ACE inhibitory activity of pangasius catfish (*Pangasius sutchi*) skin and bone gelatin hydrolysate

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Abstract Skin and bone gelatins of pangasius catfish (*Pangasius sutchi*) were hydrolyzed with alcalase to isolate Angiotensin Converting Enzyme (ACE) inhibitory peptides. Samples with the highest degree of hydrolysis (DH) were separated into different fractions with molecular weight cut-off (MWCO) sizes of 10, 3 and 1 kDa, respectively and assayed for ACE inhibitory activity. Skin and bone gelatins had highest DH of 64.87 and 68.48 % after 2 and 1 h incubation, respectively. Results from this study indicated that by decreasing the molecular weight of fractions, ACE inhibitory activity was increased. Therefore, F₃ permeates (MWCO < 1 kDa) of skin (IC₅₀ = 3.2 µg/ml) and bone (IC₅₀ = 1.3 µg/ml) gelatins possessed higher ACE inhibitory activity compared to their untreated gelatins and corresponding hydrolyzed fractions. In this study, the major amino acids were Glycine followed by Proline with an increased amount of hydrophobic amino acid content in F₃ permeates of skin (4.01 %) and bone (5.79 %) gelatin. Digestion stability against gastrointestinal proteases did not show any remarkable change on ACE inhibition potency of these permeates. It was concluded that alcalase hydrolysis of *P. sutchi* by-products could be utilized as a part of functional food or ingredients of a formulated drug in order to control high blood pressure.

Keywords Enzymatic hydrolysis · ACE inhibitory · *Pangasius sutchi* · Gelatin · High blood pressure

Introduction

Catfish is a common farm-raised, warm-water fish, supplying large quantity of fish skins and bones annually. *Pangasius sutchi*, known locally as “patin”, is one of the most popular freshwater fish sources in Malaysia (See et al. 2010). Large amounts of by-product materials including skin and bone are disposed from *P. sutchi* processing industry, which are sources of high quality protein. Fish processing by-products are potential sources of collagen and gelatin. Gelatin is a heterogeneous mixture of high molecular weight proteins derived from collagen (Giménez et al. 2009; Gómez-Guillén et al. 2011). In recent years, there has been an increasing demand to produce protein hydrolysates containing peptides with specific physiological properties, which could be marketed as functional food ingredients (Korhonen and Pihlanto-Leppala 2003). Some of these physiological properties include antioxidative, opioid, antimicrobial and angiotensin-I-converting enzyme (ACE) inhibitory activity.

ACE inhibitory peptides are the most extensively studied peptides due to the role of ACE in high blood pressure and cardiovascular disorders. Several natural ACE inhibitory peptides have been isolated from collagen and gelatin enzymatic hydrolysate of various food protein sources such as porcine skin collagen (Ichimura et al. 2009), bovine skin gelatin (Kim et al. 2001), fish skins (Byun and Kim 2001; Nagai et al. 2006; Park et al. 2009), fish cartilage (Nagai et al. 2006), scales (Fahmi et al. 2004), squid (Alemán et al. 2011) and sea cucumbers protein hydrolysates (Zhao et al. 2007) as an alternative approach to the synthetic ACE inhibitory drugs.

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Bioactive peptides are small subunits of native protein containing between 3–20 amino acid residues. These peptides are inactive in the sequence of parent proteins and could be released by enzymatic proteolysis (Kim et al. 2007). A number of commercial proteases have been used for the production of gelatin hydrolysates with bioactive properties. Alcalase, a broad specificity microbial protease, is one of the most common commercial enzymes used for production of functional fish gelatin hydrolysates. Its usage has resulted in very high protein recovery, excellent end-product functional properties and less bitterness (Hoyle and Merritt 1994; Kristinsson and Rasco 2000a). This enzyme manifested extensive proteolytic activity during the hydrolysis of skin gelatin from alaska pollack (Byun and Kim 2001) and sole and squid (Giménez et al. 2009), generating potent ACE inhibitory peptides with very low molecular weight peptidic fractions.

The molecular weight distribution is used in the selection of special types of gelatin hydrolysate for particular applications or for obtaining certain functional properties (Jeon et al. 1999; Park et al. 2001). The versatility of applications of fish gelatin and its hydrolysate as functional food ingredients depend on their properties and are governed mainly by the amino acid composition and molecular weight distribution. Therefore, after enzymatic hydrolysis, using an ultrafiltration membrane system is a suitable method for production of bioactive peptides with a desired molecular size. This technique is a low cost and easy method, and has the main advantage that the molecular size of the peptide can be controlled by adoption of an appropriate ultrafiltration membrane (Jeon et al. 1999).

