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A Comparative Study between Tilapia (*Oreochromis niloticus*) By-product and Tilapia Protein Hydrolysate on Angiotensin I-converting Enzyme (ACE) Inhibition Activities and Functional Properties

(Kajian Perbandingan antara Hidrolisat Protein Bahan Sampingan Tilapia dan Otot Tilapia (*Oreochromis niloticus*) terhadap Perencatan Enzim Pengubah Angiotensin (ACE) dan Sifat Kefungsian)

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ABSTRACT

*Tilapia is a popular freshwater fish and among the important cultured fish grown worldwide. In this study, fish protein hydrolysate was produced from tilapia (*Oreochromis niloticus*) by-product (TB) and tilapia muscle (TM) through enzymatic hydrolysis using alcalase. The TB and TM protein hydrolysates were evaluated for its characteristics in terms of angiotensin I-converting enzyme (ACE) inhibition activity, peptide size distribution, and functional properties. Hydrolysis for 1 h for TB and TM successfully produced low molecular weight peptides (<14.2kDa) with the highest ACE inhibitory activities. The findings also demonstrated that both samples have high nitrogen solubility (>80% at pH2-9) and good emulsifying, water and oil holding capacities. The study indicated that tilapia protein hydrolysates have the potential to be used as functional food products.*

Keywords: Aangiotensin I-converting enzyme (ACE) inhibition activity; functional properties; Tilapia by-product protein hydrolysate; tilapia muscle protein hydrolysate

ABSTRAK

*Tilapia (*Oreochromis niloticus*) merupakan ikan air tawar terkenal dan antara ikan terpenting yang diternak di seluruh dunia. Dalam kajian ini, hidrolisat protein ikan telah dihasilkan daripada bahan sampingan tilapia (TB) dan otot tilapia (TM) melalui hidrolisis berenzim menggunakan alkalase. Hidrolisat protein TB dan TM dinilai ciri-cirinya daripada segi aktiviti perencatan enzim pengubah angiotensin (ACE), taburan saiz peptida dan sifat kefungsian. Hidrolisis selama 1 jam berjaya menghasilkan peptida dengan berat molekul rendah (<14.2kDa) dengan aktiviti perencatan ACE yang tertinggi. Keputusan penemuan juga menunjukkan bahawa kedua-dua hidrolisat protein mempunyai kelarutan nitrogen yang tinggi (>80% pada pH2-9), dan kapasiti mengemulsi, memegang air dan memegang minyak yang baik. Kajian ini menunjukkan bahawa hidrolisat protein tilapia mempunyai potensi untuk digunakan sebagai produk makanan berfungsi.*

Kata kunci: Hidrolisat protein bahan sampingan tilapia; hidrolisat protein otot tilapia; perencatan aktiviti enzim pengubah angiotensin (ACE); sifat kefungsian

INTRODUCTION

A large amount of by-products is generated during tilapia processing, including skins, bones, frames and tails. These by-products are normally disposed without any attempts to make them useful. Fish by-products from various fish species contain considerable quantities of protein that is known to possess high nutritional value in terms of essential amino acid (AA) composition (Venugopal et al. 1996) and rich in protein content that varying from 13% up to 61% (Fahmi et al. 2004; Je et al. 2004; Sathivel et al. 2003; Wasswa et al. 2007). These fish by-products are potentially to be converted into value added products because it's can be a useful source of bioactive compounds such as bioactive peptides (Chalamiah et al. 2012; Kim & Mendis 2006). Normally, enzyme technology is widely used in fish protein modification, applied for improving production efficiency and quality as well as developing new food ingredients or products (Kristinsson & Rasco 2000; Sathivel et al. 2003; Thiansilakul et al. 2007). Alcalase was found to be an efficient enzyme for fish protein hydrolysis (Adler-Nissen 1986; Aspmo et al. 2005) because of its ability to attain a high degree of hydrolysis under mild conditions in a short time (Aspmo et al. 2005). The resulting fish protein hydrolysate possesses good nutritional and functional properties (Amiza et al. 2013, 2011; Diniz & Martin 1997; Foh et al. 2011; Klompong et al. 2007; Kristinsson & Rasco 2000; See et al. 2011; Wasswa et al. 2007).

In recent years, the production of fish protein hydrolysates (FPH) from fish by-products has received a significant attention due to their abundance in essential nutrients and bioactive peptides that offers physiological functions, such as antihypertensive activity for the

applications in healthcare and pharmaceutical products (Je et al. 2004; Jung et al. 2006; Kim et al. 2001). Isolation and description of bioactive peptides from fish by-products have been reported by several researchers and specific peptide capable of inhibiting angiotensin I-converting enzyme (ACE) activity have been identified (Bougatef et al. 2008; Je et al. 2004; Jung et al. 2006; Kim et al. 2001; Lee et al. 2010). ACE shows a significant function in the regulation of blood pressure and hypertension (Raghavan & Kristinsson 2009) by converting the inactive form of decapeptide angiotensin I into the potent vasoconstrictor octapeptide angiotensin II. In addition, this product deactivates the vasodilating nonapeptide bradykinin. For that reason, inhibition of ACE activity is a good target for antihypertension treatment. At this time, the most common methods for such treatment employ synthetic drugs, such as captopril, enalapril, alacepril and lisinopril (Ondetti 1977). However, these synthetic drugs can cause adverse effects, such as cough, taste disturbances and skin rashes (Je et al. 2004). Thus, it is very desirable and indeed necessary to find suitable alternatives from natural sources that provide safe and economical applications in place of synthetic drugs.

