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Fractionation and Angiotensin I-converting Enzyme (ACE) Inhibitory Activity of Gelatin Hydrolysates from by-products of Alaska Pollock Surimi

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Gelatin hydrolysates with a high inhibitory activity against angiotensin I-converting enzyme (ACE) were fractionated from Alaska pollock surimi refiner discharge. The ACE-inhibitory activity, expressed as IC_{50} (mg/mL), was highest (0.49 mg/mL) in gelatin hydrolysates formed by sequential 2-hr treatments of Pronase and Flavourzyme. After fractionation through four different membrane filters with molecular weight cut-offs of 3, 5, 10, and 30 kDa, the highest ACE-inhibitory activity (0.21 mg/mL) was observed with the 3-kDa filtrate.

Key words: Alaska pollock, Alaska pollock by-products, Refiner discharge, Fish gelatin, Fish gelatin hydrolysate

Introduction

Refiner discharge from surimi processing contains 4-8% whole fish (Wendel, 1999) and significant amounts of collagen (Morrissey et al., 2000). However, the use of refiner discharge as a human food has not been widely examined. Most refiner discharge is conventionally used to produce fish meal and fertilizer or is directly discharged into estuaries, resulting in environmental pollution (Ciarlo et al., 1997). Converting this waste stream to food-grade ingredients such as collagen and gelatin presents new challenges.

The peptides regulating blood pressure are potent inhibitors of angiotensin I-converting enzyme (ACE). ACE plays an important role in regulating blood pressure by converting an inactive decapeptide (angiotensin I) to a potent octapeptide vasoconstrictor (angiotensin II) and by inactivating the catalytic activity of bradykinin, which acts as a depressor (Byun and Kim, 2001). ACE belongs to a class of zinc proteases that require both zinc and chloride for its enzymatic activity.

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The enzymatic digestion of gelatin is an efficient means of producing peptides with enhanced bioactivity, such as ACE-inhibitory and anti-oxidative activities (Byun and Kim, 2001; Kim et al., 2001a). This suggests that gelatin could be used in both ordinary and health-functional foods, *i.e.*, foods with beneficial bioactivity, as well as in pharmaceutical and other industrial applications (Cho et al., 2004). Most commercial gelatin (95%) is made from the hides of porcine and bovine animals (Cho et al., 2005). Modern, health-conscious consumers, however, are reluctant to ingest gelatin extracted from land animals due to the recent outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease, and avian flu.

For application in various industrial products, such as common foods, cosmetics, and pharmaceutics, fish gelatin must have similar rheological properties to that obtained from land animals. However, the gelatin obtained from cold water fish such as Alaska pollock, cod, haddock, and Pacific whiting does not typically exhibit similar rheological properties as bovine or porcine gelatin due to a lower content of imino acid, such as proline and hydroxyproline (Cho et al., 2005). Therefore, in the aforementioned applications, the use

of fish gelatin would require chemical or enzymatic modification, both of which have major impacts on safety and/or cost. Improving the functionality of refiner-discharge gelatin with inexpensive comercial enzymes (Kim et al., 1994) has received a great deal of attention as a consumer-friendly potential resource for health-functional foods (Mendis et al., 2005).

Although efforts have been made to utilize fish skin gelatin (Byun and Kim 2001; Kim et al., 2001b; Mendis et al., 2005) and bovine skin gelatin (Kim et al., 2001a) as a food resource, no research has been conducted regarding the utilization of refiner-discharge gelatin. The objective of this study was to obtain gelatin hydrolysates via enzymatic digestion of Alaska pollock refiner discharge and fractionate gelatin hydrolysates that exhibit a high ACE-inhibitory activity.

Materials and Methods

Materials

Refiner discharge from surimi processing of Alaska pollock was provided by a commercial surimi processing plant (Pacific Seafoods, Warrenton, OR, USA) in January 2006 and stored at -40°C until use. Purified ACE and a substrate peptide (Hip-His-Leu), and Pronase E (from *Streptomyces griseus*, type XIV) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Alcalase (2.4 AU/g, from *Bacillus licheniformis*, optimum temperature 55-70°C, optimum pH 6.5-8.5), Flavourzyme (500 LAPU/g, from *Asperigillus oryzae*, optimum temperature 50°C, optimum pH 5.0-7.0), and Neutrase (0.8 AU/g, from *Bacillus amyloliquefaciens*, optimum temperature 45-55°C, optimum pH 5.5-7.5) were obtained from Novo Nordisk (Bagsvaerd, Denmark). All other substances used in this study were reagent-grade chemicals.