Gelatin is a unique protein compared to the muscle proteins. This uniqueness of gelatin lies in its amino acid content which is rich in non-polar amino acids such as Gly, Ala, Val and Pro. On the other hand, gelatin peptides have repeated unique Gly–Pro–Ala sequence in their structure. It is presumed that the observed functional properties of gelatin peptides can be associated with their unique amino acid compositions (Gómez-Guillén et al. 2011; Karim and Bhat 2009). Therefore, application of these by-products as potential source of bioactive peptides can be investigated.

In the present study, the extracted gelatins from skin and bone of *P. sutchi* were hydrolyzed with alcalase. The hydrolyzed gelatins were then purified using 10, 3 and 1 kDa ultrafiltration membranes, respectively and then assayed for ACE-inhibitory activity. The amino acid composition and the gastrointestinal stability of high potent fractions were also investigated.

Materials and methods

Materials *P. sutchi*, 1.2 to 1.5 kg, was obtained from a farm fish located in Penang, Malaysia. Amino acid standards

were purchased from Pierce (Rockford, IL, USA). Alcalase from *Bacillus licheniformis* (EC 3.4.21.14, activity of 2.4 AU/g), Pepsin (EC 3.4.23.1), Pancreatin (EC 3.2.1.1), Hippuryl-L-Histidyl-L-Leucine (Hip-His-Leu), angiotensin-converting enzyme of rabbit lung (ACE, EC 3.4.15.1), the AccQ-Fluor reagent kit, AccQ-Tag eluent and other chemicals of analytical grade were purchased from sigma (Sigma-Aldrich Chemical Co., USA). Ultrafiltration membranes of 10 kDa (Vivaflow 200), 3 and 1 kDa (Vivaflow 50) molecular weight cut off (MWCO) were purchased from Sartorius (Vivaflow, Sartorius, Germany).

Extraction of gelatin from *P. sutchi* skin and bone Gelatin was extracted from *P. sutchi* skin according to the method described by Montero and Gómez-Guillén (2000) with some modifications. The cleaned skin (30 g) was first pre-treated with eight volumes (w/v) of NaOH (0.16 M) for 60 min to remove the non-collagen proteins. Alkaline-treated skin was then drained off and rinsed 3 times with tap water. The skin was then soaked in acetic acid (0.08 M) with a ratio of 1:8 (w/v) for 60 min, drained off and washed 3 times with tap water. The pre-treated samples were mixed with distilled water (1:8, w/v) and incubated at 63 °C for 3.5 h in a shaking water bath. The gelatin solution was separated from residual skin using cheesecloth.

Gelatin extraction procedure from *P. sutchi* bone was carried out according to Alfaro et al. (2009) method with some modifications. The degreased bone (30 g) was demineralized using 3 % HCl with a ratio of 1:8 (w/v) for 21 h. The leached bone (ossein) was drained off and rinsed with tap water and then neutralized using NaOH. The neutralized ossein was incubated with distilled water (1:8, w/v) at 75 °C for 5 h, centrifuged for 20 min at 4000 × g at 4 °C (Thermo Scientific, High Speed Centrifuge, Sorvall HS23, Germany) and the soluble aqueous fractions were collected. All the extractions were performed at 4 °C. Both extracted gelatins from skin and bone were then concentrated with a rotary vacuum evaporator and freeze-dried for further experiments.

Hydrolysis of extracted gelatin For enzymatic hydrolysis, the freeze-dried skin and bone gelatins were adjusted to optimal pH and temperature for alcalase (pH 7.0; 50 °C). Gelatins were mixed with sodium phosphate (0.1 M) with a ratio of 1:100 (w/v) and the hydrolysis was performed for 0–3 h in a shaking water-bath incubator (Yang et al. 2008). At the end of the reaction, the enzyme was inactivated at the temperature of 90 °C for 10 min. The samples were then centrifuged at 6000 × g for 20 min and the soluble fraction decanted and freeze-dried. The ratio of enzyme to fish skin and bone proteins was 1:100 (v/w).