FPH can be prepared in controlled conditions in order to improve the biological activity, physicochemical and functional properties that can be used as functional foods. Protein hydrolysate from tilapia muscle has been extensively studied and shown to possess ACE -inhibitory activity (Raghavan & Kristinsson 2009), antioxidant activity (Dekkers et al. 2011; Shamloo et al. 2012) and good physicochemical properties (Foh et al. 2011). However, study on the production of protein hydrolysate from tilapia by-product (TB) is still considered less especially from its frame, bone, tail and head. Only studies on the production of FPH from tilapia skin (Wasswa et al. 2008; Yang et al. 2009) and tilapia scale (Ngo et al. 2010) have been reported. Therefore, the aim of this study was to evaluate ACE -inhibition activity and functional properties of FPH produced from TB using alcalase and TM will be used as control. These findings are significant as it provides beneficial information concerning tilapia protein hydrolysates for numerous uses such as for food and pharmaceutical products.

Materials and Methods

Preparation of Raw Material

Fresh red tilapia (*Oreochromis niloticus*) were obtained live from a local fish farm in Rawang, Selangor. The samples were then brought to the laboratory and immediately processed (washed, eviscerated and hand filleted). Then, the muscle and by-product (head, frames and tail) were separated. 1 kg of tilapia managed to generate approximately 600 g (60%) of tilapia by-product (TB) and 400 g (40%) of tilapia muscle (TM). TB and TM were then minced using a blender, packed in small polyethylene plastic bags, frozen, and stored at –20 until further use.

Enzymatic Hydrolysis Reaction

Minced TB and TM were thawed overnight in a cold room (4). To prepare TB hydrolysate, 15% w/v of minced TB was mixed with 50 mL of 50 mM of PBS (pH7.5) and the mixture was preincubated at 60 for 20 min prior to adding the 2.5% w/w alcalase enzyme (in solution form with declared activity of 2.4 activity units [AU]/mL and a density of 1.18 g/mL), to initiate the enzymatic hydrolysis reaction (Roslan et al. 2015). As for TM, 2.5% w/v of minced TM was mixed with 50 mL of 50 mM phosphate buffer solution (PBS, pH7.5) and the mixture was pre-incubated at 50 for 20 min prior to adding the 4.0% w/w Alcalase enzyme (Roslan et al. 2014). Hydrolysis was carried out at various reaction time ranging from 0.5 to 12 h and the degree of hydrolysis (DH) was determined.

Determination of Degree of Hydrolysis (DH)

Degree of hydrolysis (DH) is described as the percentage of cleaved peptide bond in protein hydrolysate:

$$DH = \frac{h}{h_{tot}} \times 100 \quad (1)$$

where h_{tot} is the total number of peptide bonds in the substrate and h is the number of hydrolyzed bonds. In the present study, the OPA method was used to measure DH, as described by Church et al. (1983) and Nielsen et al. (2001) with some modification. Briefly, the OPA reagent was prepared by dissolving 7.62 g sodium tetraborate decahydrate and 200 mg sodium dodecyl sulfate in 150 mL deionized water. Then, the solution was mixed with 160 mg o-phthaldehyde 97% in 4 mL ethanol followed by adding 400 µL of β-mercaptoethanol into the solution and finally made up to 200 mL using deionized water. A standard solution was prepared using 50 mg serine in 500 mL deionized water (0.9516 µmole/L). A total of 400 µL of serine standard or protein hydrolysate was added with 3 mL OPA reagent and mixed for 5 s. At room temperature, the mixtures were incubated for 2 min and a UV-VIS spectrophotometer was used to measure the absorbance at 340 nm. The concentration of peptide bond released was determined from the calibration curve.

Tricine SDS-PAGE Analysis

All the samples that was obtained from reaction time at 0.5 – 12 h were analysed using Tricine-SDS-PAGE which was conducted by following a method explained by Schagger and von Jagow (1987) with some modification. 4% of stacking and 16% of separating gels were prepared using gel buffer (3M Tris-HCl, 0.3% SDS, pH8.45), acrylamide/bisacrylamide (48% acrylamide, 1.5% bisacrylamide), ammonium persulfate, glycerol, distilled water and TEMED. Sample solution was mixed with the sample buffer (1M Tris-HCl, pH6.8, containing 20% SDS, glycerol and Coomassie Brilliant Blue) followed by heating at 90°C for 10 min prior to loading about 8 µL of aliquots into individual wells. Protein standards between 1.06 kDA and 26.6 kDA were also analysed on

the gels. After electrophoresis, the gels were mixed with fixing solution (50% methanol, 10% acetic acid), staining solution (Coomassie Blue, 10% acetic acid) and destaining solution (10% acetic acid). After the desired bands were appeared, the samples were matched with the known of protein standards bands.

