Comparative Enzymatic Hydrolysis of Desalted Duck Egg White Using Proteases for Functional Hydrolysates

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Abstract

The valorization of salted duck egg white (SDEW), a high-salt by-product of the egg industry, was investigated through desalting and enzymatic hydrolysis to produce protein hydrolysates with enhanced functional properties. The desalting process resulted in a 97.13% reduction in sodium content while maintaining protein integrity, confirming desalted SDEW (DS-DEW) as a suitable substrate for enzymatic modification. Two proteases with distinct catalytic properties were compared: Protease M "Amano" SD (MSD, a dual endopeptidase and exopeptidase) and Thermoase GL30 (GL, an endopeptidase). Both enzymes showed time-dependent increases in the degree of hydrolysis (DH), with GL achieving a higher final DH at 6 hours, while MSD exhibited a faster initial hydrolytic rate. Total amino acid (TAA) content increased in both groups, with MSD yielding consistently higher levels of free amino acid. Predominant amino acids included glutamic acid, aspartic acid, glycine, and leucine, along with increased levels of hydrophobic and aromatic amino acids such as tyrosine, phenylalanine, and tryptophan. Antioxidant activity evaluated by the DPPH assay was significantly higher in MSD-treated samples, peaking at 3 hours (88.36%). In contrast, GL-treated samples exhibited lower activity, which declined over time. These results demonstrate the potential of controlled enzymatic hydrolysis of DS-DEW for producing antioxidant-active hydrolysates and support the development of value-added functional ingredients, contributing to the sustainable and industrial-scale utilization of egg industry by-products within the bio-circular-green economic model.

Keywords: Desalted egg white, Protein hydrolysate, Enzyme, Free amino acids, Antioxidant activity

Introduction

The salted egg industry generates a considerable amount of waste by-products, particularly salted duck egg white (SDEW), which presents challenges in terms of utilization and environmental impact. While the yolks are often separated and used in high-value food products such as mooncakes and savory dishes, the remaining egg whites are frequently discarded due to their high salt content (typically 7-12% sodium chloride) and unpleasant odor. With the increasing demand for salted egg yolk products, it has been reported that more than 10,000 tons of SDEW are generated annually in China alone, leading to substantial losses of high-quality protein resources and raising concerns regarding food waste and environmental pollution (Du et al., 2022). These characteristics negatively affect the sensory quality and functionality of SDEW, rendering it unsuitable for direct food applications (Thamamsena & Liu, 2020). Improper disposal of SDEW contributes to food waste and represents a lost opportunity for protein recovery and value-added utilization. To address this challenge, various desalination

approaches—including ultrafiltration, electrodialysis, ion exchange, and membrane separation—have been investigated to reduce sodium content and improve the organoleptic properties of SDEW. Such strategies not only enhance its suitability for food applications but also support the Bio-Circular-Green (BCG) economic model by promoting the valorization of food industry by-products (Guo et al., 2020; Wang et al., 2015; Zhou et al., 2015). These advances position desalted duck egg white (DS-DEW) as a promising raw material for further processing, particularly in the development of functional protein ingredients.

Egg white is a high-quality protein source that is easily digestible and rich in essential amino acids. It contains several major proteins, including ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme, and its protein content typically ranges from 10-12% (w/w) (Mine, 1995). These proteins not only provide nutritional benefits but also serve as excellent precursors for the production of bioactive peptides—short amino acid sequences that may contribute to health promotion. In recent years, enzymatically hydrolyzed egg white proteins have been reported to exhibit antioxidant, antihypertensive, antimicrobial, and anti-inflammatory activities. These bioactivities, along with their nutritional value, have led to the classification of such hydrolysates as *functional hydrolysates*, which are gaining increasing interest as ingredients in health-oriented food and nutraceutical applications (Thamamsena & Liu, 2020).

Enzymatic hydrolysis is a widely adopted technique for producing protein hydrolysates with targeted bioactivities. Compared with chemical hydrolysis, enzymatic methods offer greater specificity, safety, and control over peptide profiles. Protein hydrolysates *derived* from various sources such as soy, fish, milk, and egg have been extensively studied, demonstrating improved bioactivity and digestibility. Among these, egg white protein hydrolysates are particularly attractive due to their small peptide size, high digestibility, strong antioxidant potential, and hypoallergenic properties. Furthermore, egg whites are naturally free of fat and cholesterol, making them suitable for health-conscious consumers and ideal for the development of clean-label functional food products. The bioactivity and quality of protein hydrolysates are influenced by various factors, including substrate concentration, hydrolysis time, temperature, pH, and especially the type of protease used. Proteolytic enzymes are generally classified into endopeptidases, which cleave internal peptide bonds, and exopeptidases, which release amino acids from terminal ends. Selecting an appropriate enzyme or enzyme combination can significantly enhance hydrolysis efficiency and generate peptides with desirable bio-functional properties (Benedé & Molina, 2020; Johny et al., 2022).

