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Angiotensin I-converting enzyme (ACE) inhibitory activities of sardinelle (*Sardinella aurita*) by-products protein hydrolysates obtained by treatment with microbial and visceral fish serine proteases

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ABSTRACT

The angiotensin I-converting enzyme (ACE) inhibitory activities of protein hydrolysates prepared from heads and viscera of sardinelle (*Sardinella aurita*) by treatment with various proteases were investigated. Protein hydrolysates were obtained by treatment with Alcalase®, chymotrypsin, crude enzyme preparations from *Bacillus licheniformis* NH1 and *Aspergillus clavatus* ES1, and crude enzyme extract from sardine (*Sardina pilchardus*) viscera. All hydrolysates exhibited inhibitory activity towards ACE. The alkaline protease extract from the viscera of sardine produced hydrolysate with the highest ACE inhibitory activity (63.2 ± 1.5% at 2 mg/ml). Further, the degrees of hydrolysis and the inhibitory activities of ACE increased with increasing proteolysis time. The protein hydrolysate generated with alkaline proteases from the viscera of sardine was then fractionated by size exclusion chromatography on a Sephadex G-25 into eight major fractions (P_1–P_8). Biological functions of all fractions were assayed, and P_4 was found to display a high ACE inhibitory activity. The IC_{50} values for ACE inhibitory activities of sardinelle by-products protein hydrolysates and fraction P_4 were 1.2 ± 0.09 and 0.81 ± 0.013 mg/ml, respectively. Further, P_4 showed resistance to *in vitro* digestion by gastrointestinal proteases. The amino acid analysis by GC/MS showed that P_4 was rich in phenylalanine, arginine, glycine, leucine, methionine, histidine and tyrosine. The added-value of sardinelle by-products may be improved by enzymatic treatment with visceral serine proteases from sardine.

1. Introduction

Hypertension is related to the incidence of coronary heart disease and its treatment is effective in reducing the risk of the disease (Collins et al., 1990; MacMahon et al., 1990). The angiotensin I-converting enzyme (EC 3.4.15.1; ACE) plays an important physiological role in the regulation of blood pressure (Skeggs, Kahn, & Shumway, 1956). The ACE can increase blood pressure by converting the inactive decapeptide angiotensin-I to the potent vasoconstrictor angiotensin-II (an octapeptide). The ACE is a multifunctional enzyme which also catalyses the degradation of bradykinin (a vasodilating nonapeptide) (Erdös, 1975). Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of high blood pressure, since it reduces the activity of angiotensin-II and increases the level of bradykinin. Besides, several ACE inhibitors may also have beneficial effects on glucose and lipid metabolism (Pollare, Lithell, & Berne, 1989). Several effective oral ACE inhibitors have been developed, namely, captopril, enalapril, and lasinopril and all are currently used as clinical antihypertensive drugs (Kuster & Marshall, 2005). Although synthetic ACE inhibitors are effective as antihypertensive drugs, they cause adverse side effects such as coughing, allergic reactions, taste disturbances, and skin rashes. Therefore, research and development to find safer, innovative and economical ACE inhibitors is necessary for the prevention and remedy for hypertension.

Since the discovery of ACE inhibitors in snake venom (Ferreira, Bartelt, & Greene, 1970), several reports have been published on the ACE inhibitory activity of peptides from food proteins, like casein (Silva & Malcata, 2005), rapeseed (Marczak et al., 2003), mushroom (Lee, Kim, Park, Choi, & Lee, 2004), whey protein (Vermeirssen, 2004), porcine and chicken muscle (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001; Fujita, Yokoyama, &

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Yoshikawa, 2000), bovine skin gelatin (Kim, Byun, Park, & Shahidi, 2001), soybean (Kuba, Tana, Tawata, & Yasuda, 2005), sake and sake lees (Saito, Wanezaki, Kawato, & Imayasu, 1994) and fish proteins (Fahmi et al., 2004; Ichimura, Hu, Aita, & Maruyama, 2003).

Proteases such as pepsin, chymotrypsin and trypsin are frequently used in hydrolysis to produce ACE inhibitory peptides (Costa, Gontijo, & Netto, 2007; Hyun & Shin, 2000; Yu, Hu, Bai, Du, & Lin, 2006). Microbial alkaline proteases are also utilized in the production of ACE inhibitors from food proteins such as sardine (*Sardina pilchardus*) (Matsui et al., 1993) and rapeseed (Marczak et al., 2003). However, there are a small number of reports on the use of digestive enzymes from fish in the producing bioactive peptides.