ACE INHIBITORY ACTIVITY OF TB HYDROLYSATE

ACE inhibition activity was measured by monitoring the release of hippuric acid (HA) from the hydrolysis of the substrate hippuryl-histidyl-leucine (HHL) using ACE solution. This method was conducted following the method explained by Jimsheena and Gowda (2009) with some modification. HHL was prepared in 0.05 M of potassium phosphate buffer (pH8.2) containing of 0.3 M NaCl. About 50 µL of TB hydrolysate and 50 µL of ACE solution was mixed and preincubated at 37 for 10 min. Then, 150 µL of HHL solution was mixed and incubated at 37 for 60 min. The reaction was stopped with addition of 250 µL of 1.0 M HCl. Then, 400 µL of pyridine was mixed followed by 200 µL of BSC. The solution was mixed using a vortex mixer and then cooled on ice. After the yellow color was developed, then it was measured at 410 nm using spectrophotometer. The ACE inhibition percentage was calculated as follows:

$$\text{ACE – inhibitory activity (\%)} = \frac{A - B}{A - C} \times 100 \quad (2)$$

where A is the absorbance of solution without sample; B is the absorbance of solution with sample; and C is the absorbance of blank solution (without ACE solution and sample).

DETERMINATION OF FUNCTIONAL PROPERTIES

NITROGEN SOLUBILITY

Nitrogen solubility of TB and TM protein hydrolysates were measured according to the method described by Diniz and Martin (1997) with a slight modification. Briefly, 2.5 g of sample was dissolved in 25 mL of distilled water and the pH of the mixtures were adjusted in the range of 2 to 9 with either 0.5 N HCl or 0.5 N NaOH. The mixtures were constantly stirred at room temperature for 35 min. After that, the mixture was centrifuged at 2800 × g for 35 min at 4. 1 mL of the supernatant was analyzed for Nitrogen (N) content by the Kjedahl method. The nitrogen solubility was calculated as follows:

$$\text{Nitrogen solubility (\%)} = \frac{\text{Supernatant (N) concentration}}{\text{Sample (N) concentration}} \times 100\%. \quad (3)$$

WATER HOLDING CAPACITY (WHC)

The water holding capacity (WHC) of TB and TM protein hydrolysates were evaluated according to the method of Diniz and Martin (1997) with slight modification. About 0.5 g of sample was placed into 50 mL centrifuge tube and 10 mL of distilled water was added. The mixtures were vortexed for 30 s and the dispersions were then allowed to stand at room temperature for 60 min and centrifuged at 2800 g for 25 min at 25. The supernatant was filtered with Whatman No. 1 filter paper in order to remove the soluble aqueous fraction. The recovered volume of mixtures after filtration was measured. The difference between the initial volume of distilled water added to the protein sample and the volume of the supernatant was determined. The water holding capacity was expressed as mL of water absorbed per g of protein sample.

OIL HOLDING CAPACITY (OHC)

The oil holding capacity (OHC) of TB and TM protein hydrolysates were determined according to the oil titration method by Diniz and Martin (1997). About 0.5 g of each sample was taken into 50 mL centrifuge tube and 10 mL canola oil was added. The mixture was vortexed for 30 s in triplicate. The oil dispersion was centrifuged at 2800 g for 25 min at 25. After centrifugation, the canola oil was decanted. The oil holding capacity of sample was measured from the volume difference. The oil holding capacity was calculated as the amount of oil absorbed per g of sample.

EMULSIFYING CAPACITY (EC)

The emulsifying capacity of TB and TM protein hydrolysates were determined using the method described by Diniz and Martin (1997). 0.25 g of sample and 15 mL of canola oil were added to 30 mL of NaCl and homogenized with the Homogenizer Wisemix HG 515A (Translab Malaysia) at 9500 rpm for 30 min in order to produce emulsion. After that, another 15 mL of canola oil was added over 1.5 min and homogenized for 30 s. The mixture was transferred to centrifuge tubes, held in water bath at 85 for 15 min and then centrifuged at 3000 × g for 20 min. EC was calculated as in equation:

$$EC = \frac{V_A - V_R}{W_s},$$

where is the volume of oil added to form an emulsion; is the volume of oil released after centrifugation; and is the weight of the sample.

STATISTICAL ANALYSIS

All the experiments were conducted in triplicate (n=3) and were subjected to statistical analysis using the Statistical Analysis System (1989) with ANOVA and Duncan's multiple range test were used for multiple comparison. Standard deviation was calculated using the same software.

