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Preparation of Skipjack Tuna (*Katsuwonus pelamis*) Protein Hydrolysate Using Combined Controlled Enzymatic Hydrolysis and Glycation for Improved Solubility and Emulsifying Properties

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Abstract Preparation of the hydrolysate from under-utilized skipjack tuna (*Katsuwonus pelamis*) protein with improved functional properties using combined controlled enzymatic hydrolysis and glycation was investigated. Five enzymes including alcalase, flavorase, neutrase, trypsin and protamex were performed to hydrolysis under the optimum conditions. The results showed that 5-hour hydrolysis led to increases in degree of hydrolysis (DH) and amino acids content, and change in soluble protein content. To obtain considerable small-size peptides, a controlled enzymatic hydrolysis (2.5 h) by trypsin was carried out. This condition produced nearly 80% soluble protein which could not be precipitated by 10% TCA. The resulting hydrolysate was then subjected to glycation with alginate at 60 °C and 65% relative humidity for 3 hours. Functional properties assay showed that glycation significantly increased (P<0.05) the solubility, emulsifying activity index (EAI) and emulsion stability index (ESI). This work suggested that the controlled enzymatic hydrolysis in combination with glycation would effectively improve the functional properties of fish protein hydrolysates.

Keywords: *fish protein hydrolysates, skipjack tuna, enzymatic hydrolysis, glycation, solubility, emulsifying properties*

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1. Introduction

Fish processing by-products and the under-utilized discards are traditionally recognized as low-value resources with negligible market value. Many of them are developed as plant fertilizers and livestock feeds [1]. The others are subjected to inappropriate disposal which is a major cause of environmental pollution [2]. However, these rest raw materials consist of valuable components such as fish oil, proteins, collagen and gelatine, enzymes and minerals, and it has been estimated that if some components are recovered and used in the development of human food products, their value increases five times [3]. Skipjack tuna (*Katsuwonus pelamis*) is one of the most important commercial fish species distributed in both offshore waters and open seas in tropical and temperate regions around the world, including the Pacific, Atlantic, and Indian Oceans [4]. Recently, the main purse-seine fishery regions of skipjack tuna are distributed in the western and central Pacific Ocean. The purse-seine skipjack tuna catch was increased from 818,442 tonnes in 2000 to 1,381,070 tonnes in 2010 [5]. Skipjack tuna is commonly used for canning production, and approximately one-third of the whole fish is not utilized. It has been reported that the skipjack tuna viscera is a good source for recovery of trypsin and pepsin [6,7], but the recovery of under-utilized protein is seldom studied.

Enzymatic hydrolysis is one of the methods for protein recovery and modification from fish under-utilized protein. Fish protein hydrolysates have good functional properties such as water holding, texture, gelling, whipping, solubility and emulsifying properties when added to food [8,9,10]. For example, Gbogouri et al. [11] stated that salmon byproducts hydrolysates with high degree of hydrolysis (DH) had the best solubility, but greater emulsifying capacity and emulsion stability were noticeable when DH was low. These functional properties were improved because of the changed molecular size, hydrophobicity and polar groups of the hydrolysate influenced by hydrolysis [12]. Glycation (or glycosylation) is an effective method for improving the functional properties of food proteins, and it proceeds at a faster rate under the "dry" conditions than the "wet" conditions [13]. Saeki and Inoue [14] reported that the solubility of carp myofibrillar proteins was improved after glycated with glucose at 40 °C and 65% relative humidity for 48 h. Sato et al. [15]

claimed that glycoconjugates between carp myofibrillar proteins and alginate oligosaccharide (50 °C and 35% relative humidity for 120 h) showed excellent emusion-formation ability. As for glycated hydrolysates, Decourcelle et al. [16] stated that glycation of shrimp (*Pandalus borealis*) hydrolysates with xylose significantly enhanced the emulsifying properties. However, there is little information about functional properties of glycoconjugates of fish protein hydrolysates. The combination of glycation and controlled enzymatic hydrolysis might be a promising way for the functionality modification of food proteins, but limited information is available so far [17].