In Tunisia, sardinelle (*Sardinella aurita*) catches were about 13,300 tonnes in 2002 (FAO., 2004). During processing, solid wastes including heads and viscera are generated and constitute 30% of the original material. These wastes, which represent an environmental problem to the fishing industry, constitute an important source of protein. Traditionally, protein-rich by-products are converted to fish meal for animal feed (Ström & Eggum, 1981) and fish silage. However, most of these products possess low economic value. Novel means of processing are required to convert the underutilized fish and fish by-products into more marketable and acceptable form. An interesting alternative is to transform sardinelle proteins by-products into biologically active peptides by protease treatments.

In the present study, we report on the digestion of sardinelle (*S. aurita*) protein by-products by microbial and visceral serine proteases from sardine (*Sardina pilchardus*) and the preparation of fish protein hydrolysates with angiotensin-I-converting enzyme inhibitors.

2. Materials and methods

2.1. Materials

Sardinelle (*S. aurita*) and sardine (*S. pilchardus*) were purchased from the fish market of Sfax city, Tunisia. They were washed twice with water; heads and viscera were separated, and then stored in sealed plastic bags at −20 °C until used.

Angiotensin I-converting enzyme from rabbit lung and the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemicals Co (St Louis, MO, USA). Acetonitrile was of HPLC grade. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. Other chemicals and reagents used were of analytical grade.

The enzymes used were: Alcalase® from Novozymes® (Bagsvaerd, Denmark); pepsin (from porcine gastric mucous), chymotrypsin (from bovine pancreas) and trypsin (from bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); crude enzyme preparations from *Aspergillus clavatus* ES1 (Hajji, Kanoun, Nasri, & Gharsallah, 2007) and *Bacillus licheniformis* NH1 (El Hadj-Ali et al., 2007), and crude enzyme from sardine viscera (*S. pilchardus*) (Bougatef, Souissi, Fakhfakh, Triki-Ellouz, & Nasri, 2007) were prepared in our laboratory.

2.2. Preparation of crude enzyme extract from sardine viscera

Viscera from *S. pilchardus* (250 g) were homogenized for 30 s with 500 ml extraction buffer (10 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$). The mixture was centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was discarded and the supernatant was lyophilized and stored at −20 °C until use.

2.3. Preparation of protein hydrolysates from sardinelle by-products

Heads and viscera (500 g), in 500 ml distilled water, were first minced then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked heads and viscera sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to the optimum activity value for each enzyme, then, the heads and viscera proteins were digested with enzymes. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH solution. After the required digestion time the reaction was stopped by heating the solution at 80 °C during 20 min to inactivate the enzyme. The sardinelle by-products protein hydrolysates were then centrifuged at 5000*g* for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1-2). Samples were stored as hydrolyzed sardinelle protein powders. Hydrolysis conditions are summarized in Table 1.

2.4. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds in the substrate studied (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) as given below.

$$\mathrm{DH}\ (\%) = \frac{h}{h_{\mathrm{tot}}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{\mathrm{tot}}} \times 100$$

where B is the amount of base consumed (ml) to keep the pH constant during the reaction. Nb is the normality of the base, MP is the mass (g) of protein (N × 6.25), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as

$$\alpha = \frac{10^{\mathrm{pH-pK}}}{1 + 10^{\mathrm{pH-pK}}}$$

where pH and pk are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in a fish protein concentrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

2.5. Chemical analysis

The moisture and ash content were determined according to the AOAC (1995) standard methods 930.15 and 942.05, respectively. Total nitrogen content of fish protein hydrolysates was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

Table 1
Hydrolysis conditions of sardinelle by-products proteins, ACE inhibitory activities and IC_{50} values of SHVPH obtained with various proteases treatment

Enzyme	Optimum conditions		DH (%)	ACE inhibition (%)	IC_{50} (mg/ml)
	Temperature (°C)	pH			
Proteases NH1	50	10.0	11	47.4 ± 1.5	2.1 ± 0.36
Alcalase®	50	8.0	8	43 ± 2	2.3 ± 0.24
Sardine proteases	40–50	8.0	8	63.2 ± 1.75	1.2 ± 0.09
Chymotrypsin	40	7.5	6.5	55.8 ± 1.25	1.8 ± 0.13
Proteases ES1	40	8.0	4.7	13.2 ± 0.65	7.4 ± 0.62