Despite these advances, a clear research gap remains: few studies have directly compared proteases with distinct catalytic mechanisms in the hydrolysis of DS-DEW. In particular, the differences between dual-activity enzymes (endo-exopeptidases, such as MSD) and enzymes with strictly endopeptidase activity (such as GL) have not been systematically evaluated in terms of hydrolysis behavior, amino acid release, and antioxidant potential. Understanding these differences is essential for selecting the most appropriate enzyme for producing functionally enhanced DS-DEW hydrolysates. We therefore hypothesized that Protease M "Amano" SD (MSD), which possesses both endopeptidase and exopeptidase activities, would generate peptides with higher antioxidant activity and greater free amino acid release thanThermoase GL30 (GL), which functions solely as an endopeptidase.

Therefore, the objective of this study (1) compare the hydrolysis patterns of MSD and GL when applied to DS-DEW; (2) evaluate the resulting differences in degree of hydrolysis, amino acid composition, and antioxidant activity; and (3) identify hydrolysis conditions that maximize the functional properties of DS-DEW protein hydrolysates. This approach supports the valorization of food industry by-products and contributes to the development of sustainable health-oriented ingredients.

Materials and Methods

Sample preparation and preliminary characterization of desalted egg white samples

Desalted duck egg white (DS-DEW) was prepared from salted duck egg white (SDEW) obtained from PC Intertrade Limited Partnership (Nakhon Pathom, Thailand). The desalting process was carried out using a proprietary in-house method currently under petty patent application in Thailand (Application No. 2203003406), and detailed procedures are withheld due to intellectual property protection. This DS-DEW served as the primary raw material for enzymatic hydrolysis in this study.

To establish baseline values and verify the effectiveness of the desalination process, untreated SDEW and fresh duck egg white (DEW) were included in the preliminary analyses. were included as reference samples. All three samples—SDEW, DS-DEW, and DEW—underwent preliminary analyses to determine moisture content, salt content, and degree of hydrolysis (DH), as summarized in Table 1. All measurements in the preliminary characterization were performed in triplicate (n = 3).

Moisture content was determined according to the method described by AOAC (2000). Samples were dried in a hot-air oven at 105 °C for 24 hours. Each sample was analyzed in triplicate, and the moisture content was calculated using Equation (1):

$$\text{Moisture content } (\%) = \frac{W1 \times W2}{W2} \times 100 \qquad \text{Eq (1)}$$

Where: W_1 = weight of the sample before drying (g)

W_2 = weight of the sample after drying (g)

The sodium chloride (NaCl) content was determined according to the AOAC method (1990). A 5 g sample was weighed into a 250 mL Erlenmeyer flask, followed by the addition of 100 mL of boiling distilled water. The mixture was stirred thoroughly until the sample was completely dissolved, then allowed to cool to approximately 50-55 °C. After that, 2 mL of potassium chromate solution (K_2CrO_4) was added as an indicator. The solution was titrated with 0.1 N silver nitrate solution ($AgNO_3$) until a persistent reddish-brown color appeared and remained for at least 30 seconds, which was considered the endpoint. The sodium chloride content was calculated using Equation (2):

$$\text{Sodium chloride } (\%) = \frac{\text{Volume of AgNO3 (mL)}}{\text{Sample weight (g)}} \times 0.585 \qquad \text{Eq (2)}$$

The determination rate can be calculated using the following equation:

$$\text{Desalination rate } (\%) = \frac{S0 - S1}{S0} \times 100 \qquad \text{Eq (3)}$$

Where: S_0 = salt content before the desalting process

S_1 = salt content after the desalting process

For the preliminary comparison between DEW and DS-DEW, the degree of hydrolysis (DH) was determined using the pH-stat titration method under acidic conditions, as described by Brodkorb et al. (2019). The DH was calculated according to the formula of Mat et al. (2018), as shown in Equation (4):

$$DH = 100 \times \frac{V \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH}} \qquad \text{Eq (4)}$$

Where: V = Volume of titrant (mL)

N = Normality (meq/mL)

m = Protein mass (g)

h_{tot} = Number of peptide bonds per gram of proteins

α_{COOH} Degree of dissociation of the carboxylic groups produced

Preparation of hydrolysis

To investigate the influence of protease specificity on hydrolysis efficiency and the resulting peptide characteristics, two proteolytic enzymes with distinct mechanisms were selected. Protease M "Amano" SD (MSD), derived from *Aspergillus oryzae*, possesses both endopeptidase and exopeptidase activities, with a minimum enzyme activity of 40,000 U/g, enabling it to cleave peptide bonds both internally and at terminal ends. In contrast, Thermoase GL30 (GL30), derived from *Bacillus stearothermophilus*, functions primarily as an endopeptidase with a minimum enzyme activity of 300,000 U/mL, targeting only internal peptide linkages. The comparison of these two enzymes was intended to elucidate how different catalytic specificities affect the degree of hydrolysis, amino acid composition, and antioxidant properties of protein hydrolysates obtained from DS-DEW. Both enzymes were obtained from Amano Enzyme Asia Pacific Co., Ltd., Thailand.