Degree of hydrolysis Degree of hydrolysis (DH) was calculated according to percent of trichloroacetic acid (TCA)

method as described by Hoyle and Merritt (1994). After hydrolysis, 20 ml of protein hydrolysate was added to 20 ml of 20 % (w/v) TCA to produce 10 % TCA soluble material. The mixtures were left to stand for 30 min to allow precipitation, followed by centrifugation at $7800\times g$ for 15 min. The supernatant was analyzed for soluble nitrogen using kjeldahl method (Kjeltec 2100, Foss, Denmark) (AOAC 2005). The percent DH is expressed as follows:

$$\%DH = (10\% \text{ TCA} - \text{soluble N in sample} / \text{total N in sample}) \times 100$$

SDS-PAGE analysis The skin and bone extracted gelatins and their hydrolysates were prepared for sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) analysis according to the method described by Ghassem et al. (2011a) with some modification. Samples were mixed with 20 mL treatment buffer (125 mM Tris, 2 % SDS, 10 % glycerol) and heated in a 50 °C water bath for 20 min and then centrifuged at $1000\times g$ for 30 min at 4 °C. Protein concentration was determined using Bradford and diluted to 3.5 mg/ml using treatment buffer containing 10 % mercaptoethanol and 0.001 % bromophenol blue. Samples were well mixed and heated at 50 °C for 10 min and then stored at –80 °C for subsequent SDS-PAGE.

Protein patterns of skin and bone gelatin samples were analyzed using (SDS-PAGE), according to the method of Schagger and von Jagow (1987). A 4 % stacking gel and a 16 % polyacrylamide separating gel (acrylamide: N,N'-bis-methylene acrylamide, gel buffer containing 0.3 % SDS (w/v), TEMED, ammonium persulfate) were used in a tris-tricine system to detect the *P. sutchi* skin and bone protein hydrolysate patterns. A volume of 20 µL of each sample was loaded on 16 % gel (Mini Protein II unit, Bio-Rad Laboratories) and run using discontinuous tris-tricine buffer at a constant current setting of 25 mA/gel and a constant voltage of 100 V for 3 h. Proteins were visualized by 0.02 % (w/v) Coomassie blue G250 staining and destaining by soaking in several changes of 40 % (v/v) methanol–10 % (v/v) acetic acid until a clear background resulted.

Fractionation of gelatin hydrolysate Ultrafiltration is the process of separating extremely small particles and dissolved molecules from fluids. The hydrolysate solutions of gelatins with the highest DH were first filtered by 0.2 µm membrane and separated into large and low molecular weight fractions by ultrafiltration at 4 °C using 10 kDa Molecular Weight Cut-Off (MWCO) membrane followed by 3 and 1 kDa MWCO membranes to enrich specific peptide fractions. These membranes were activated by spinning 100 mL of deionized water prior to use. These permeates were designed as F₁, F₂ and F₃, corresponding to the small peptides with molecular weight fraction less than 10, 3

and 1 kDa, respectively, and assayed for ACE inhibitory activity.

Measurement of protein solubility and peptide content The soluble contents of skin and bone gelatins and their hydrolysates were determined by the Folin-Lowry method (Lowry et al. 1951) and their peptide contents measured by method of Church et al. (1983) using o-phthaldialdehyde (OPA) spectrophotometric assay. Each hydrolysate, containing 5–100 µg protein, was mixed with OPA reagent and the absorbance measured at 340 nm.

Determination of ACE inhibition activity The ACE inhibitory activity was assayed with RP-HPLC technique modified from the spectrophotometric method described by Wu et al. (2002). A volume of 25 µL containing different concentrations of samples or borate for control were added to 100 µL solution containing 5 mM hippuryl-L-histidyl-L-leucine (HHL). The samples and HHL were prepared in 100 mM Na-borate buffer, pH 8.3, containing 300 mM NaCl. After incubation at 37 °C for 10 min, a 10 µL of ACE (100 mU/mL) was added and samples were incubated for 30 min at 37 °C with continuous agitation. The enzyme reaction was stopped by the addition of 100 µL of 1 M HCl. The solution was filtered through a 0.45 µm nylon syringe filter and injected directly onto Agilent ZORBAX C₁₈ column (4.6 mm×250 mm, 5 µm, Waters). The column was eluted with 50 % methanol in water (v/v) containing 0.1 % TFA at a flow rate of 0.5 mL/min and the absorbance was measured at 228 nm. ACE inhibition rate was calculated as follows:

$$\text{ACE inhibition (\%)} = (B - A / B - C) \times 100$$

Where A is the relative area of HA peak generated in the presence of ACE inhibitor component, B the relative area of HA peak generated without ACE inhibitors and C is the relative area of HA peak generated without ACE.