The aim of the present study was to effectively recover and modify proteins from the under-utilized skipjack tuna protein. Therefore, the combined controlled enzymatic hydrolysis and glycation method was expected to obtain the hydrolysate with improved functional properties. These functional properties including solubility, emulsion activity and emulsion stability of the non-glycated and glycated hydrolysate with a model saccharide alginate under a "dry" condition were studied. The present study would provide a possible way out to the problem regarding skipjack tuna under-utilized protein recovery, and provide production of protein hydrolysate with high-quality functional properties applied in food industry.

2. Materials and Methods

2.1. Materials and Reagents

Frozen skipjack tuna (*Katsuwonus pelamis*) caught by purse-seine was provided by Zhejiang Ocean Family Co. Ltd. (Zhejiang, China). Alcalase was purchased from Shanghai Yuanju Biotechnology Co. Ltd. (Shanghai, China). Flavorase, neutrase, trypsin and protamex were purchased from Beijing Dingguo Biotechnology Co. Ltd. (Beijing, China). Bovine serum albumin was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai China). Coomassie brilliant blue R-250 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals and reagents were of analytical grades.

2.2. Enzymatic Hydrolysis

The skipjack tuna meat was minced using a grinder and the mince was stored in a plastic bag at -20 °C until used. The mince were thawed at room temperature for 0.5 h. The lipids were removed according to the method of Bligh and Dyer [18] by chloroform-methanol (2:1, v/v) extraction three times and centrifugation at 4000 rpm. Subsequently, the thawed mince was hydrolysed with commercially available enzymes, alcalase, flavorase, neutrase, trypsin and protamex, under their corresponding optimum hydrolysis conditions as listed in Table 1. Enzymatic hydrolysis was performed in a water batch with constant stiring. The pH of the slurry was constantly maintained by the addition of 1 M NaOH. The resulting hydrolysates were heated in boiling water for 10 min to inactivate the enzyme. After cooling to room temperature, the hydrolysates were centrifuged at 10,000 rpm for 10 min to remove impurities. Half of the hydrolysates were stored in a beaker at -20 °C until used, and another half of the hydrolysates were freeze-dried, sealed, and stored in a desiccator.

Table 1. Hydrolysis conditions of different commercial enzymes

	Alcalase	Flavorase	Neutrase	Trypsin	Protamex
Solid-liquid ratio (w/w)	1:2	1:2	1:2	1:2	1:2
Teperature (ºC)	55	55	50	50	50
pH	8	7	7	8	7
Enzyme dosage (U/g)	20000	20000	20000	20000	20000
Time (h)	5	5	5	5	5

2.3. Determination of the Degree of Hydrolysis

The degree of hydrolysis (DH) was determined according to the method described by Hoyle and Merritt [19] with some modifications. An aliquot of 10 mL hydrolysate was thoroughly mixed with 10 mL of 10% trichloroacetic acid (TCA), and the mixture was standed for 5 min. Then the mixture was subjected to centrifugation at 4000 rpm for 10 min and the supernatant was removed. The 10% TCA soluble nitrogen and total nitrogen content was determined by the Kjeldahl method [20]. The DH was calculated using the following equation:

$$DH = \frac{N_2 - N_1}{N_0 - N_1} \times 100\% \quad (1)$$

where N_2 is 10% TCA soluble nitrogen after hydrolysis; N_1 is 10% TCA soluble nitrogen before hydrolysis; N_0 is the total nitrogen.

2.4. Amino Acid Composition Analysis

An aliquot of 5 mL of each hydrolysate was deproteinized with 5 mL of 4% 5-sulfosalicylic acid dihydrate, and the hydrolysates were filtrated by a 0.45 μm Millipore filter. The amino acid composition was measured with an automatic amino acid analyzer (S-433D, Sykam Co., Eresing, Germany). Tryptophan was not determined.

2.5. Determination of Soluble Protein Content

Soluble protein contents of the hydrolysates were determined by the TCA precipitation method. Briefly, an aliquot of 5 mL of hydrolysate was added to 5 mL of 10% TCA. The mixture was centrifuged at 5,000 rpm for 10 min. The soluble protein concentration of supernatant was evaluated by the Biuret method [22] using bovine serum albumin as a standard protein. The content of soluble protein was calculated as:

$$\text{Soluble protein content} = \frac{M_1 - M_2}{M_1} \times 100\% \quad (2)$$

where M_1 is total protein content; M_2 is protein content in supernatant.