2.6. Determination of the angiotensin I-converting enzyme (ACE) inhibition activity

The ACE inhibition activity was assayed as reported by Nakamura et al. (1995). A volume of 80 μl containing different concentrations of protein hydrolysates were added to 200 μl containing 5 mM hippuryl-L-histidyl-L-leucine (HHL), and then preincubated for 3 min at 37 °C. Sardinelle heads and viscera protein hydrolysates (SHVPH) and HHL were prepared in 100 mM borate buffer, pH 8.3, containing 300 mM NaCl. The reaction was then initiated by adding 20 μl of 0.1 U/ml ACE from rabbit lung prepared in the same buffer and incubated for 30 min at 37 °C. The enzyme reaction was stopped by the addition of 250 μl of 0.1 M HCl. The released hippuric acid (HA) was quantified by RP-HPLC on a Vydac C_{18} column connected to a system composed of a Waters TM 600 automated gradient controller pump module, a WaterWisp 717 automatic sampling device and a Waters 996 photodiode array detector. The sample was then eluted using an acetonitrile gradient from 0% to 28% and from 28% to 47% in 0.1% trifluoroacetic acid (TFA) (v/v) for 50 and 20 min, respectively. The eluate was followed at 226 nm. Spectral and chromatographic data were stored on a NEC image 446 computer. Millennium software was used to acquire, analyze and plot chromatographic data. The average value from three determinations at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE inhibition } (\%) = \left[\frac{B-A}{B-C}\right] \times 100$$

where A is the absorbance of HA generated in the presence of ACE inhibitor component, B the absorbance of HA generated without ACE inhibitors and C is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay).

The IC_{50} value was defined as the concentration of hydrolysate (mg/ml) required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

2.7. Stability study

The stability of the ACE inhibitory peptides solution against gastrointestinal proteases was assessed *in vitro*. Solutions (10 mg/ml) were individually incubated with pepsin (pH 2.0), or trypsin (pH 8.0) for 3 h at 37 °C. In successive digestion test, the sample was first incubated with trypsin for 3 h, heat treated for 5 min in boiling water, and then incubated 3 h at 37 °C with chymotrypsin. The reaction solutions were heated for 5 min in boiling water to terminate the reaction and then centrifuged at 10,000*g* for 10 min, the supernatants were used for measurement of ACE inhibitory activity.

2.8. Fractionation of hydrolysate obtained by treatment with alkaline proteases from sardine viscera with Sephadex G-25 gel filtration

The freeze-dried hydrolysate (1 g), with a DH of 6%, was suspended in 20 ml of distilled water, then loaded onto a Sephadex G-25 gel filtration (2.5 cm × 54 cm), pre-equilibrated and eluted with distilled water. Fractions (5 ml each) were collected at a flow rate of 60 ml/h, and the absorbance was measured at 226 nm.

2.9. Reverse-phase high-performance liquid chromatography (RP-HPLC)

Peptide fractions from Sephadex G-25, which exhibited the highest ACE inhibitory activity, were further separated by RP-HPLC on a Vydac C_{18} column (10 mm × 250 mm) (Grace-Vydac, USA).

The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyse chromatographic data. The mobile phase was water/trifluoroacetic acid (1000:1, v/v) as eluent *A* and acetonitrile/trifluoroacetic acid (1000:1, v/v) as eluent *B*. Samples were filtered through 0.22 μm filters, and then applied on the C_{18} column and eluted by eluent A for 10 min then with a linear gradient of acetonitrile for 25 min. The flow rate was 1 ml/min. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software.