RESULTS AND DISCUSSION

ENZYMATIC HYDROLYSIS OF TB AND TM AT DIFFERENT TIME REACTIONS

In this study, the enzymatic hydrolysis of TB and TM was carried out using alcalase enzyme at different reaction time

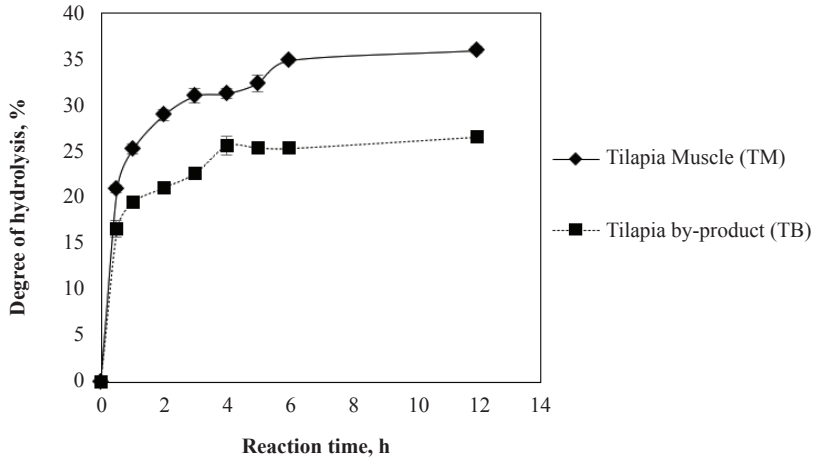

FIGURE 1. Effect of reaction time on degree of hydrolysis of tilapia by-product and tilapia muscle

(0.5 up to 12 h) in order to obtain the highest possible DH within the time range studied. The enzyme's effectiveness was monitored as the progression of the percentage of cleaved peptide bond, denoted as DH (Figure 1) (Adler-Nissen 1986).

In the initial stage (0.5-4 h), hydrolysis rate was increased significantly (p<0.05) for both TB and TM with DH values obtained from 16.58-25.59% and 20.94-31.25%, respectively. Subsequently, the rate for TM increased slowly (p<0.05) as time increased from 5 to 12 h with the DH value of 32.37% to 35.91%. As for TB, there was no significant increased (p>0.05) with DH value of 25.59-26.55%, indicating TB has relatively reached a steady state phase. The hydrolysis rate become slowly were observed for both TB and TM which might be due to the reduction in peptide bonds available for hydrolysis as time of reaction increases from 4 to 12 h. Therefore, it could be suggested that 4 and 6 h of hydrolysis is sufficient to obtain the highest DH value for TB (25.59%) and TM (34.83%) using alcalase, respectively. This is based on the results obtained, which shows that there is no significant (p>0.05) increase in DH value achieved after reaching that particular time. The results also showed that the DH value for TM is much higher compared to TB, indicating that hydrolysis on different parts of the same species of fish would essentially give different DH values. The difference in the DH value between TB and TM mostly contributed to the difference in protein content and AA compositions that affected the catalytic action of alcalase. The hydrolysis curves obtained here exhibited profiles similar to a reports for Pacific whiting solid wastes (Benjakul & Morrissey 1997), Atlantic salmon muscle (Kristinsson & Rasco 2000), yellow stripe trevally (Klompong et al. 2007), tilapia muscle (Foh et al. 2011) and capelin (Shahidi et al. 1995).

The present hydrolysis of fish protein was characterized by an initial rapid phase, during which a large number of peptide bonds were hydrolyzed. Concurrently, the enzyme was rapidly absorbed onto insoluble protein particles and polypeptide chains cleaved those that were loosely bound to the surface (Benjakul & Morrissey 1997). The rate then decreased and reached a stationary phase in which no apparent hydrolysis took place. The reduction in hydrolysis rate might be attributed to several factors, such as the decrease of available substrates over time due to the decrease in concentration of peptide bonds available for hydrolysis (Benjakul & Morrissey 1997; Guerard et al. 2001) a decrease in enzyme activity by the products formed at high degree of hydrolysis (Guerard et al. 2001; Souissi et al. 2007). In addition, high concentrations of soluble peptides in the reaction mixture can reduce both the hydrolysis rate and soluble protein recovery (Shahidi et al. 1995).

PEPTIDE MOLECULAR RANGE OF TB AND TM PROTEIN HYDROLYSATES

Enzymatic hydrolysis on fish protein can produce peptide with various sizes. Determination of FPH size distribution is required in order to recognize the chain length of peptides produced, which can relate with its functional properties. Electrophoresis shows that TB and TM protein hydrolysates possessed a distinctive peptide profile at different DH values (Figure 2(a) and 2(b)). Both preparations exhibited wide ranges of low molecular weight peptides, from 1.06 to 26.6 kDa and also, peptide sizes >26.6 kDa. Peptide sizes were observed to decrease with increasing reaction time, which were comparable to the results obtained by Benjakul and Morrissey (1997), Kristinsson and Rasco (2000) and Quaglia and Orban (1990) with most of the peptide sizes were detected at low molecular weight <14.2 kDa. From SDS-PAGE results, smaller peptide sizes, <14.2 kDa, were clearly observed at the early stages (hydrolysis for 0.5 to 2 h), but, after more than 3 h of hydrolysis, this formerly visible band became unclear. This observation was probably because of the high hydrolytic activity of alcalase enzyme, which continue to hydrolyze peptides of <14.2 kDa to even smaller sizes (<1 kDa). A similar behavior was observed for both samples. Several studies have shown alcalase's ability via a high degree of substrate hydrolysis, to produce low molecular weight