2.6. Glycation of the Hydrolysate

Glycation of the fish protein hydrolysate prepared by trypsin was performed in a controlled dry state. The hydrolysate and alginate were mixed in a ratio of 1:1 (w/w) and the mixture was incubated at 60 °C and 65% relative humidity for 3 hours in an incubator (DHP-9162, Hasuc Instrument Manufacture Co. Ltd., Shanghai, China). After reaction, the mixture was used for the following functional properties analysis.

2.7. Solubility

The samples (hydrolysate and glycated hydrolysate) were dissolved in distilled water to form final concentration of 1.5% (w/v). The pH of the sample solution was adjusted to 2, 4, 6, 8 and 10 by 1 M HCl or 1 M NaOH and standed for 30 min. Then the sample solution was centrifuged at 7,500 rpm for 15 min. The protein concentration was evaluated by the Biuret method [21] using bovine serum albumin as a standard protein. The solubility was calculated as:

$$\text{Solubility} = \frac{\text{Protein content in supernatant}}{\text{Total protein content}} \times 100\% \quad (3)$$

2.8. Emulsifying Properties

Emulsifying properties of hydrolysate and glycated hydrolysate were determined according to the method of Pearce and Kinsella [22]. Corn oil (1 mL) and 3 mL of 1% (w/v) protein solution were mixed and the pH was adjusted to 2, 4, 6, 8 and 10 by 1 M HCl or 1 M NaOH. The mixture was homogenized for 1 min. An aliquot of the emulsion (50 μL) was pipetted from the bottom of the container at 0 and 10 min after homogenization and mixed with 5 mL of 0.1% (w/v) sodium dodecyl sulphate (SDS) solution. The absorbance of the diluted solution was measured at 500 nm. The absorbances measured immediately (A_0) and 10 min (A_{10}) after emulsion formation were used to calculate the emulsifying activity index (EAI) and the emulsion stability index (ESI) as follows:

$$\text{EAI}(m^2/g) = \frac{2 \times 2.303 \times A_0}{0.25 \times \text{Protein weight(g)}} \quad (4)$$

$$\text{ESI(min)} = A_0 \times \Delta t / \Delta A \quad (5)$$

where $\Delta A = A_0 - A_{10}$; Δt = 10 min.

2.9. Statistical Analysis

All the experiments were performed with three independent trials and all the determinations were triplicated, except amino acid composition determination, which was performed once. The results were represented as mean ± standard deviation. Analysis of variance (ANOVA) was performed to identify significant differences (P<0.05).

3. Results and Discussion

3.1. Enzymatic Hydrolysis

The skipjack tuna meat was hydrolyzed by five independent enzymes under the optimum hydrolysis conditions (displaying the highest enzymatic activity) according to the manufacturers, with data listed in Table 1. The enzymes were inactivated after 5 h. The DH was determined and the results were depicted in Figure 1. The fish meat gained the lowest DH of only 2.43% with alcalase treatment, while the protamex treatment gave rise to the highest DH of 78.33%, followed by the trypsin (71.68%). Flavorase and neutrase made the fish meat a considerable DH, with values of 33.80% and 56.72%, respectively. These results were similar to Zhao et al. [23], who reported that protamex and trypsin were found to be the most efficient in terms of the hydrolytic efficiencies in contrast to flavorase, alcalase and neutrase, after rice dreg protein being hydrolyzed under the optimum hydrolysis conditions for 4 h.