2.10. Analysis of amino acids by gas-chromatography/mass spectrometry (GC/MS)

Samples were dried under a stream of nitrogen and submitted to "classical" acid hydrolysis (16 h at 110 °C in 100 μl of 6 N HCl). After that, samples were evaporated under a stream of nitrogen and submitted to a short time methanolysis (500 μl of 0.5 M HCl in anhydrous methanol, 90 min at 80 °C) to form methyl esters (Zanetta & Vincendon, 1973). After drying them under a stream of nitrogen, samples were transesterified using 200 μl of 1.5 M HCl in redistilled isoamyl alcohol overnight at 100 °C (Zanetta & Vincendon, 1973). The reagent was obtained by adding 107 μl of acetyl chloride in precooled (−20 °C) isoamyl alcohol. After drying them under a stream of nitrogen and mild heating with hair drier, samples were supplemented with 200 μl of acetonitrile and 25 μl of heptafluorobutyric anhydride (HFBAA) and heated for 30 min at 150 °C. After evaporation, samples were dissolved in 400 μl of dried acetonitrile, and an aliquot (1 μl) was injected into the Ross injector of the GC/MS.

For GC/MS analysis, the GC separation step was performed using a Carlo Erba GC 8000 gas chromatograph (Chrompack France, Les Ullis, France) equipped with a 25 m 0.32 mm CPSil5 CB Low bleed/MS capillary column, 0.25 m film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260 °C and samples were analyzed using a temperature program starting at 90 °C for 3 min, followed by an increase (5 °C/min) until 260 °C was reached. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for masses larger than 1000, to a Riber 1010 H mass spectrometer (mass detection limit 2000). Analyses were routinely performed in the electron impact mode (ionisation energy 70 eV; source temperature 150 °C) and to preserve the filament of the ionization source, GC/MS records were performed 5 min after injection. Quantitation of the various constituents was performed using the total ion count (TIC) of the MS detector and the Xcalibur software (Finnigan Corp.). To assess the mass of the various derivatives, MS analyses were also performed in the chemical ionization mode in the presence of ammonia (ionization energy 150 eV, source temperature of 100 °C). Detection was performed for positive ions.

2.11. Protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

3. Results and discussion

3.1. Preparation of sardinelle protein hydrolysates using various proteases

The biological properties of protein hydrolysates depend on the protein substrate, the specificity of the enzyme used for the

proteolysis, the conditions used during hydrolysis and the degree of hydrolysis. In the present study, Alcalase®, crude enzyme preparations from *B. licheniformis* NH1 and *A. clavatus* ES1, chymotrypsin and crude enzyme extract from sardine viscera were used for the production of protein hydrolysates from sardinelle heads and viscera. The hydrolysis curves of sardinelle by-products after 3 h of incubation are shown in Fig. 1. Enzymes were used at the same activity levels (10×10^3 U/ml) to compare hydrolytic efficiencies. The hydrolysis of the sardinelle by-products proteins was characterized by a high rate of hydrolysis for the first 1 h. The rate of enzymatic hydrolysis was subsequently decreased, and then the enzymatic reaction reached the steady-state phase when no apparent hydrolysis took place. Crude enzyme preparation from *B. licheniformis* NH1 was the most efficient, while that from *A. clavatus* ES1 was the lowest efficient. After 3 h of hydrolysis, the DH reached about 11% with crude enzyme preparation from NH1 strain, 8% with Alcalase® and enzyme extract from sardine viscera, 6.5% with chymotrypsin and 4.7% with crude enzyme preparation from *A. clavatus* ES1 (Fig. 1).

The typical hydrolysis curves were also reported for yellow stripe trevally (Klompong, Benjakul, Kantachote, & Shahidi, 2007), Pacific whiting solid wastes (Benjakul & Morrissey, 1997), herring (Liceaga-Gesualdo & Li-Chan, 1999), salmon muscle (Kristinsson & Rasco, 2000), salmon by-products (Gbogouri, Linder, Fanni, & Parmentier, 2004), capelin (Shahidi, Han, & Synowiecki, 1995) and sardine (Quaglia & Orban, 1987).

3.2. ACE inhibitory activities of sardinelle by-products protein hydrolysates obtained with various proteases

The hydrolysates obtained after 3 h of incubation were freeze-dried and assayed for ACE inhibitory activity. As shown in Table 1, all hydrolysates at 2 mg/ml, except that obtained with the crude enzyme preparation from *A. clavatus* ES1, could act as significant inhibitors of angiotensin converting enzyme. The results showed that hydrolysates digested with visceral serine proteases had much higher ACE inhibitory activity, indicating that these proteases were more suitable for marine protein digestion and production of ACE inhibitory peptides. Higher DH obtained with NH1 proteases did not guarantees a higher ACE inhibitory activity. This may be due to the fact that *B. licheniformis* NH1 extract, contains both endoprotease and exoprotease activities, which offer the ability to achieve higher DH of sardinella heads and viscera proteins than the other enzymes, but at the same time, the exoprotease may also involve the inactivation of the active peptide sequence by cutting one or more amino acids from N-terminal or C-terminal positions.