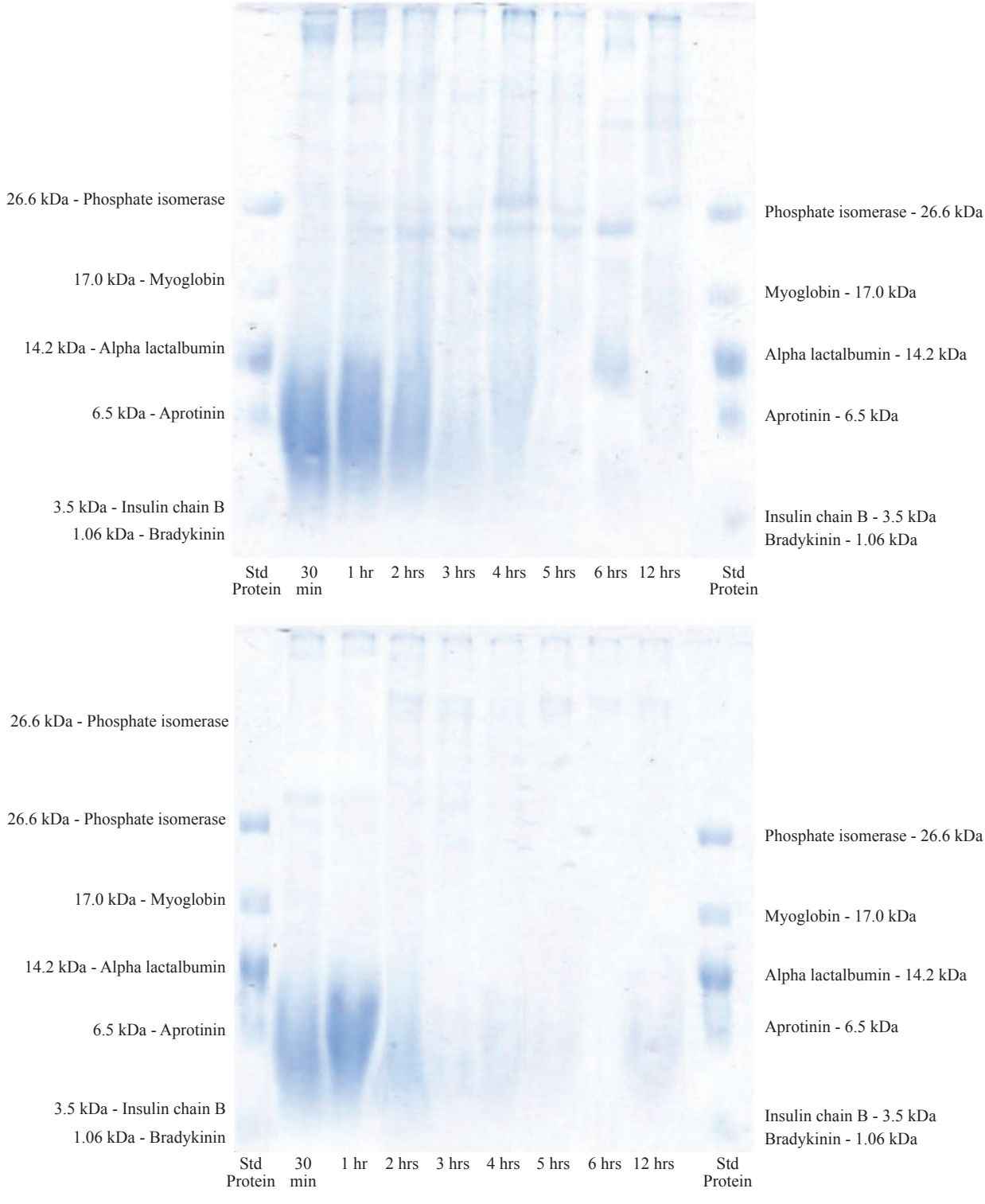

FIGURE 2. SDS-PAGE profiles of (a) TB protein hydrolysate and (b) TM protein hydrolysate

peptides (Benjakul & Morrissey 1997; Lalasidis et al. 1978; Liaset et al. 2000).

IN VITRO STUDIES ON ACE -INHIBITORY ACTIVITIES OF TB AND TM PROTEIN HYDROLYSATES

Production of ACE -inhibitory peptides from FPH has received much attention because of its ability to be used as an alternative treatment, instead of drugs for hypertension. Several studies have demonstrated that FPH very effectively inhibits ACE activity (Je et al. 2004; Jung et al. 2006; Kim et al. 2001). TB and TM protein hydrolysates were further analyzed for their ACE -inhibition activity. Table 1 shows ACE -inhibition activity of these hydrolysates at various time reactions. Both TB and TM protein hydrolysates exhibited high inhibitory activities ranging from 71-89%. At the initial stages of hydrolysis (0.5 h), ACE -inhibitory

TABLE 1. ACE-inhibitory activity of TB and TM protein hydrolysates at different time of hydrolysis

Reaction time, h	ACE inhibition activity of TB protein hydrolysate (%)	ACE inhibition activity of TM protein hydrolysate (%)
0.5	83.79 ± 0.54^{c}	85.80 ± 1.37^{a}
1	89.04 ± 1.42^{a}	87.45 ± 0.98^{a}
2	88.26 ± 0.83^{a}	87.63 ± 1.20^{a}
3	86.96 ± 1.52^{a,b}	85.75 ± 2.34^{a}
4	86.59 ± 0.74^{a}	81.51 ± 1.37^{b}
5	85.94 ± 1.24^{b}	81.32 ± 1.04^{b}
6	81.95 ± 1.53^{c}	81.04 ± 1.14^{b}
12	75.64 ± 0.61^{d}	78.14 ± 0.69^{c}