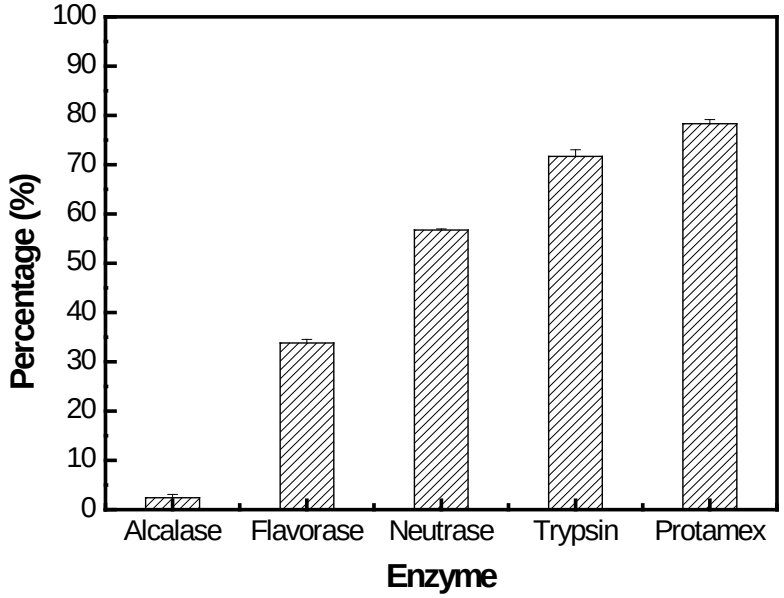

Figure 1. The degree of hydrolysis (DH) of skipjack tuna protein hydrolysates prepared by different enzymes

3.2. Amino Acid Composition Analysis

As shown in Table 2, the amino acid composition of five hydrolysates treated with different enzymes was listed. Except for tryptophan (Trp), other 17 amino acids within hydrolysates were determined. It was found that the total content of amino acids was increased as the increase of DH. For example, alcalase induced the lowest DH (2.43%, Figure 1) and corresponding hydrolysate had the lowest total content of amino acids (1083.75 mg/100 g). With the enzyme type changing to flavorase, neutrase and trypsin, the DH of corresponding hydrolysates increased, and the total content of amino acids was elevated from 2417.18 mg/100 g to 3278.18 mg/100 g. After protamex treatment, the DH of hydrolysate was enhanced to the maximum (78.33%, Figure 1), and the total content of amino acids rose up to 9366.74 mg/100 g. This was because the high DH led to peptides breakdown to a considerable mount of amino acids. His and Pro accounted for the majority in hydrolysate prepared by alcalase. After flavorase treatment, His was also the most abundant amino acid in hydrolysate, but hydrophobic aliphatic amino acid Leu increased with the content ranking next to His. In neutrase-treated hydrolysate, although Leu decreased, other hydrophobic aliphatic amino acids such as Val and Ile increased by a considerable amount. Trypsin-treated hydrolysate had a large number of His, Phe and Tyr, but the lowest content of Ser (9.00 mg/100 g). Protamex-treated hydrolysate was characterized by the highest content of Leu, followed by His and Lys. Therefore, different types of enzyme contributed to different amino acid composition of skipjack tuna meat hydrolysates.

Table 2. Amino acid composition of skipjack tuna protein hydrolysates prepared by different commercial enzymes

Amino acids	Hydrolysates prepared by five enzymes (mg/100 g)				
	Alcalase	Flavorase	Neutrase	Trypsin	Protamex
Asp	33.48	54.66	49.46	16.46	75.12
Thr	21.54	42.90	17.80	16.32	425.90
Ser	25.54	33.08	27.66	9.00	138.88
Glu	40.76	60.12	57.16	29.12	357.64
Gly	18.16	17.46	21.02	12.62	44.34
Ala	77.88	90.12	76.82	62.66	254.38
Cys	26.96	78.76	119.70	52.44	229.22
Val	32.36	82.34	105.18	42.66	645.26
Met	18.22	87.40	144.04	129.44	541.88
Ile	15.40	51.42	111.50	76.46	429.72
Leu	40.14	214.36	123.38	220.10	1340.16
Tyr	18.46	77.58	123.52	298.26	762.40
Phe	22.70	176.76	11.06	444.62	926.64
His	489.14	915.58	1278.58	1337.90	1079.52
Lys	48.64	150.10	85.18	194.00	1010.00
Pro	144.36	139.56	200.28	161.30	170.50
Arg	10.01	144.98	58.68	174.82	935.18
Total	1083.75	2417.18	2611.02	3278.18	9366.74