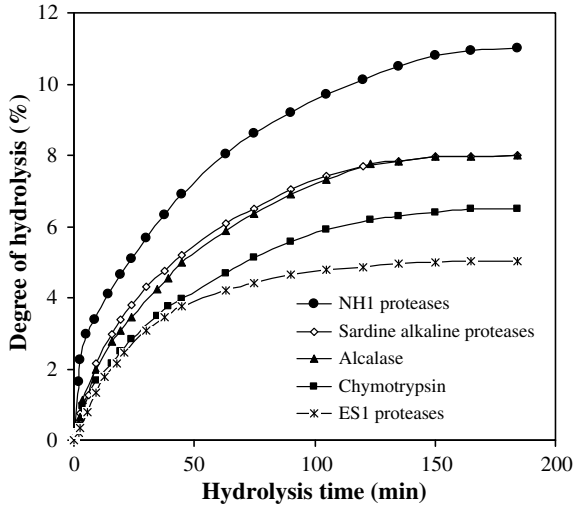

Fig. 1. Hydrolysis curves of sardinelle heads and viscera proteins treated with various enzyme preparations. Reaction conditions are illustrated in Table 1.

The IC_{50} values for ACE inhibition of all hydrolysates varied between 1.24 and 7.4 mg/ml (Table 1). The hydrolysate with the highest inhibition was obtained with sardine crude enzyme extract, reaching an IC_{50} value of 1.2 mg/ml, approximately 7-fold more powerful than the product obtained using the ES1 enzyme preparation. The IC_{50} value of sardinelle protein hydrolysate was lower than those of hydrolysates from oyster, scallop, codfish skin, and codfish bone whose presented an IC_{50} greater than 10 mg/ml (He et al., 2007), whereas it is higher than those of β-conglycinin and glycinin hydrolysates (0.126 and 0.148 mg/ml, respectively) (Kuba et al., 2005).

The obtained results indicated that the sardinelle by-products protein hydrolysates contained ACE inhibitory peptides. Considering the inhibition effects on ACE, sardinelle protein by-products treated with endogenous enzymes from sardine viscera was employed for the characterization of ACE inhibitory activity.

The proximate composition of the freeze-dried sardinelle by-products protein hydrolysate showed that the hydrolysate had high protein content (73%) and could be an essential source of proteins. The high protein content was a result of the solubilisation of protein during hydrolysis, the removal of insoluble undigested non-protein substances and the partial removal of lipid after hydrolysis (Benjakul & Morrissey, 1997). The ash and lipid contents of the hydrolysate were 12.6% and 7.3%, respectively.

3.3. Effect of the degree of hydrolysis on ACE inhibitory activity

The degree of hydrolysis is a measure of the extent of hydrolysis degradation of a protein, and it is the most widely used indicator for comparison among different protein hydrolysates. During hydrolysis, a wide variety of larger, medium and smaller peptides are generated, depending on enzyme specificity. In order to study the effect of the DH on the evolution of ACE inhibitory activity, sardinelle proteins were hydrolyzed by the crude enzyme extract from sardine viscera, and the degree of hydrolysis and the ACE inhibition activity were measured. As shown in Fig. 2, undigested sardinelle by-products proteins exhibited low ACE inhibitory activity (about 3%), whereas, the DH and ACE inhibitory activity increased with the increase of hydrolysis time. Higher ACE inhibition activity was obtained with DH of 6%. Further digestion up to a DH value of 6% did not result in an increase in the ACE inhibitory activity. This graph clearly shows that hydrolysis was required to release ACE inhibitory peptides from an inactive form within the sequence of sardinelle heads and viscera proteins.

The obtained result indicated that the crude enzyme from sardine viscera would be effective in the producing of strong inhibitors for the ACE reaction.