Amino acids analysis Acid, alkaline and performic acid hydrolysis of *P. sutchi* skin and bone gelatins and their hydrolysates were performed using Waters AccQ. Tag amino acid analyzer (Waters Corporation, Ireland). Acid analysis of hydrolysate samples was performed according to the Alaiz et al. (1992). Each sample was hydrolysed with 5 mL 6 M hydrochloric acid (HCl) in a closed test tube, shaken for 20 min and then kept in oven for 24 h at 110 °C. Internal standard α-aminobutyric acid (AABA) was added to the hydrolyzed 150 samples and filtered through a nylon 0.2 mm cellulose acetate membrane filter (Whatman No.1). The total content of cysteine and cystine was determined by oxidizing the protein with performic acid and then samples were hydrolyzed with 6 M HCl (Cohen et al. 1988).

Alkaline hydrolysate was performed to determine the amount of tryptophan using 4.3 M LiOH. H₂O as described by Thiansilakul et al. (2007). Each hydrolysate was then hydrolyzed with 6 M HCl and filtered.

Separation of amino acid was carried out on a C₁₈ AccQ-Tag amino acid analysis column (150×3.9 mm, Waters, USA). The column temperature was set at 37 °C with a flow rate set at 1 mL/min. The UV detector was operated at 248 nm (for peak identification), and the fluorescence detector was with a 250 nm excitation and a 395 nm emission wavelength (for amino acid quantification). The hydrolysed sample was then analysed using an automatic amino acid analyser (Waters Corporation, USA) equipped with Waters 717 Autosampler.

Effects of gastrointestinal digestion on ACE inhibitory activity The stability of the ACE inhibitory peptides solution against gastrointestinal proteases was carried out in vitro according to the method of Wu and Ding (2002). A purified hydrolysate solution of 3.5 % (w/v) in 0.1 M KCl-HCl buffer (pH 2) was incubated with 4 % (w/v) pepsin for 4 h at 37 °C. The reaction was stopped by boiling in water bath for 10 min. The mixture was then neutralized to pH 7.0, centrifuged at 10,000 × g for 30 min and the soluble fractions were assayed for ACE inhibitory activity. The remaining neutralized suspension was further digested with 4 % (w/v) pancreatin at 37 °C for another 4 h. The enzyme was inactivated by boiling for 10 min followed by centrifugation at 10,000×g for 30 min. The supernatant was collected and evaluated for ACE inhibitory activity.

Statistical analysis Experiments were performed in triplicates and data were analyzed using a one-way analysis of variance (ANOVA) using SPSS, version 14. The differences in means between the samples were determined at 5 % confidence level ($P < 0.05$).

Results and discussion

Degree of hydrolysis of gelatin hydrolysates Hydrolysis of *P. sutchi* skin and bone gelatins was carried out by Alcalase for 3 h. The degree of hydrolysis (DH) is a measure of the extent of hydrolytic degradation of a protein, and it is the most widely used indicator for comparison among different protein hydrolysates (Guérard et al. 2002). The hydrolysis of the *P. sutchi* skin and bone were characterized by a high rate of hydrolysis during the first 1–2 h (Fig. 1). The rate of enzymatic hydrolysis was subsequently decreased, until reaching a stationary phase when no apparent hydrolysis took place. This is similar to the typical hydrolysis curve reported by Bougatef et al. (2008), Giménez et al. (2009) and Klompong et al. (2007). According to Benjakul and

Fig. 1 Degree of hydrolysis (DH) of *P.sutchi* skin (●) and bone (■) gelatin with alcalase for 3 h. Each data represents average value and $\pm$ SD of triplicate determinations ($n=3$). The standard errors of means are shown as error bars

Morrissey (1997), the rate of enzymatic cleavage of peptide bond controls the overall rate of hydrolysis. However, available substrate decreases as time of reaction increases.

Figure 1 indicates faster hydrolysis rate of bone than skin gelatin with alcalase. Due to faster hydrolysis, enzymatic degradation of bone gelatin was performed over 1 h, while hydrolysis of skin gelatin took over 2 h. The DH of alcalase gelatin hydrolysates from skin and bone after 2 and 1 h incubation was 64.87 and 68.48 %, respectively (Fig. 1). The difference observed in DH between skin and bone gelatin hydrolysates could be attributed to the different availability of susceptible bonds and enzyme affinity towards different substrates, possibly due to differences in the size and structure of the protein chains that compose the parent gelatins (Ghassem et al. 2011a; Giménez et al. 2009). Enzymatic hydrolysis was performed in order to achieve the desired degree of hydrolysis to obtain biologically active peptides. Based on the results of the previous studies, ACE inhibitory activity of peptides increased with prolonged incubation with enzyme. However, longer hydrolysis time led to the peptides lost their ability to inhibit ACE (Wu et al. 2008; Xu et al. 2011).