3.3. Soluble Protein Content

Changes of the soluble protein content during the skipjack tuna meat hydrolysis were investigated to understand the peptide release properties. TCA is able to induce precipitation of protein because of the three chloro groups in the molecule [24] and it has been reported that most of the peptides left in supernatant solution contained three or four amino acid residues [25], i.e., the soluble protein contains considerable small-size peptides. As shown in Figure 2, the soluble protein content of five enzyme-treated hydrolysates was determined every 0.5 h. In the beginning of hydrolysis, approximately 20% of the native skipjack tuna protein survived 10% TCA. Afterwards, there were mainly two trends in change of the soluble protein content. One trend was dipicted by alcalase, flavorase and neutrase-treated hydrolysates, where the most soluble protein content was around 40% at 2.5-3 h. After that, the content was gradually decreased to 25%-35%. Another trend was formed by protamex and trypsin-yielded hydrolysates. Both of the hydrolysates reached the highest soluble protein content of 80% at 1.5-2.5 h, but the sharp drops were seen after that and the content stopped at < 20%. As for the first trend, lower DH was resposible for the lower soluble protein content. High molecular weight proteins were not able to be hydrolyzed to small-size peptides before precipitation. As far as protamex and trypsin-yielded hydrolysates were concerned, the initial fast hydrolysis resulted in the boom of DH. A considerable mount of soluble small-size peptides were formed and survived TCA precipitation. With increase of the hydrolysis time, these small-size peptides were further hydrolyzed to amino acids, which could not be determined by the Biuret method. These results indicated that 5-hour hydrolysis by five enzymes could not maintain the most small-size peptides. To achieve this, a controlled enzymatic hydrolysis by protamex or trypsin is required.

Because of lower price than protamex, trypsin was used to hydrolyze the skipjack tuna protein in a controlled condition: solid-liquid ratio 1:2 (w/w), teperature 50 ºC, pH 8, enzyme dosage 20000 U/g and time 2.5 h. Under

this condition, the soluble protein content of the hydrolysate would reach 80%. The hydrolysate was glycated and determined for the following functional properties.

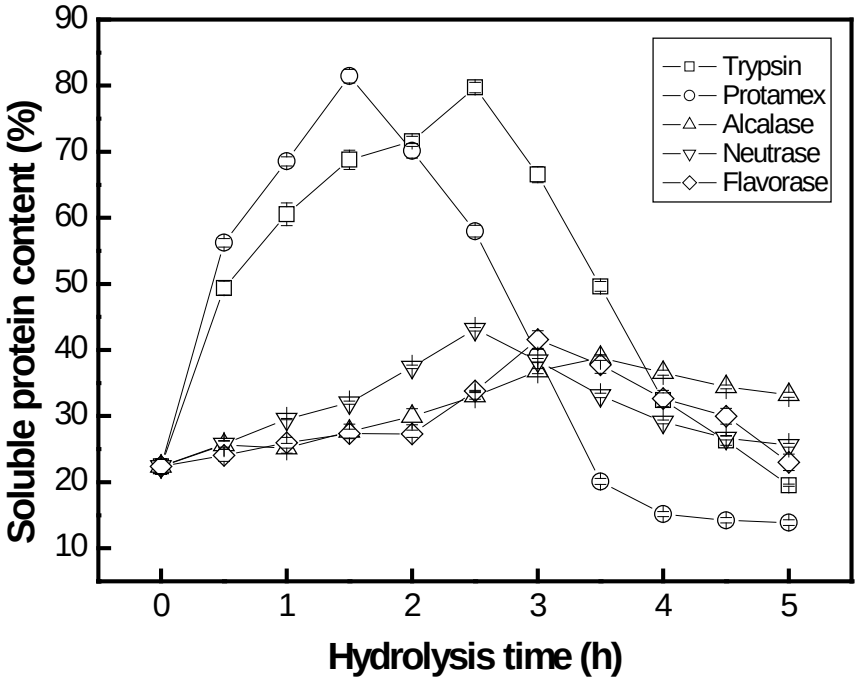

Figure 2. Changes of the soluble protein content during hydrolysis of skipjack tuna protein by different commercial enzymes

3.4. Solubility

Solubility is one of the most important functional properties of food protein hydrolysates. A low solubility may cause an unattractive appearance and a sandy mouthfeel of the final product [26]. Figure 3 (A) describes the solubility of non-glycated and glycated skipjack tuna protein hydrolysate in the pH range of 2-10. Both of the non-glycated and glycated hydrolysate were soluble over a wide pH range with more than 82.37% solubility. Starting from pH 2, there was a steady increase in solubility of non-glycated and glycated hydrolysate, and the maximum appeared at pH 8, from which the solubility of two samples decreased. At pH 8, the solubility of non-glycated and glycated hydrolysate was 96.68% and 98.80%, respectively. It is worthwhile to note that the solubility at pH 8-10 was higher than that at pH 2-6. The result revealed that the basic pH provided better solubility of hydrolysate. This is a general consencus which is also validated by other researchers [2,27,28]. pH is an important factor influencing the solubility of hydrolysate, because the pH affects the charge on the weakly acidic and basic sidechain groups and hydrolysate generally show low solubility at their isoelectric points [27].