3.4. Fractionation of the hydrolysate obtained by treatment with alkaline proteases from sardine viscera by gel filtration G-25

The hydrolysate, obtained by treatment with crude enzyme from sardine viscera, which displayed the highest ACE inhibitory activity, was further fractionated by gel filtration chromatography on a Sephadex G-25 column. Eight fractions were separated and designated as P_1–P_8 (Fig. 3a). These fractions were pooled, freeze-dried, and the inhibition against ACE was determined. As shown in Fig. 3b, all fractions displayed ACE inhibitory activity. Fraction P_3 exhibited the highest level of ACE inhibitory activity (76.6 ± 1.25) followed by fraction P_4 (74.5 ± 1.33) at 1 mg/ml.

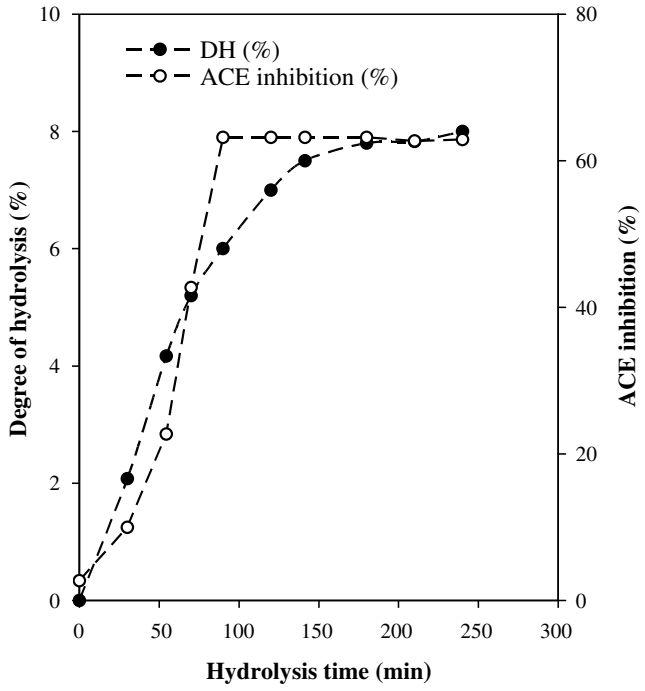

Fig. 2. Comparison of ACE inhibitory activity and degree of hydrolysis of SHVPH as a function of hydrolysis time. The reaction conditions were as follows: reaction volume, 500 ml; [substrate], 100% (w/v); pH 8.0; temperature 50 °C and E/S 0.27% w/w, protein).

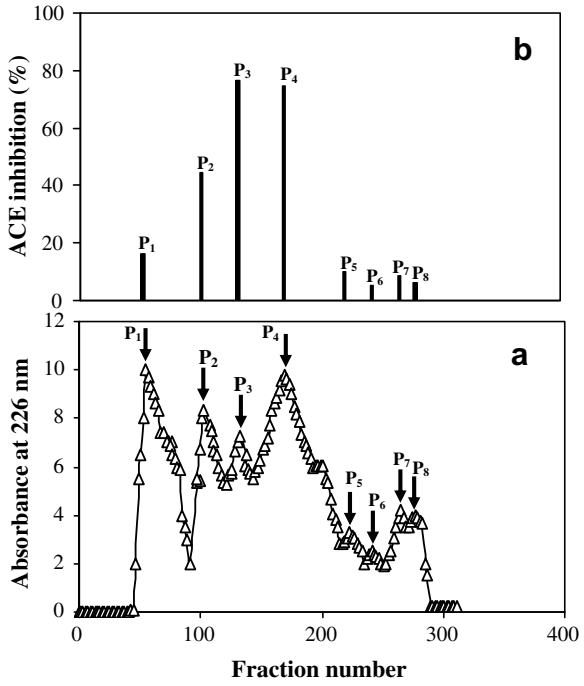

Fig. 3. Elution profile of SHVPH obtained with alkaline proteases extract from sardine viscera separated by size exclusion chromatography on Sephadex G-25 (lower panel) and the ACE inhibitory activities of the separated fractions (P_1–P_8) (upper panel).

3.5. RP-HPLC analysis

Semi preparative RP-HPLC column-C_{18} was used for further fractionation of fractions P_3 and P_4 obtained from Sephadex G-25. The elution profiles of the hydrolysates can be grouped into three categories of hydrophobicity of the eluted peptides (low, medium and high) (Fig. 4). The alkaline proteases from sardine viscera resulted in a hydrolysate having higher concentration of high-hydrophobicity peptides than fractions P_3 and P_4 (Fig. 4a). Whereas, the fraction P_4 contained high concentration of low hydrophobic peptides and a low concentration of high hydrophobic peptides (Fig. 4c) than P_3 (Fig. 4b) and the initial hydrolysate.