Protein hydrolysates were prepared by mixing DS-DEW with distilled water at a 2:1 volume ratio (DS-DEW: water), followed by pH adjustment to 7.0 using 5% acetic acid. Protease enzymes were added at a concentration of 1% (w/w) based on the weight of DS-DEW as adapted from a patented enzymatic hydrolysis method (US Patent No. 8,796,009 B2). The hydrolysis reaction was conducted in a shaking water bath at 50 ± 2 °C with continuous agitation for 0, 1, 3, and 6 hours. At each time point, the enzymatic activity was terminated by heating the mixture at 95 ± 2 °C for 15 minutes. The hydrolysates were then centrifuged at 10,000 rpm for 10 minutes at 4 °C, and the supernatants, representing the desalted duck egg white hydrolysates (DS-DEWH), were collected in tightly sealed tubes and stored at -20 °C until further analysis. All hydrolysis experiments and sampling points were conducted in triplicate (n = 3).

Determination of the degree of hydrolysis

For the main determination of the degree of hydrolysis (DH) in this study, the O-phthalaldehyde (OPA) spectrophotometric method, modified from Bavaro et al. (2021), was employed due to its high sensitivity, accuracy, and specificity for detecting free amino groups released during enzymatic hydrolysis. This method is widely recognized for its reliability in assessing the extent of protein degradation in hydrolysates.

The OPA reagent was prepared by mixing 50 mL of 100 mM sodium tetraborate, 5 mL of 20% (w/w) sodium dodecyl sulfate, and 2 mL of ethanol containing 80 mg of OPA. The mixture was then brought to a final volume of 100 mL with distilled water. An aliquot of 500 µL of appropriately diluted DS-DEWH was mixed with 1,000 µL of OPA reagent and incubated in the dark at room temperature for 5 minutes. Absorbance was then measured at 340 nm using a UV-Visible spectrophotometer. L-leucine was used as a standard to generate the calibration curve. The degree of hydrolysis (%) was calculated based on the method of Spellman et al. (2003), as presented in Equation (5). DH was determined at 0, 1, 3, and 6 hours of hydrolysis to assess the enzymatic activity and time-dependent hydrolysis profiles. DH determination was performed for each hydrolysis time (0, 1, 3, 6 h) in triplicate (n = 3).

$$DH\ (\%) = \frac{\Delta Abs \times 1.833 \times d}{c} \qquad \text{Eq (5)}$$

Where: ΔAbs is the Abs of test sample at 340 nm - Abs unhydrolyzed sample at 340 nm

d is the dilution factor

c is the protein concentration (gL^{-1})

Determination of Total Amino Acid Content

Amino acids identification and quantification were performed by acid hydrolysis followed by amino acid analysis. Each DS-DEWH was hydrolyzed with 6 M hydrochloric acid (HCl) at 110 °C for 22 hours under vacuum-sealed conditions to prevent oxidative degradation of amino acids. After hydrolysis, the acid was evaporated under reduced pressure, and the residue was re-dissolved in 0.02 N HCl. The amino acid composition was analyzed using an amino acid analyzer (L-8900, Hitachi, Japan) equipped with a cation-exchange column (4.6 × 60 mm). All samples were analyzed in triplicate (n = 3).

Determination of Antioxidant Activity

To comprehensively evaluate the antioxidant properties of the protein hydrolysates, DPPH radical scavenging activity was employed. The antioxidant activity of DS-DEW-derived protein hydrolysates was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, following the method of Cui et al. (2022), with slight modifications to suit a 96-well microplate reader system. DS-DEWH was dissolved in 95% ethanol at a ratio of 2:1 (v/v). A 0.1 mM DPPH solution was prepared in 70% ethanol under dark conditions at ambient temperature. Then, 100 µL of the DS-DEWH sample was mixed with 100 µL of the DPPH solution in each well of the microplate and incubated at 25 °C for 30 minutes. After incubation, the absorbance was measured at 517 nm using a microplate reader. The DPPH radical scavenging activity was expressed as the percentage of inhibition according to Equation (5):

$$\text{DPPH radical scavenging activity } (\%) = \frac{Ab - As}{Ab} \times 100 \quad \text{Eq (5)}$$

Where: Ab is the absorbance of the control (without antioxidant)

As is the absorbance of the sample

Statistical analysis

The differences between treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's new multiple range test (DMRT) for pairwise comparisons. Statistical significance was considered at $p < 0.05$. All statistical analyses were performed using IBM® SPSS® Statistics version 25 (IBM Corp., Armonk, NY, USA).