SDS-PAGE pattern SDS-PAGE is a very powerful technique for small-scale separation of polypeptides and for assigning molecular weights to these molecules. The method described by Laemmli (1970) cannot separate polypeptides with masses less than 15 kDa. The most generally used technique is the one developed by Schagger and von Jagow (1987) with the use of high concentration gels and the incorporation of materials to resolve the polymers of low molecular weight. Representative SDS-PAGE electrophoregrams of *P. sutchi* skin and bone gelatin hydrolysates are shown in Fig. 2. As shown in Fig. 2, the untreated gelatin of both samples exhibited a number of high molecular

Fig. 2 SDS-PAGE pattern of skin (**a**) and bone (**b**) gelatins and of their hydrolysates. In both SDS-PAGE profiles, lane 1 represents protein marker; lane 2, control (untreated samples); lane 3, alcalase gelatin hydrolysate with the highest DH

weight bands. SDS-PAGE pattern presents intense bands corresponding to the α_1 and α_2 chains (~116 kDa) as well as β -components (~200 kDa) in Lane 2. However, gelatin extracted from *P. sutchi* bone showed considerable amount of low molecular weight peptides (below 97 kDa) which did not appear in the skin gelatin. Therefore, the bone gelatin seems to be more accessible to the alcalase attack due to the greater amount of peptides with molecular weight lower than α -chains, which can also explain the higher DH of bone gelatin than that of skin. This observation is in accordance with Giménez et al. (2009) who observed that the sole skin gelatin contained highly crosslinked aggregates and protein fractions >200 kDa than the squid skin gelatin as a result of which lower DH observed in that gelatin.

The both untreated gelatins were digested by alcalase to produce lower molecular weight fragments. As shown in Fig. 2, the proteolytic degradation of *P. sutchi* skin gelatin by alcalase exhibited high and low molecular weight bands ranging from 66 to 45 kDa, while the *P. sutchi* bone gelatin hydrolysate contained two low molecular weight bands near 31 kDa. Therefore, the differences observed between molecular weight of gelatin hydrolysates could be due to the different molecular weight distribution of the parent gelatins. The presence of the low molecular weight bands in both gelatin hydrolysates in this study may result in production of bioactive peptides with potent physiological properties.

IC₅₀ value, peptide content and protein solubility of gelatin hydrolysates Gelatin hydrolysates are generally obtained by enzymatic proteolysis. A number of commercial proteases have been used for the production of these hydrolysates and peptides (Lin and Li 2006; Mendis et al. 2005; Yang et al. 2008). Protease specificity affects size, amount, free amino acid composition and, peptides and their amino acid sequences, which in turn influences the biological activity of the

hydrolysates (Chen et al. 1995; Jeon et al. 1999; Wu et al. 2003). Considering the fact that microbial-derived proteinases are low-cost and safe, and the product yields are very high, the use of these enzymes to hydrolyze food proteins is valuable (Phelan et al. 2009). Alcalase, which is a commercial protease from a microbial source, has been used in numerous studies dealing with gelatin/collagen hydrolysis because of its broad specificity as well as the high degree of hydrolysis that can be achieved in a relatively short time under moderate conditions (Benjakul and Morrissey 1997; Diniz and Martin 1996). Therefore, in the present study, *P. sutchi* skin and bone gelatins were hydrolyzed by alcalase as a microbial protease. In the present study, *P. sutchi* skin and bone gelatine hydrolysates with the highest DH were ultrafiltrated using 10, 3 and 1 kDa MWCO ultrafiltration membranes and fractionated into peptidic permeates defined as F₁ (MW <10 kDa), F₂ (MW <3 kDa) and F₃ (MW <1 kDa). The ACE-inhibitory activity of all permeates were assayed and calculated as IC₅₀ (amount of peptide required to inhibit 50 % of the ACE activity). Table 1 shows IC₅₀ values of *P. sutchi* skin and bone gelatins and their hydrolysates with different molecular weight fractions with respect to protein solubility and peptide content.