Enzymatic treatment of food proteins may result in a bitter taste due to the formation of a low molecular weight peptides composed mainly of hydrophobic amino acids. Thus, the formation of peptides with bitterness is the most serious problem in the practical use of food protein hydrolysates. In the present work, we attempted to produce a hydrolysate with low bitterness from sardinelle containing powerful ACE inhibitory activity. Thus, P_4, which contained higher concentration of low hydrophobic peptides, was evaluated as an effective fraction with slightly bitterness and high ACE inhibitory activity. The IC_{50} value of P_4 was determined with logarithmic linearization (Jérôme, Laurent, & Jean-Luc, 2002) to be 0.81 mg/ml. The molecular masses of peptides in P_4 were estimated to be between 200 and 600 Da.

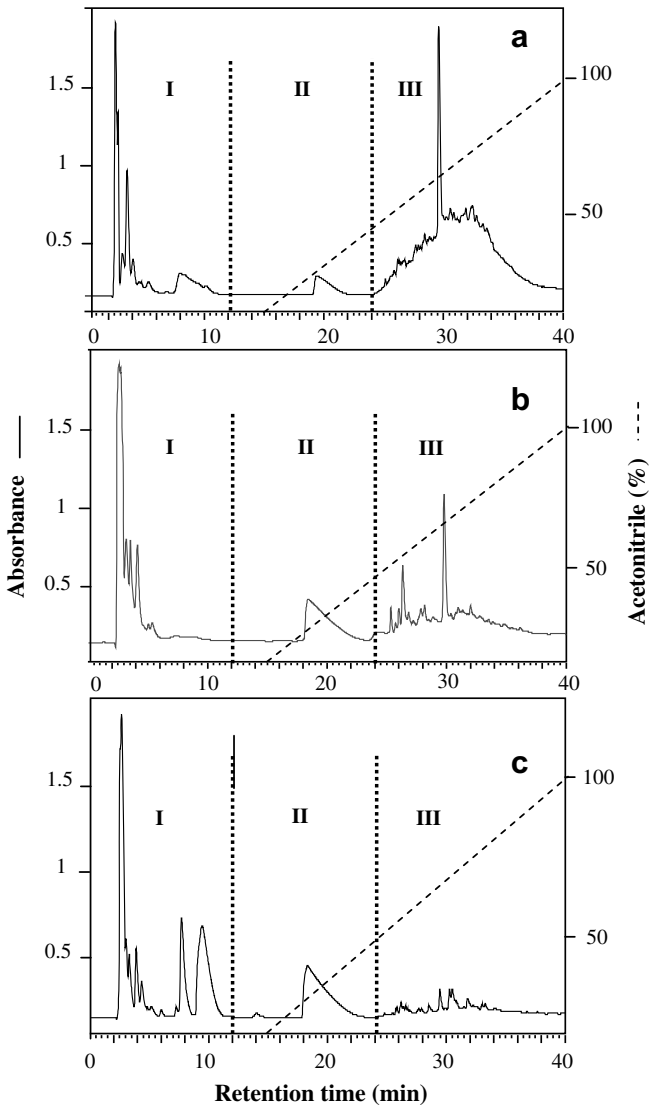

Fig. 4. RP-HPLC elution profiles pattern on a Vydac C18 column of the SHVPH (a), fractions P3 (b) and P4 (c) obtained from Sephadex G-25. The chromatograms regions I, II and III indicate the hydrophobicity of eluted peptides (low, medium and high, respectively).

These data show that the ACE inhibitory peptides are present in the hydrolysate and their inhibitory activity against ACE increased after gel filtration. Compared with reports on proteolytic hydrolysates of various proteins, which showed IC_{50} for ACE inhibitory activities in the range of 0.18–246.7 mg/ml (Lee, Kwon, Shin, & Yang, 1999; Mullally, Meisel, & FitzGerald, 1997), the sardinelle protein hydrolysate had moderate ACE inhibitory activity.