Results

Physicochemical Properties of Salted Duck Egg White Before and After Desalting

Table 1 presents the moisture content, salt content, and degree of hydrolysis of three types of duck egg white samples used in this study: fresh duck egg white (DEW), salted duck egg white (SDEW), and desalted duck egg white (DS-DEW). The moisture content of SDEW (81.54 ± 0.11%) and DS-DEW (81.57 ± 0.61%) samples showed no significant difference. However, DEW exhibited a slightly higher moisture content (87.20 ± 0.13%). Regarding salt content, SDEW contained significantly higher sodium levels (46.25 ± 0.54%) than both DEW (0.47 ± 0.19%) and DS-DEW (1.33 ± 0.19%) ($p < 0.05$), confirming that the desalting process effectively reduced sodium chloride by approximately 97% relative to the untreated SDEW. The DH values of DEW and DS-DEW were comparable (2.34 ± 0.19, 2.41 ± 0.12%), indicating that desalting did not affect the susceptibility of the protein to enzymatic hydrolysis. Due to its high salt content, SDEW was excluded from enzymatic hydrolysis, as it would remain unsuitable for food applications even after treatment.

Table 1 Moisture content, salt content, and degree of hydrolysis of duck egg white samples before and after desalting

Sample	Moisture content (%)	Salt content (%)	Degree of hydrolysis (DH, %)
Duck Egg White (DEW)	87.20 ± 0.13	0.47 ± 0.19	2.34 ± 0.19
Desalted Duck Egg White (DS-DEW)	81.57 ± 0.61	1.33 ± 0.19[b]	2.41 ± 0.12
Salted Duck Egg White (SDEW)	81.54 ± 0.11	46.25 ± 0.54[a]	ND

Different superscript letters in the same column indicate significant differences ($p < 0.05$).

ND = Not Determined

Degree of Hydrolysis

The degree of hydrolysis (DH) of DS-DEW treated with Protease M "Amano" SD (MSD) and Thermoase GL30 (GL) at various hydrolysis times is shown in Fig. 1. In both enzyme treatments, the DH significantly increased over the 6-hour hydrolysis period ($p < 0.05$). At 1 hour, the GL treated samples exhibited a higher DH value (3.58%) compared to the MSD-treated samples (2.21%). However, from 3 hours onward, the MSD group showed a greater hydrolytic rate, reaching a plateau of approximately 6.96% by 6 hours. In contrast, the GL-treated group showed a sharp initial increase, followed by a slower rise between 1 and 3 hours, and a steeper

increase again from 3 to 6 hours, which indicated a delayed activation or shift in the enzyme's hydrolytic efficiency over time. These distinct hydrolytic patterns may reflect differences in enzyme specificity: GL, an endopeptidase, exhibited a time-dependent but non-linear increase in DH, while MSD, which has both endo- and exopeptidase activities, showed rapid early hydrolysis followed by an early plateau. Statistical differences within each enzyme group over time are indicated by lowercase letters (a-d), and differences between enzyme groups at each time point are indicated by uppercase letters ($p < 0.05$). These results highlight MSD's effectiveness in early-phase hydrolysis, while GL showed a more prolonged and late-phase increase in DH. This comparison provides insight into how protease types influence hydrolysis progression, which is critical for optimizing enzyme selection in bioactive peptide production.

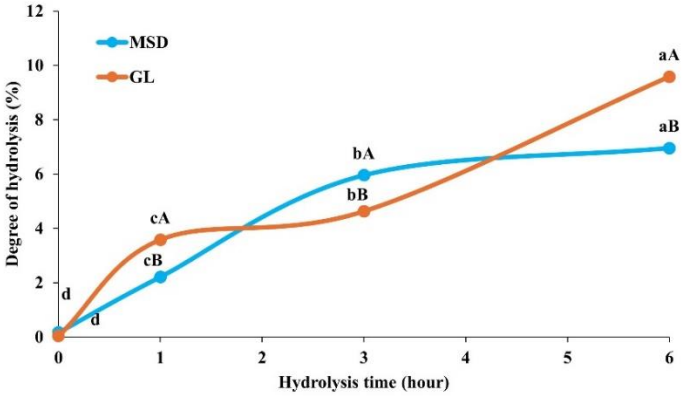

Figure 1 Degree of hydrolysis (%) of desalted duck egg white (DS-DEW) hydrolyzed with Protease M "Amano" SD (MSD) and Thermoase GL30 (GL) at 0, 1, 3, and 6 hours. Different lowercase letters (a-d) indicate significant differences ($p < 0.05$) among hydrolysis times within the same enzyme group, and different uppercase letters (A-B) indicate significant differences between enzyme groups at the same time point

