the radical scavenging strengths of the samples.

Conclusion

The present study found that using various protein hydrolysates improved the functional properties and antioxidant capacities of the salted egg white. This study also found that prolonged curing with salt lowered the functionalities of egg white protein hydrolysates. A consistent decrease in foaming and emulsification properties was observed during the prolonged curing period. Furthermore, the major protease enzymes, specifically neutrase, protamex and alcalase have given similar hydrolysate properties, particularly in foaming and emulsification. Overall, hydrolysis by neutrase gave the best results in terms of the degree of hydrolysis, α -ANC, foaming and emulsion properties, and radical scavenging abilities. This study concludes that neutrase is a highly suited protease enzyme for the hydrolysis of salted duck egg white.

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