All data expressed as mean ± standard deviation (n=3), Different superscript letters within each column indicate significant differences ($p < 0.05$), Similar superscript letters within each column were not significantly different ($p > 0.05$)
ACE=Angiotensin I-converting enzyme

activities were around 83.79 and 85.80% with DH values of 16.58 and 20.94% for TB and TM protein hydrolysates, respectively. High ACE -inhibitory activities were found in TB protein hydrolysates at hydrolysis time from 1-4 h with values ranging from 86.96-89.04%. As hydrolysis prolonged to 5–12 h, ACE -inhibitory activities decreased gradually from 85.94 to 75.64%. A similar trend was found in ACE -inhibitory activities for TM protein hydrolysates. Hydrolysis for 1-3 h, high ACE -inhibitory activities were obtained for TM protein hydrolysates with values ranging from 85-87%, but these values did not show any significant differences (p>0.05). However, ACE -inhibitory activities decreased significantly (p<0.05) as hydrolysis time was increased from 4 to 12 h (81.51-78.14%).

This result clearly demonstrated that hydrolysis time greatly influences ACE -inhibitory activity and that high ACE -inhibitory activity can be obtained from around 1 to 4 h of hydrolysis which is at low DH value for both TB protein hydrolysate (19.51-25.59%) and TM protein hydrolysate (25.20-31.25%). The longer the hydrolysis time (high DH value), the lower the ACE -inhibitory activity. Most studies demonstrated that DH values as well as ACE inhibitory activity increased as the hydrolysis progressed (Balti et al. 2010; Je et al. 2004; Jung et al. 2006; Lee et al. 2010; Raghavan & Kristinsson 2009) which possibly due to the high concentration of small peptides-sizes produced at the longer hydrolysis time. However, a few studies have found that ACE inhibitory decreased as the hydrolysis progressed (Bougatef et al. 2008; Theodore & Kristinsson 2007). This suggested that small peptide-sizes did not necessarily give a high ACE inhibitory activity.

In this study, although the presence of smaller peptides was clearly observed as hydrolysis proceeded, inhibitory activity did not increase but instead decreased. Enzymes such as alcalase contain both endopeptidase and exopeptidase activities, which may be involved in the inactivation of the active peptide sequence, particularly exopeptidase by cutting one or more amino acids from N-terminal or C-terminal positions (Bougatef et al. 2008). As DH increases, there is a possibility for the enzyme to cleave certain amino acids with highly potent inhibitory of ACE such as tryptophan, phenylalanine, tyrosine, or proline at their C-terminal and branched aliphatic amino acids at the N-terminal (Bougatef et al. 2008) thus lowering inhibition efficiency (Theodore & Kristinsson 2007).

The results have shown that the highest ACE -inhibitory activity can be achieved when hydrolysis was carried out for 1 h, which indicating that a short hydrolysis duration is sufficient to produce TB and TM hydrolysates with high ACE -inhibitory activity. The hydrolysates produced at 1 h was selected for the amino acid compositions and functional properties analyses. The TB and TM protein hydrolysates were freeze-dried prior to the next analyses.

Functional Properties of TB and TM Protein Hydrolysates

Nitrogen solubility of TB and TM protein hydrolysates

High solubility is a crucial criteria for proteins to be utilized in food systems, especially in liquid foods and beverages to improve other functional properties such as emulsification and foaming (Kristinsson & Rasco 2000). Generally, protein solubility is affected by amino acid composition and sequence, molecular weight, and conformation of polar and nonpolar groups in amino acids (Gbogouri et al. 2004; Klompong et al. 2007; Turgeon et al. 1991). It is important to utilize the amino acid composition and conformation of proteins, including hydrophilic and hydrophobic properties that influence protein solubility. Nitrogen solubility values for TB and TM protein hydrolysates were evaluated at pH range of 2-9 (Figure 3) and all hydrolysates were soluble at more than 80%. High solubility of protein hydrolysates are usually related with the degradation of protein structures into low molecular weight peptides during enzymatic hydrolysis which lead to unfolding of protein molecules and exposed both polar and non-polar amino acid that buried inside protein molecules (Klompong et al. 2007; Kristinsson & Rasco 2000). Smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, which increase the ability to form hydrogen bonds with water (Gbogouri et al. 2004; Klompong et al. 2007). Consequently, hydrolysates with