Amino Acid Composition

The amino acid composition of DS-DEW hydrolyzed with MSD and GL at 0, 1, 3, and 6 hours is shown in Fig. 2. Overall, the contents of most amino acids increased with hydrolysis time in both enzyme treatments, reflecting progressive protein degradation. At the initial stage (0 h), MSD-treated samples already contained slightly higher amounts of leucine, Threonine, and valine than GL. After 1 h, both enzyme treatments showed a sharp rise in EAAs, with leucine, Phenylalanine, Methionine, and valine being the most abundant. At 3 and 6 h, MSD-treated samples exhibited significantly higher concentrations of leucine (51.58 mg/g), isoleucine, lysine, and valine when compared with GL samples. These EAAs are particularly important as they not only enhance the nutritional value of the hydrolysates but are also associated with bioactivities such as muscle protein synthesis and antioxidant potential. The consistent increase in EAAs, especially in MSD-treated hydrolysates, reflects the broader proteolytic action of MSD (endo and exopeptidase), which facilitated more effective release of nutritionally and functionally relevant amino acids compared to GL, which acts mainly as an endopeptidase. The comparative profiles demonstrate MSD's superior capacity to release essential amino acids with known bioactivities, underscoring the enzyme's potential for generating functionally enriched hydrolysates.

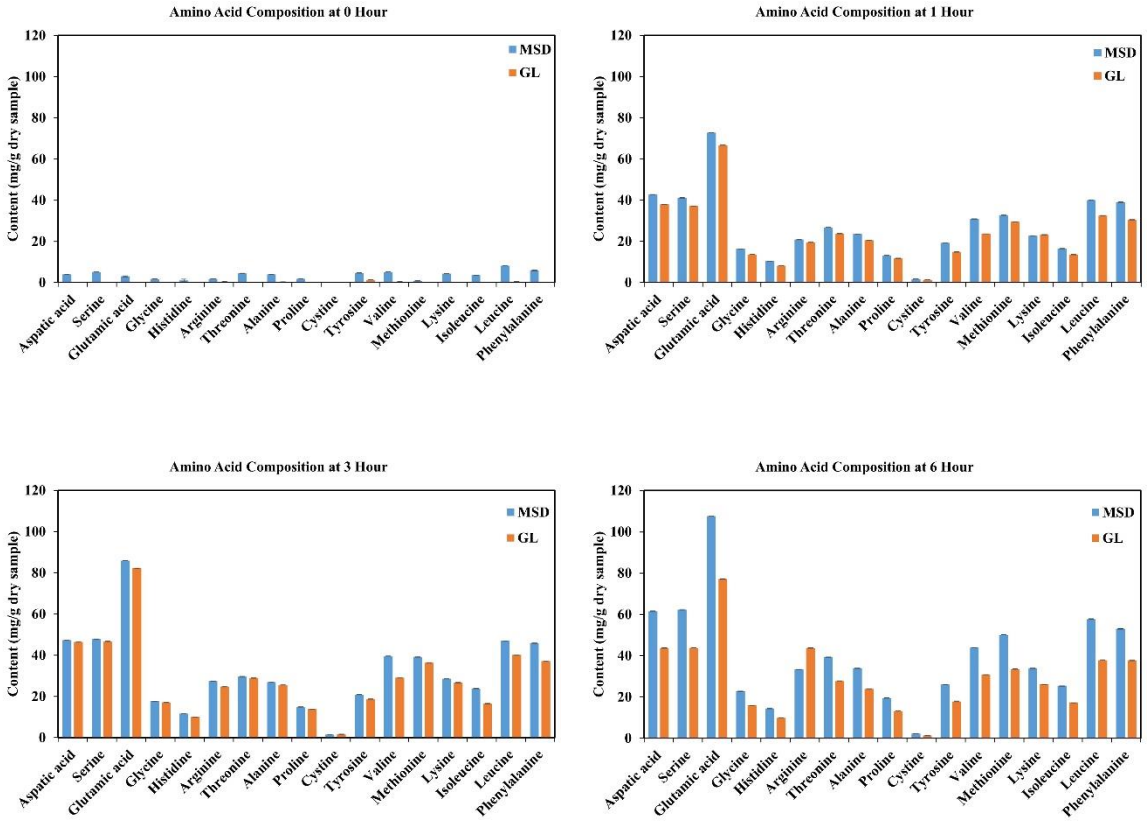

Figure 2 Amino acid composition (mg/g sample, dry sample) of desalted duck egg white (DS-DEW) hydrolyzed with Protease M "Amano" SD (MSD) and Thermoase GL30 (GL) at 0, 1, 3, and 6 hours