There were statistically significant differences of IC₅₀ values in untreated, hydrolyzed and ultrafiltrated gelatin fractions ($P < 0.05$). As shown in Table 1 all the gelatin fractions exhibited ACE inhibitory activity. Inhibition potencies of alcalase digest of skin (IC₅₀=870 μ g/ml) and bone (IC₅₀=710 μ g/ml) gelatin before fractionation were significantly higher than untreated skin (IC₅₀=1680 μ g/ml) and bone (IC₅₀=1550 μ g/ml) gelatin (Table 1). The ACE inhibition activity of skin and bone gelatin hydrolysates in the present study were almost similar with that of alaska pollack skin (IC₅₀=629 μ g/ml) (Byun and Kim 2001), skate skin (IC₅₀=680 μ g/ml) (Lee et al. 2011), sardinella by-product (IC₅₀=810 μ g/ml) (Bougatef et al. 2008) and sea bream scales (IC₅₀=570 μ g/ml) (Fahmi et al. 2004) protein hydrolysates. Results of this study indicated that by increasing the MWCO size, ACE inhibition activity of fractions were increased. As illustrated in Table 1, F₃ permeates of both skin and bone gelatin hydrolysates possessed higher ACE inhibitory activity (IC₅₀=3.2 and 1.3 μ g/ml, respectively) than F₁ and F₂ permeates.

Enzyme-hydrolysed gelatin plays an increasingly important role in various products and applications due to its functional properties. A large number of studies have investigated enzymatic hydrolysis of collagen or gelatin for the production of bioactive peptides for the application in food industry. Besides exploring diverse types of bioactivities such as ACE inhibitory activity, studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of isolated peptides from gelatin hydrolysates (Gómez-Guillén et al.

Table 1 Protein solubility, peptide content and IC₅₀ value of *P.sutchi* skin and bone gelatins and their hydrolysates

Samples		Membrane cut-off MW ^a (kDa)	Protein solubility (mg/g)	Peptide content (mg/g)	IC ₅₀ ^b (μg/mL)
(n=3)	Skin	Untreated gelatin	19.6±0.74	11±0.69	1680
		Gelatin hydrolysate ^c	170.9±0.44	98.9±0.61	870
		< 10	261.1±0.49	157±0.66	124
		< 3	371.6±1.70	201.2±0.59	70
		< 1	520.8±2.92	280.8±2.31	3.2
^a MW Molecular Weight	Bone	Untreated gelatin	26.3±0.40	13.4±0.61	1550
		Gelatin hydrolysate ^c	210.1±1.54	113.7±0.81	710
		< 10	316.±1.88	196.9±1.69	81
		< 3	414.4±0.65	221.3±0.91	52
		< 1	611.2±1.94	311.7±1.62	1.3

^b IC₅₀=concentration of sample that inhibits 50 % of ACE activity

^c Gelatin alcalase hydrolyaste of skin and bone with highest DH

2011). The enrichment of fractions or specific peptides with the aim of ultrafiltration has been widely applied in order to produce hydrolysates with improved nutritional or functional properties within specific molecular weight ranges from high molecular mass residues (Harnedy and Fitzgerald 2012; Picot et al. 2010). Alcalase manifested extensive proteolytic activity during the hydrolysis of skin gelatin from Alaska pollack, squid *Todarodes pacificus* and giant squid, producing hydrolysates with low average molecular weight which exhibited high ACE inhibitory capacity (Giménez et al. 2009; Nam et al. 2008). Several reports have indicated that ultrafiltrated hydrolysates composed of short-chain peptides with very low molecular weights contained remarkably high in vitro ACE inhibitory activity (Ghassem et al. 2011b; Li and Aluko 2010; Pan et al. 2012; Segura-Campos et al. 2011), which coincided well with the current report. Similar findings were also observed for enzymatic digests of whey proteins where ultrafiltrated peptide fractions with molecular weight less than 3 kDa (Mullally et al. 1997) and 1 kDa (Fujita et al. 2000) possessed higher ACE inhibitory activity than their hydrolysates.

Protein solubility and peptide content of the skin and bone gelatins and their hydrolysate fractions are also shown in Table 1. Ultrafiltrated F₃ permeates of both skin and bone gelatin hydrolysates had the highest protein solubility and peptide content among other fractions. However, significant differences were observed in protein solubility and peptide content of the skin and bone gelatin and their corresponding hydrolysate fractions. Results pointed out that these values rose by increasing the MWCO of sizes of both skin and bone gelatin fractions. It has been suggested that an increase in the solubility of protein hydrolysate over that of the original protein is due to the increased hydrophilicity of the hydrolysate and the enzymatic release of smaller size peptides (Kristinsson and Rasco 2000b; Radha et al. 2008).