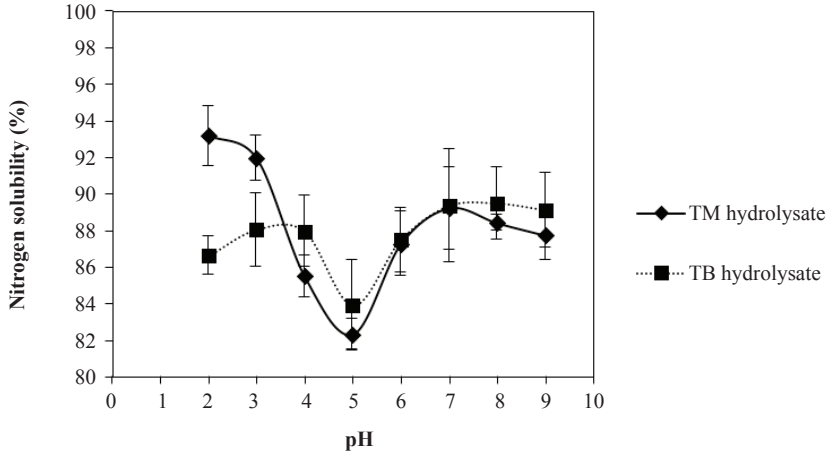

FIGURE 3. Nitrogen solubility of TB and TM protein hydrolysates at different pH

smaller peptides have higher solubility (Gbogouri et al. 2004; Kristinsson & Rasco 2000).

The lowest solubility of TB and TM protein hydrolysates were found at pH5 and the pH at which the minimum solubility occurred can be considered as isolelectric point. Hydrolysates generally exhibit their lowest solubility at their isoelectric points and different sources of protein hydrolysates have a different in Ip (Diniz & Martin 1997; Krisstinsson & Rasco 2000). Ip is the pH at which the net charge of a protein is zero and charge repulsion is minimized, increasing the tendency for protein-protein interactions and aggregation via hydrophobic interaction, resulting in insolubility and reducing protein-water interactions (Diniz & Martin 1997; Kristinsson & Rasco 2000).

TB and TM protein hydrolysates exhibited high solubility values at both acidic and alkaline conditions, as displayed with the U-shaped curve. TM protein hydrolysate showed a higher nitrogen solubility (>90%) under acidic conditions (pH2-3), as compared to basic condition (pH8-9). This probably due to large ratio of surface hydrophilic residues to surface nonpolar groups, contributing to the high protein solubility. High electrostatic repulsion between peptide with positive net charge residues at acidic condition, could also promote its solubilization. Nitrogen solubility slightly increase with the increase of pH from pH6 to 9 with values ranging from 87-89%. Lower nitrogen solubility was observed for TB protein hydrolysate under acidic conditions (pH2-3) with values ranging from 86.62-88.04% as compared to TM protein hydrolysate (91.94-9320%). This is could be due to the higher content of aspartic acid and glutamic acid in TB protein hydrolysate (161.43 mg g^{-1} - unpublished data) than TM protein hydrolysate (101.78 mg g^{-1} - unpublished data) which exhibited a lower solubility at acidic condition as a result of the lack of repulsion. According to Damodaran (2008), most of food proteins exhibited a minimum solubility at acidic condition when the sum of aspartic acid and glutamic acid residues is greater than the sum of lysine, arginine and histidine. From our data (unpublished), the total content of aspartic acid and glutamic acid residues is higher (161.43 mg g^{-1}) compared to the total content of lysine, arginine and histidine (93.82 mg g^{-1}) in TB protein hydrolysate and this could leads to a lower solubility. A slight decrease of nitrogen solubility at pH4 and then significantly (p<0.05) decreased when pH was changed to pH 5 (83.91%), which is supposed to be the isoelectric point of TB protein hydrolysate. At pH 6-8, nitrogen solubility showed increase and reached the highest value at pH8 (89.48%). Above the Ip (basic condition), the net charge is negative and solubility is enhanced. Most proteins are highly soluble at alkaline conditions (Damodaran 2008). A fluctuated nitrogen solubility for both TM and TB at each pH studied (pH2-9) is could be due to differences in amino acid compositions and conformation of polar and non-polar groups in amino acids as a result of electrostatic and hydrophobic interaction between polar and non-polar groups of amino acids. Solubility is increased if electrostatic repulsion between the molecules is higher than hydrophobic interaction. High solubility of TB and TM protein hydrolysates at a wide range of pH, indicates that both samples have potential applications in many food systems.

WATER HOLDING CAPACITY (WHC)

Water-protein interactions strongly influence protein functional properties. Water-holding capacity (WHC) represents a protein's water-absorption ability, which also retains its position within a protein matrix and it is positively associated with water-binding capacity (Damodaran 2008). According to Diniz and Martin (1997), a good WHC value of proteins is considered to reach up to 15 mL/g. The WHC results for TB and TM protein hydrolysates is shown in (Table 2), which TM protein hydrolysate has significantly higher (p<0.05) in WHC value (4.33 mL g^{-1}) as compared to TB protein hydrolysate (4.07 mL g^{-1}). These values are rather high compared with the WHC results reported from Atlantic salmon muscle hydrolysates (0.92 -1.24 mL g^{-1}) (Kristinsson & Rasco 2000), Grass carp skin hydrolysates (2.0-4.9 mL g^{-1}) (Wasswa et al. 2007) and minced tilapia meat hydrolysates (1.77-2.10 mL g^{-1}) (Foh et al. 2011). However, the present results were quite similar to the results (4-5 mL g^{-1}) reported by Diniz and