Antioxidant Activities

The antioxidant activities of protein hydrolysates derived from DS-DEW were evaluated using the DPPH radical scavenging assay, as shown in Fig. 3. Hydrolysates produced with MSD exhibited significantly higher DPPH scavenging activity at all time points compared to those treated with GL ($p < 0.05$). For MSD-treated samples, activity increased from 69.23% at 1 hour to a peak of 88.36% at 3 hours, then declined slightly to 79.15% at 6 hours. In contrast, GL-treated hydrolysates showed their highest activity at 1 hour (21.24%), followed by a gradual decrease to 13.16% at 6 hours. This trend was consistent with the total amino acid (TAA) contents: the MSD group showed a continuous increase from approximately 470 mg/g to nearly 690 mg/g, while the GL group's TAA values plateaued after 1 hour. These results suggest a correlation between hydrolysis time, amino acid release, and antioxidant potential, particularly for MSD-treated hydrolysates.

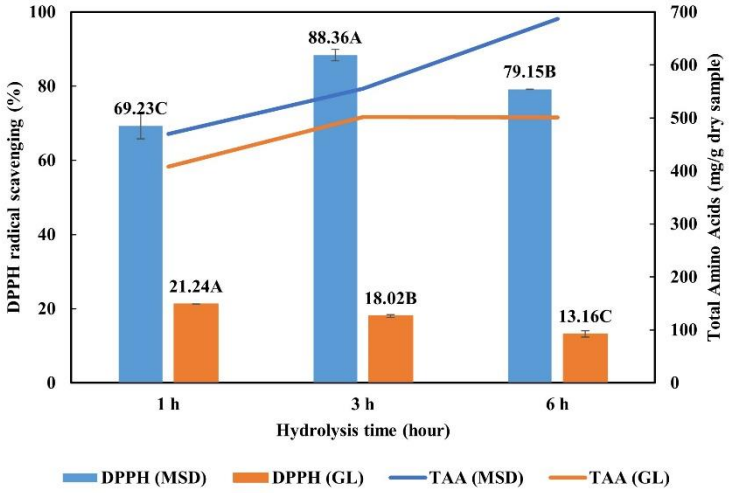

Figure 3 DPPH radical scavenging activity (%) of protein hydrolysates from desalted duck egg white (DS-DEW) hydrolyzed with Protease M "Amano" SD (MSD) and Thermoase GL30 (GL) at 0, 1, 3, and 6 hours. Different lowercase letters (a–d) indicate significant differences ($p < 0.05$) among hydrolysis times within the same enzyme group, and different uppercase letters (A–B) indicate significant differences between enzyme groups at the same time point

Discussion

The desalination of salted duck egg white is a crucial step in preparing suitable raw materials for enzymatic hydrolysis. In this study, the desalting process, adapted from the patented method, successfully reduced the salt content from 46.25% to 1.33%, while the moisture content remained relatively stable (Table 1). This indicates that the desalting process did not result in substantial dehydration, thereby helping to preserve the overall physical state of the protein material. To assess whether the desalting procedure affected protein susceptibility to enzymatic hydrolysis, fresh duck egg white (DEW) was compared to that of desalted duck egg white (DS-DEW), the main experimental substrate in this study. The DH values of DS-DEW ($2.41 \pm 0.12\%$) and DEW ($2.34 \pm 0.19\%$) were not significantly different ($p > 0.05$), suggesting that the protein hydrolytic potential was retained following desalting. These findings are consistent with previous reports stating that desalting improves usability while maintaining protein structural integrity (Guo et al., 2020; Thamamsena & Liu, 2020). These results support the use of DS-DEW as a viable substrate for further enzymatic treatment.