In addition, Figure 3 (A) reveals another result that glycated hydrolysate exhibited significantly higher solubility (P<0.05) than the non-glycated counterpart at all pHs. For example, non-glycated and glycated hydrolysate displyed the highest solubility at pH 8, but the solubility of the glycated (98.80%) was significantly higher (P<0.05) than the non-glycated (96.68%). It has been widely accepted that food proteins after glycation would increase their solubility [14], but whether glycation would increase the solubility of food protein hydrolysates is unclear. Liu et al. [13] reported that the isoelectric points of the glycated proteins often shift toward more acidic pH, this might be responsible for an improved solubility of the glycated fish protein hydrolysate discovered in the present study.

3.5. Emulsifying Properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of both non-glycated and glycated skipjack tuna protein hydrolysate as affected by pH are shown in Figure 3 (B) and Figure 3 (C), respectively. As for non-glycated hydrolysate, the lowest EAI and ESI were found at pH 2, where the solubility was also the lowest. Mutilangi et al. [29] deemed that the higher EAI of hydrolysates accompanied their higher solubility, because hydrolysates with high solubility can rapidly diffuse and adsorb at the interface. The highest EAI and ESI of non-glycated hydrolysate were found at pH 8 and 10, respectively. This suggested that the basic pH gives the better emulsifying properties. The result was in agreement with Pacheco-Aguilar et al. [30], who reported that Pacific whiting (*Merluccius productus*) muscle protein hydrolysate displayed the highest EAI and ESI at pH 10 than the low pHs. This might be because molecular repulsion generated among protein/peptides due to negative charge density at basic pH resulted in a more stable and cohesive interface, thus retarding emulsion coalescence [30].

Figure 3 (B) and Figure 3 (C) also reveal that glycated hydrolysate exhibited significantly higher EAI and ESI (P<0.05) than the non-glycated counterpart at all pHs, except for EAI at pH 6, where EAI showed almost the same value between the non-glycated and the glycated hydrolysate. This indicated that the emulsifying properties of skipjack tuna protein hydrolysate were improved after glyction with alginate. It has been reported that the emulsifying properties of fish myofibrillar protein were greatly elevated after glycation [31]. To the best of our

knowledge, only Decourcelle et al. [16] reported the emulsifying properties of glycated hydrolysate, in which a glycated shrimp hydrolysate with xylose was significantly enhanced (P<0.05) in emulsion activity. Nakamura et al. [32] pointed out the reason for elevated emulsifying properties might be that the polysaccharide chains attached to the protein/peptides suppress the association of each oil droplet or the aggregation among protein/peptides. On the other hand, the alginate used for glycation in the present study is branched and long chain length saccharide, which provides more steric hindrance, preventing coalescence of the coated fat droplets [33].