TABLE 2. Functional properties of TB and TM protein hydrolysates

	TB hydrolysate	TM hydrolysate
Water holding capacity (mL g^{-1})	4.07 ± 0.12^{b}	4.33 ± 0.12^{a}
Oil holding capacity (mL g^{-1})	3.93 ± 0.12^{a}	4.00 ± 0.20^{a}
Emulsifying capacity (mL g^{-1})	21.33 ± 2.31^{b}	34.00 ± 2.00^{a}

All data expressed as mean ± standard deviation (n=3)
Different superscript within each row indicate significant differences (p<0.05)

Martin (1997). Based on the WHC value of fish proteins (Diniz & Martin 1997), TB and TM protein hydrolysates have shown low abilities to bind and hold water, indicating that both samples may not be as good as protein as water-binding agents. These results were in agreement with previous findings (Diniz & Martin 1997), in which shark protein hydrolysates have lower WHC compared with the original protein substrate. Degradation of protein structure through enzymatic hydrolysis results in diminishment of the sample's absorption capacity (Diniz & Martin 1997).

OIL HOLDING CAPACITY (OHC)

Another important food functional property is the ability to absorb oil that can influence product taste. Product with good oil-holding capacity (OHC) is usually used as a functional ingredient in meat and confectionary industries. Several researchers have evaluated OHC value on a few types of proteins such as egg albumin, soy protein concentrate and fish muscles (Diniz & Martin 1997; Kristinsson & Rasco 2000). They found that OHC values for proteins varied with values ranging from 2.36-6.80 mL/g. As presented in Table 2, there is no significant difference (p>0.05) in OHC for TB and TM protein hydrolysates with values of 4.00 and 3.93 mL g^{-1}, respectively. These values were slightly higher than the results from tilapia meat hydrolysates (2.27 mL g^{-1}) (Foh et al. 2011) and catfish frame hydrolysates (2.9-3.5 mL/g) (Amiza et al. 2013), but comparable with the results from Atlantic salmon muscle hydrolysates (3.86-5.98 mL/g) (Kristinsson & Rasco 2000) and shark protein hydrolysates (3.6-4.4 mL/g) (Diniz & Martin 1997). It can be considered that TB and TM protein hydrolysates have good OHC values, in which the results obtained are still within the range of OHC values from protein sources (Diniz & Martin 1997; Kristinsson & Rasco 2000).

EMULSIFYING CAPACITY

Formation of a stable emulsion is related to protein-lipid interactions in food systems. Greater number of peptide molecules and exposed hydrophobic AA residues, as a result of protein hydrolysis, could improve the emulsion formation. TM protein hydrolysate was found to have higher (p<0.05) emulsifying capacity compared with TB protein hydrolysate (34.0 mL and 21.33 mL g^{-1}, respectively, Table 2). Higher emulsification capacity of TM protein hydrolysate might be due to low level degradation of TM by alcalase, thus larger peptides are available at oil-water interface, which increase the protein surface area, consequently improving emulsion formation. However, these values were considered lower compared to other fish protein reported by Diniz and Martin (1997) and Kristinsson and Rasco (2000) who managed to obtain emulsifying capacity ranging from 44-60 and 50.7-70.3 mL/g, respectively, but comparable with the results from catfish frame hydrolysates (17.2-29.8 mL/g) (Amiza et al. 2013). Lower emulsifying capacity for TB and TM protein hydrolysates as compared to other fish species might be due to both samples have undergone excessive hydrolysis which based on high DH values (Table 1) and resulting more peptides with low molecular weight (Figure 2).

This is an agreement with previous studies (Gbogouri et al. 2004; Kristinsson & Rasco 2000; Quaglia & Orban 1990) which reported that excessive hydrolysis leads to loss of emulsifying properties. In addition, peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties (Klompong et al. 2007). The mechanism to generate the emulsion system is attributed to the adsorption of peptides on the surface of freshly formed oil droplets during homogenization and the formation of a protective membrane that inhibits coalescence of the oil droplet (Klompong et al. 2007). Hydrolysates are surface-active materials and promote oil-in-water emulsion because of their hydrophilic and hydrophobic groups with their associated charges (Gbogouri et al. 2004). Thus, hydrolysates with a higher DH have poor emulsifying capacity and stability due to their small peptide size. Small peptides migrate rapidly and adsorb at the surface, but show less efficiency in decreasing the interface tension since they cannot unfold and reorient at the interface like large peptides to stabilize emulsions (Gbogouri et al. 2004; Klompong et al. 2007).

CONCLUSION

The findings from this study has contributed to the existing knowledge with regards to ACE -inhibition activity, amino acid compositions and functional properties of tilapia protein hydrolysates. Through enzymatic hydrolysis using alcalase, the hydrolysates of TB and TM have successfully produced small peptide size (<14.4 kDa) with high ACE inhibitory activity within a short period of time (1 h). TB and TM protein hydrolysates exhibited good functional properties, which are based on high solubility and other properties such as water-holding, oil-holding and emulsifying capacities that are comparable with protein functional properties. This study indicated that tilapia protein hydrolysates could potentially serve as a good

source of bioactive peptides as well as food ingredients in many food systems to improve food product quality.

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