To explore enzymatic performance, DS-DEW was subjected to hydrolysis using two different proteases: Protease M "Amano" SD (MSD) and Thermoase GL30 (GL), which differ markedly in catalytic properties. MSD, derived from *Aspergillus oryzae*, exhibits both endopeptidase and exopeptidase activities with a minimum enzymatic activity of 40,000 U/g, enabling it to cleave peptide bonds both at internal sites and terminal residues. This dual mechanism facilitates rapid release of peptides during the initial stages of hydrolysis (Hinnenkamp & Ismail, 2021). In contrast, GL30—an endopeptidase from *Bacillus stearothermophilus*—is a thermolysin-type neutral metalloprotease with a substantially higher activity (≥300,000 U/mL). Although it lacks exopeptidase activity, its high catalytic efficiency contributes to effective cleavage at the N-terminal side of hydrophobic or aromatic residues (Olagunju et al., 2021). As illustrated in Fig. 1, the progression of hydrolysis revealeddistinct kinetic patterns between the two enzymes. Overall, DH increased significantly with time for both enzymes ($p < 0.05$), consistent with general trends in protein hydrolysis. MSD exhibited faster hydrolysis during the

early phase (0–1 h), likely due to its dual enzymatic action. However, GL gradually surpassed MSD and reached a significantly higher DH value at 6 hours (10.47 ± 0.27%), compared to 6.66 ± 0.25% for MSD. These results reflect the greater catalytic efficiency and stability of GL, particularly during prolonged hydrolysis. Interestingly, GL displayed a non-linear hydrolytic trend, with a sharp increase in DH at the initial stage (0–1 h), a noticeable plateau between 1 and 3 hours, and a renewed rise thereafter. This biphasic pattern may be attributed to initial cleavage of readily accessible peptide bonds, followed by substrate saturation or intermediate peptide inhibition, before conformational changes later expose new cleavage sites. The hydrolytic behavior observed in this study aligns with previous findings. For example, Lyu et al. (2023) reported that neutral protease exhibited rapid and effective hydrolysis of egg white protein (EWP), with DH reaching nearly half of its final value after only 15 minutes. Similarly, Asaithambi et al. (2023) showed that papain-like MSD exhibited early-stage efficiency due to its dual activity, followed by high DH at extended times. Together, these observations emphasize the importance of selecting proteases according to their catalytic profiles and desired hydrolysis timeframes, particularly for functional ingredient development.

In addition to DH, total amino acid (TAA) content was assessed to reflect the extent of peptide bond cleavage and generation of free amino acids (Fig. 2). Despite having slightly lower DH values, MSD-treated samples consistently showed higher TAA content at all time points, supporting the notion that dual enzyme action not only promotes faster cleavage but also enhances free amino acid release. In contrast, GL tended to generate larger peptide fragments, which may not be fully cleaved into free amino acids under the experimental conditions. Among the released amino acids, glutamic acid, aspartic acid, glycine, and leucine were predominant—each playing essential roles in flavor development and bioactivity. Glutamic and aspartic acids exhibit strong antioxidant properties through metal-chelating and electron-donating mechanisms. Leucine, a branched-chain amino acid (BCAA), plays a vital role in both muscle synthesis and the regulation of oxidative stress. In our study, increasing the hydrolysis time led to a significant enhancement in the total amino acid content, particularly hydrophobic and aromatic amino acids such as alanine, valine, methionine, leucine, tyrosine, and phenylalanine. These trends agree with previous findings reported by Ruíz-Henestrosa et al. (2009) and Wang et al. (2022), who also observed an increase in hydrophobic amino acids during enzymatic hydrolysis of plant and animal proteins. Furthermore, the proportion of hydrophobic amino acids in our study (approximately 25% of total amino acids) was comparable to that reported in their studies. The elevated levels of aromatic amino acids observed in both studies further support the link between amino acid composition and antioxidant activity. As supported by Asaithambi et al. (2023), Gijsman (2012), and He et al. (2021), peptides rich in these amino acids-especially di- and tripeptides-exhibit potent antioxidant properties. Our findings align with these reports and further demonstrate the potential of egg white protein hydrolysates as sources of bioactive compounds. Notably, MSD's consistently higher TAA levels, despite lower DH, suggest that peptide length and cleavage pattern —rather than DH alone—play a crucial role in determining amino acid release.

Antioxidant activity, as evaluated by the DPPH radical scavenging assay, further revealed distinct patterns between the enzymes (Fig. 3). MSD-treated hydrolysates demonstrated significantly higher scavenging activity at all time points compared to GL. The highest antioxidant capacity was observed in the MSD group at 3 hours (88.36 ± 0.78%), followed by a slight decline at 6 hours (79.15 ± 1.23%), despite a continuous increase in total amino acids (TAA). This observation suggests that antioxidant capacity was not solely dependent on TAA, but rather influenced by the nature and composition of peptides and free amino acids generated during hydrolysis.