The search for in vitro ACE inhibitors is the most common strategy followed by selection of potential antihypertensive peptides derived from proteins of different food

sources. However, identification of peptide structures and chemical synthesis of potentially active peptides are needed to be investigated to confirm the in vivo activity of these peptides. On the other hand, in the view of an industrial application, main considerations of protein hydrolysates containing antihypertensive peptides would be the organoleptic characteristics of these ingredients and the evaluation of the resistance of the active peptides to processing conditions. The interactions between food matrices and bioactive peptides may affect to its structure, bioactivity, and its bioavailability (Hernández-Ledesma et al. 2011; Wu and Aluko 2007). Thus, the stability of antihypertensive peptides incorporated in foods and the effects of processes on bioactive peptides in food matrices has to be evaluated. Applications of isolated bioactive ACE-inhibitory peptides from catfish by-products as nutraceutical ingredients in the functional foods largely depends on the physiological activity of their specific amino acids after digestion and absorption in the gastrointestinal tract. Therefore, further studies are required to determine the in vivo antihypertensive activity of the purified potent ACE inhibitory peptides.

Amino acid compositions The specific amino acid composition of a peptide is a critical factor for its ACE inhibitory activity. High inhibition activity of these peptides is due to the interaction between C-terminal sequences of inhibitors with three subsites of active sites of ACE. The presence of tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp), alanine (Ala), glycine (Gly) and proline (Pro) at the C-terminus of a peptide contributes to its higher activity (Lee et al. 2009). Tryptophan exerts the strongest influence on the ACE inhibitory activity of peptides. Proline is one of the crucial amino acid residues involved in ACE inhibitory activity (Meisel 2005). The occurrence of amino acid Pro at the C-terminal sequence of several strong ACE inhibitory peptides, isolated from different protein hydrolysates have been reported (Fujita et al. 2000; Jimsheena and Gowda 2011; Majumder and Wu 2011; Wijesekara et al. 2011).

Table 2 Amino acid composition (%) of untreated, hydrolyzed and F₃ permeate gelatins of *P. sutchi* skin and bone

Amino acids	Skin			Bone		
	Gelatin	GH ^a	F ₃ ^b	Gelatin	GH ^a	F ₃ ^b
Asp	5.28±0.13	4.34±0.09	3.43±0.12	4.48±0.13	3.78±0.21	3.26±0.14
Ser	3.79±0.07	3.24±0.11	2.18±0.07	3.97±0.13	3.13±0.08	2.16±0.09
Glu	7.83±0.10	5.61±0.05	4.43±0.09	8.16±0.07	5.82±0.14	3.76±0.21
Gly	31.23±0.17	32.33±0.20	38.22±0.22	33.09±0.08	34.42±0.11	39.53±0.13
His	0.71±0.04	0.61±0.04	0.54±0.11	0.81±0.03	0.56±0.06	0.38±0.08
Arg	5.08±0.17	6.14±0.12	5.22±0.11	5.14±0.09	7.03±0.12	5.74±0.18
Thr	3.4±0.14	3.08±0.05	2.13±0.09	2.6±0.14	2.43±0.13	1.21±0.09
Ala	9.45±0.11	9.01±0.15	8.15±0.13	9.31±0.15	8.17±0.08	7.02±0.12
Pro	11.98±0.12	12.82±0.09	14.7±0.13	11.42±0.12	12.32±0.08	14.16±0.12
Tyr	0.61±0.04	0.72±0.05	1.45±0.08	0.6±0.08	1.08±0.05	1.95±0.04
Val	2.76±0.09	2.84±0.11	2.17±0.15	2.18±0.12	2.45±0.11	2.02±0.04
Met	2.63±0.18	4.74±0.29	6.51±0.36	3.79±0.17	5.16±0.14	6.97±0.16
Lys	3.45±0.17	2.65±0.13	1.25±0.23	3.24±0.11	2.51±0.17	1.03±0.06
Ile	1.64±0.05	1.25±0.07	1.33±0.15	1.23±0.14	1.14±0.09	1.16±0.06
Leu	2.74±0.06	2.6±0.14	1.62±0.09	2.26±0.11	2.13±0.05	1.71±0.16
Phe	1.17±0.02	1.98±0.05	2.47±0.05	1.7±0.08	2.03±0.12	2.84±0.11
Cys	0.02±0.01	0.01±0.02	0.03±0.01	0.01±0.02	0.01±0.02	0.02±0.02
Trp	0.01±0.01	0.03±0.01	0.09±0.03	0.01±0.03	0.03±0.01	1±0.03
Hyp	6.31±0.09	6±0.08	4.13±0.06	6.07±0.09	5.83±0.15	4.12±0.07

(n=3)

^a Gelatin hydrolysates of skin and bone^b The most active fraction obtained from ultrafiltration membrane