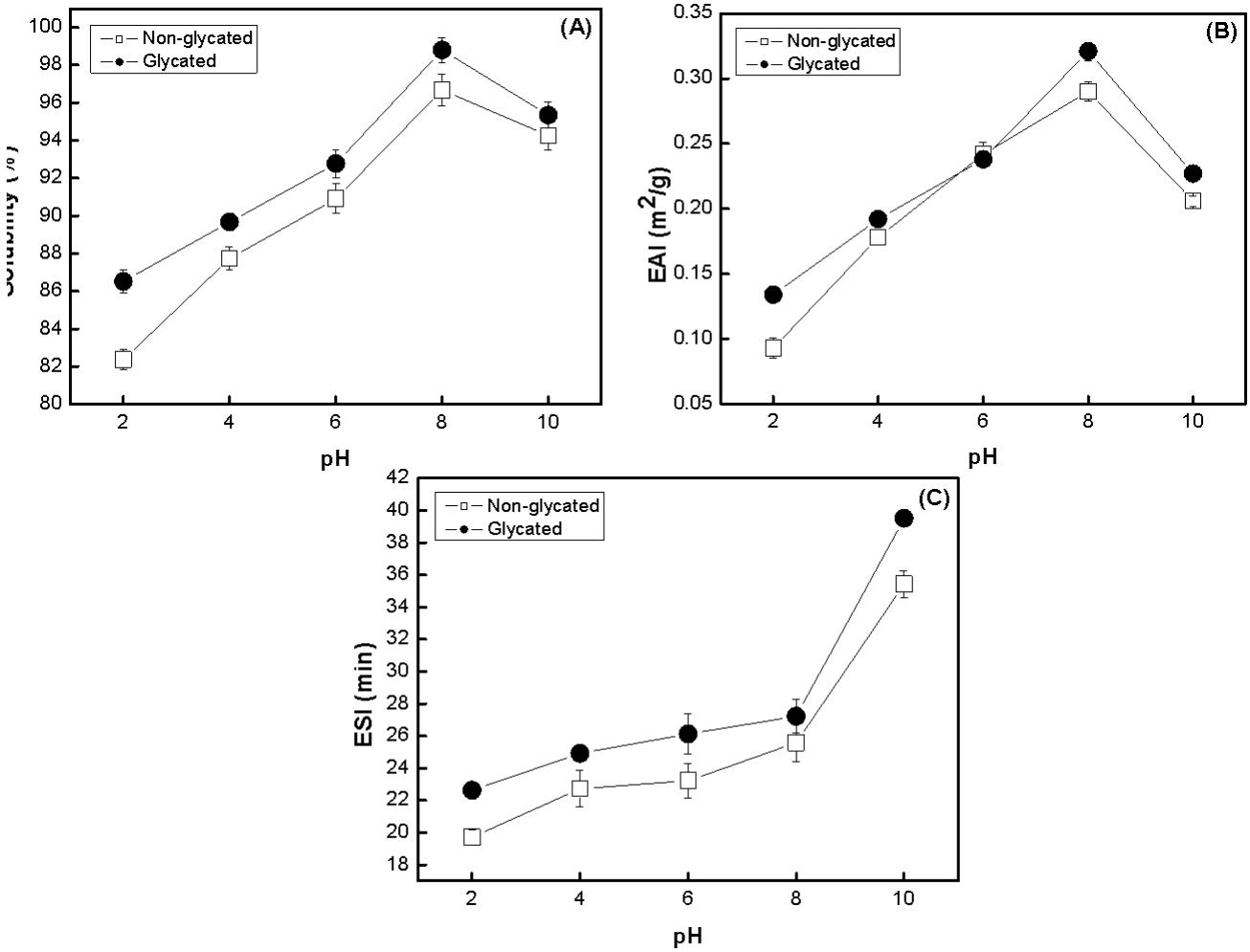

Figure 3. Functional properties of non-glycated and glycated hydrolysate prepared by trypsin under the controlled enzymatic hydrolysis condition: solid-liquid ratio 1:2 (w/w), teperature 50 ºC, pH 8, enzyme dosage 20000 U/g and time 2.5 h. Effect of pH on the (A) solubility, (B) emulsifying activity index (EAI) and (C) emulsion stability index (ESI) of non-glycated and glycated hydrolysate

4. Conclusion

Commercially available enzymes of alcalase, flavorase, neutrase, trypsin and protamex under the optimum hydrolysis conditions dramatically modified the molecular properties of under-utilized skipjack tuna protein. However, 5-hour hydrolysis could not obtain considerable small-size peptides. Therefore, a controlled enzymatic hydrolysis by trypsin was implemented: solid-liquid ratio 1:2 (w/w), teperature 50 ºC, pH 8, enzyme dosage 20000 U/g and time 2.5 h. This condition produced the 80% soluble protein within the hydrolysate. Glycation significantly enhanced (P<0.05) the solubility, emulsifying activity and emulsion stability of hydrolysate. The basic pH contributed to the increases of these properties. The present study suggested that the controlled enzymatic hydrolysis by trpsin and glycation with alginate would greatly improve the functional properties of food protein hydrolysates. This combined method would also be applied for other food protein modification. Further study will focus on the interaction between protein hydrolysate and saccharide, structure of glycoconjugate, and mechanisms of improved functional properties.

Acknowledgments

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