MSD's superior performance can be attributed to its dual enzymatic action (endo- and exopeptidase), which facilitates the release of smaller peptides and free amino acids—many of which are known to exhibit high antioxidant potential. Peptides enriched with hydrophobic and aromatic amino acids (e.g., Tyr, Phe, Trp), as well as basic amino acids (e.g., Lys, Arg, His), are particularly effective in neutralizing free radicals by donating hydrogen atoms or electrons to DPPH• radicals (He et al., 2021; Zhao et al., 2022). Additionally, the presence of phenolic groups and imidazole rings in these amino acids enhances their ability to stabilize radicals via resonance mechanisms (Gijsman, 2012; Phongthai et al., 2016). These structural features are likely responsible for the peak antioxidant activity observed at 3 hours in the MSD-treated hydrolysates—representing an optimal phase where active antioxidant peptides were abundant before potential degradation or aggregation at longer hydrolysis times. These tendencies strongly support a functional linkage between peptide composition and antioxidant activity, beyond the extent of hydrolysis itself.

In contrast, GL-treated samples showed a different pattern. The highest DPPH activity was detected at 1 hour (21.24 ± 0.36%), followed by a continuous and significant decline over time (18.02% at 3 hours and 13.16% at 6 hours). This decrease in antioxidant activity, despite relatively stable TAA levels, suggests that the peptides generated by GL—an endopeptidase with narrow cleavage specificity—were less effective in contributing to radical scavenging. It is possible that the peptides produced by GL were predominantly medium-sized, less hydrophobic, or lacked key residues associated with antioxidant function. Some studies have suggested that an excess of medium-sized or partially hydrolyzed peptides can sterically hinder interaction with DPPH radicals, especially if hydrophobic residues are buried within the peptide chain (Zhou et al., 2022) This may explain the lower and declining antioxidant activity observed in GL-treated hydrolysates, despite relatively high DH values and stable TAA levels. The divergence between DH and antioxidant trends further reinforces that hydrolysis efficiency and bioactivity are not directly proportional but are strongly enzyme dependent.

These findings are consistent with previous reports of egg white proteins indicating that low-molecular-weight peptides (<1 kDa) possess superior antioxidant properties due to better accessibility to reactive sites (Liu et al., 2015; Zheng et al., 2020; Zhou et al., 2022). The comparative performance of MSD and GL in this study highlights how enzymatic specificity shapes the functional properties of protein hydrolysates. Taken together, while MSD demonstrates a clear advantage in generating antioxidant-active components during the early phase of hydrolysis, GL's high catalytic efficiency and sustained TAA levels may offer other functional benefits, such as improved yield of target peptides or bioactivity under different physiological conditions. These complementary properties suggest potential for synergistic effects if the two enzymes are applied in combination. A sequential or co-enzymatic hydrolysis strategy—leveraging the broad-spectrum cleavage of MSD followed by the high-efficiency action of GL—could be a promising approach to enhance both peptide diversity and biofunctional quality. Further investigation into such enzyme blending strategies is warranted to optimize antioxidant capacity and tailor the hydrolysates for specific functional food applications. Future studies should also incorporate peptide profiling (LC-MS/MS) and molecular weight distribution analysis to verify the structural basis of the observed bioactivities, as well as statistical correlation analyses to more clearly define relationships among DH, TAA, and antioxidant activity.

Conclusion and Suggestions

This study demonstrated the feasibility of using desalted duck egg white (DS-DEW) as a raw material for enzymatic hydrolysis. The removal of excessive salt did not compromise protein hydrolytic potential, supporting the valorization of this underutilized by-product. Among the tested enzymes, Protease M "Amano" SD (MSD) generated higher levels offree amino acids and small peptides, resulting in higher antioxidant activity, particularly at 3 hours of hydrolysis, despite exhibiting slightly lower DH values thanThermoase GL30 (GL). In contrast, GL achieved higher DH but showed a decline in antioxidant potential over time. These findings emphasize that functional outcomes depend more on peptide composition than on hydrolysis extent alone. Hydrolysis with MSD for approximately 3 hours appears to be the optimal condition for maximizing antioxidant activity and free amino acid release, whereas GL may be more suitable for applications requiring more extensive protein hydrolysis. Accordingly, these results provide practical guidance for selecting appropriate enzyme-time combinations based on targeted functional outcomes. Each enzyme exhibited distinct advantages—MSD favored early-stage antioxidant peptide production, while GL was more effective in prolonged hydrolysis. Future research should explore the combined use of endo- and exopeptidases to enhance both peptide yield and functional properties. In particular, characterization of peptide structure and statistical correlation between DH, amino acid release, and antioxidant capacity will help validate the mechanistic basis of the observed bioactivity. Moreover, synergistic enzyme strategies—such as sequential or co-hydrolysis—may optimize bioactive peptide production for high-value applications, especially in the functional food and nutraceutical industries, where antioxidant-stable ingredients are in growing demand.

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Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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