The amino acid compositions of *P. sutchi* skin and bone gelatins and their corresponding hydrolysate fractions are summarized in Table 2. The major constituent amino acids of all fractions were Gly, Pro, Ala and Met. However, some differences were remarkable. The results indicated that both potent F₃ fractions from skin and bone gelatins contained increased amount of Pro content than their hydrolyzed gelatins (Table 2). Moreover, the noticeable differences were in the contents of hydrophobic (particularly aromatic) amino acids such as Phe, Tyr, Trp, which increased in fractions F₃ of skin (4.01 %) and bone (5.79 %) gelatin compared to untreated gelatin and their hydrolysates (Table 2). Incorporation of hydrophobic amino acids in the peptide sequence may enhance the ACE inhibition activity of peptide by binding to the catalytic active sites of the somatic form of the ACE and block the angiotensin II formation (Gómez-Ruiz et al. 2004).

According to Wu et al. 2008, reduction in the amount of positively charged amino acids in the protein hydrolysates may contribute to the high inhibition activity of the peptides. Table 2 shows a lower content of positively charged amino acids (Arg, Lys, His) in F₃ fractions of *P. sutchi* skin and bone gelatins in comparison to their untreated gelatins and hydrolysates. These results were in agreement with those reported by Wu et al. 2008 and Ghassem et al. 2011b. Therefore, the high ACE inhibitory potency of F₃ fractions

of skin (IC₅₀=3.2 µg/ml) and bone (IC₅₀=1.3 µg/ml) gelatins could be due to the high content of hydrophobic amino acid residues in their peptidic fragments.

To conclude, a structural analysis of peptides representing a variety of bioactivities showed that bioactive motifs mostly occupy random coil or helical parts of the protein chain. Some of the peptides matched a combination of coil and helix structures. These peptides were rich in proline or aromatic residues, which also seem to be crucial in the context of the structure-activity relationship of peptides. More studies are required to investigate the amino acid sequence of peptides in fraction F₃.

Table 3 ACE inhibitory activity of hydrolysates after digestion by gastrointestinal proteases

Enzymes	F ₃ ^a (%)	F ₃ ^b (%)
Control	46.7±1.32	57.1±3.31
Pepsin	45.4±3.81	55.9±1.70
pepsin + pancreatin	43.7±2.10	54.3±4.13

(n=3)

^a The most active fraction obtained from ultrafiltration membrane of alcalase digested of skin gelatin^b The most active fraction obtained from ultrafiltration membrane of alcalase digested of bone gelatin

Stability study Although the ACE inhibitory peptides exhibited potent ACE-inhibitory activity in vitro, it has been reported that some of them failed to show antihypertensive effects in vivo, which is possibly due to hydrolysis by ACE or gastrointestinal enzymes (Wu et al. 2008). To evaluate the resistance of F₃ fractions of skin and bone gellins against gastrointestinal proteases, the digestion stability were measured by incubating the hydrolysates (5 mg/mL) with proteases and assessed for ACE-inhibitory activity. The ACE-inhibitory activity of F₃ fractions of *P. sutchi* skin and bone gelatins showed negligible changes on IC₅₀ by in vitro incubation with gastrointestinal proteases (Table 3). It was observed that after digestion by pepsin and mixture of pepsin and pancreatin, ACE inhibitory potencies of fractions were almost the same as the control samples (Table 3). Wu et al. (2008) and Tsai et al. (2008) reported that short-chained peptides with very low molecular weights were resistant to gastrointestinal peptidases due to their fast absorption in the small intestine. The findings from present study also recommended that potent ACE inhibitory peptides extracted from *P. sutchi* skin and bone gelatins could possibly maintain their inhibition activity after being orally administered.

Conclusion

In this study, functional bioactive peptides with in vitro ACE-inhibitory activity have been isolated from skin and bone gelatin hydrolysate of catfish *P. sutchi*. Results indicated that peptidic fractions of skin (IC₅₀=3.2 µg/ml) and bone (IC₅₀=1.3 µg/ml) gelatins with MW less than 1 kDa contained the highest inhibition activity. These ACE inhibitory fractions obtained from skin and bone gelatin hydrolysates (< 1 kDa) also showed high resistance toward gastrointestinal proteases in vitro. Due to production of potent ACE-inhibitory peptides from alcalase *P. sutchi* gelatin by-products hydrolysates, it is suggested that gelatin protein hydrolysates could be used as health promoting ingredients in functional foods to prevent hypertension. However, further study should be done to evaluate in vivo antihypertensive effect and amino acid sequence of gelatin hydrolysate

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