3.6. Stability study of P4 peptides

In order to exert an antihypertensive effect *in vivo*, the ACE inhibitory peptides must be absorbed in their intact form from intestine and further be resistant to plasma peptidases degradation to reach their target sites. To investigate the resistance of peptides in fraction P_4 against gastrointestinal proteases, the digestion stability was evaluated, by incubating the hydrolysate with different proteases and testing the residual ACE inhibitory activity. The ACE inhibitory activity of the fraction P_4 was not affected by *in vitro* incubation with gastrointestinal proteases. The ACE inhibitory activity of the fraction P_4 after digestion by pepsin, trypsin and mixture of trypsin and chymotrypsin were 73.5%, 73.3% and 71.6%, respectively, and that of the control was 74.5%. This result suggests that bioactive peptides present in fraction P4 may be resistant to digestion in the gastrointestinal tract. These findings are in accordance with that reported by Yu et al. (2006) showing the stability of globin hydrolysate against gastrointestinal proteases.

3.7. Amino acids analysis

The identification of the different amino acids was based on their spectra obtained according to Pons et al. (2002). The amino acid analysis by GC/MS of the fraction P_4 was presented in Fig. 5. Seven peaks are considered major, based on the % area (Fig. 5). Protein hydrolysate was rich in arginine, histidine, glycine, methionine, phenylalanine, leucine and tyrosine which % molar accounted for 35.6%, 20.68%, 8.11%, 7.26%, 6.52%, 5.07% and 2.4%, respectively (Table 2). Under the conditions of the used acid hydrolysis, Trp was destroyed. So, we can not detect its presence. Shahidi et al. (1995) reported that sensitive amino acids, such as methionine and tryptophan, were present in smaller amounts after hydrolysis of capelin proteins. Many studies have shown that peptides with high potent inhibitory of ACE have tryptophan, phenylalanine, tyrosine, or proline at their C-terminal, and branched aliphatic amino acid at the N-terminal (Li, Le, Shi, & Shrestha, 2004).

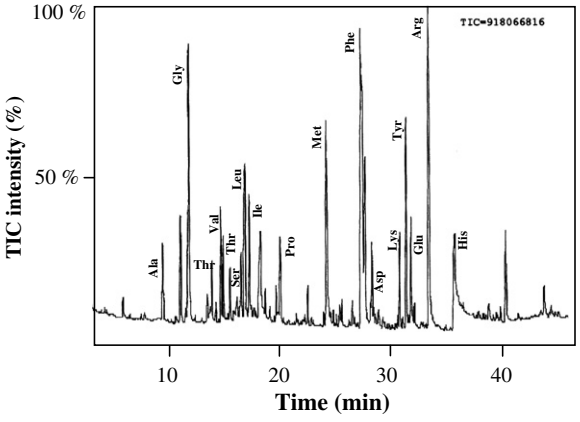

Fig. 5. Chromatogram of the GC/MS (EI mode of ionization) of the fraction P_4 after hydrolysis and derivatisation.

Table 2
Amino acids analysis of the fraction P_4 by GC/MS

Amino acids	Retention time (min)	Molar %
Ala	11.08	2.36
Gly	11.43	*8.11*
Val	13.49	2.23
Thr	13.58	1.08
Ser	14.25	0.95
Leu	15.27	*5.07*
Ile	15.44	2.44
Pro	17.47	1.91
Met	20.57	*7.26*
Phe	23.18	*6.52*
Asp	24.01	1.02
Lys	25.52	0.93
Tyr	26.2	*2.4*
Glu	26.38	1.46
Arg	27.52	*35.6*
His	29.32	*20.68*

4. Conclusions

In this study, hydrolysates from sardinelle by-products obtained by treatment with various proteolytic preparations were analyzed for their ACE inhibitory activities. The untreated sardinelle by-products proteins exhibited low ACE inhibitory activity (3%). When sardinelle by-products proteins were digested by proteases, the ACE inhibitory activity of the hydrolysates was markedly increased. Among five proteolytic preparations, sardinelle hydrolysis with the crude enzyme extract from sardine viscera resulted in the production of the hydrolysate with the highest ACE inhibitory activity.

This study suggests that ACE inhibitors derived from sardinelle by-products could be utilized to develop functional foods for prevention of hypertension. Further works should be done to isolate and characterize potent antihypertensive peptides from sardinelle protein hydrolysates, and to demonstrate their antihypertensive activity *in vivo*.

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