Preparation of ultrafiltrates from refiner-discharge gelatin hydrolysate

For the preparation of gelatin, Alaska pollock refiner discharge was washed with cold tap water for 10 min. The material was then sieved to remove soluble proteins and foreign materials. The washing process was repeated three additional times. The washed discharge was pretreated with 10 volumes (v/w) of 0.32 M $Ca(OH)_2$ at 5°C in an automatic stirrer (No. 1; Lab Co., Daejeon, Korea) for 1 hr to remove non-collagenous materials and to inactivate enzymes. $Ca(OH)_2$ was then removed by washing with cold tap water, neutralized with 0.15 M acetic acid, and the neutralized mixture was rewashed with cold tap water. Three volumes (v/w) of distilled water were added to the pretreated refiner discharge and gelatins were extracted at 50°C for 4 hr in a shaking water bath (BS-21; Jeio Tech, Daejeon, Korea). The extracted gelatin solution was decolorized and deodorized on activated carbon, which was then removed by centrifugation at 5,300×*g* and 20°C for 15 min. The supernatant was vacuum-filtered through filter paper (No. 3; Whatman, Maidstone, Kent, UK) and the filtered solution was dried at 50°C for 14-20 hr in a hot-air blast dryer (WFO-601SD; EYELA, Tokyo, Japan) to obtain the final gelatin product.

Gelatin hydrolysates were prepared by one- and two-step enzymatic hydrolysis reactions yielding first and second hydrolysates, respectively. To prepare the first hydrolysates, the gelatin was dissolved in a buffer solution and hydrolyzed with one of four commercial proteases (Alcalase, Neutrase, Flavourzyme, or Pronase E) at a ratio of 100:2 (w/w) protein substrate to enzyme. Each hydrolysis reaction was performed for 0.5-12.0 hr at the optimal temperature for each of the enzymes and then heated at 98°C for 10 min to inactivate the proteases. To prepare the second hydrolysates, the first hydrolysates were re-hydrolyzed for 2.0 hr with either Alcalase, Neutrase, or Flavourzyme. Again, the ratio of protein substrate to enzyme was 100:2 (w/w) and the optimal temperatures for each enzyme were used. The resulting hydrolysate solution was heated at 98°C for 10 min to inactivate the proteases.

Ultrafiltrates with a high ACE-inhibiting activity were obtained by sequentially fractionating the second hydrolysates through membrane filters with 3-, 5-, 10-, and 30-kDa molecular weight cut-offs (MWCOs). The fractionated proteins were lyophilized and stored at -20°C until use.

Degree of hydrolysis

The degree of hydrolysis was evaluated according to the method described by Hoyle and Merritt (1994). A 50-mL aliquot of hydrolysate solution was mixed with an equal volume of 20% trichloroacetic acid (TCA) and centrifuged at 2,560×*g* for 15 min. The resulting supernatant was subjected to nitrogen analysis and the degree of hydrolysis was calculated according to the following equation:

$$DH\,(\%) = (10\%\ \text{TCA-soluble nitrogen/total nitrogen}) \times 100.$$

Angiotensin I-converting enzyme (ACE) inhibitory activity

The ACE-inhibitory activity of fish gelatin hydrolysates was assayed by measuring the concentration

of hippuric acid liberated from hippuryl-His-Leu as described by Horiuchi et al. (1982) with some modification. For each assay, 15 uL of sample solution was mixed with 50 uL of 60 mU/mL rabbit lung ACE and 100 uL of sodium borate buffer (pH 8.3), pre-incubated at 37°C for 5 min, and then incubated with 125 uL of substrate solution (5 mM hippuryl-His-Leu in 0.1 M sodium borate buffer containing 0.6 M NaCl at pH 8.3) at 37°C for 30 min. The reaction was stopped by adding 20 uL of 10% trifluoroacetic acid. The hippuric acid concentration was determined by a HPLC (model 1100; Hewlett Packard, Palo Alto, CA, USA) on a Zorbax 300SB C_8 column (4.6 mm×150 mm; Agilent, Santa Clara, CA, USA). The IC_{50} value was defined as the concentration of fish gelatin hydrolysate required to inhibit 50% of the ACE-inhibitory activity.

Molecular weight distribution

The molecular weight distribution of the hydrolysates was determined by gel filtration chromatography using a Sephadex G-50 column (1.6×95 cm; Roche Applied Science, Penzberg, Germany) and 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Chromatography was carried out with 2 mg of each hydrolysate fraction at room temperature and a flow rate of 40 mL/hr. The absorbance of the effluent was monitored at 280 nm. A standard distribution was determined against those of aprotinin (6,500 Da) and cytochrome (12,400 Da), which are considered chromatographic standards.

Amino acid composition

The amino acid composition was determined using an amino acid analyzer (Biochrom 30; Pharmacia Biotech, Uppsala, Sweden). Gelatin samples were hydrolyzed in 6 N HCl in evacuated, sealed tubes at 110°C for 16 hr. The acid hydrolysate was evaporated to dryness in a vacuum evaporator at 40°C and diluted with lithium citrate buffer to accommodate analyses of samples containing hydroxyproline and hydroxylysine.

Results and Discussion

Characterization of the first hydrolysates

The degree of hydrolysis in the first hydrolysates increased markedly within the first 2 hr for all of the enzyme systems (Fig. 1). This initial increase was followed by very little change or a slight decrease with further hydrolysis. The highest degree of hydrolysis was observed after a 2-hr digestion with Pronase E, followed in descending order by Alcalase,

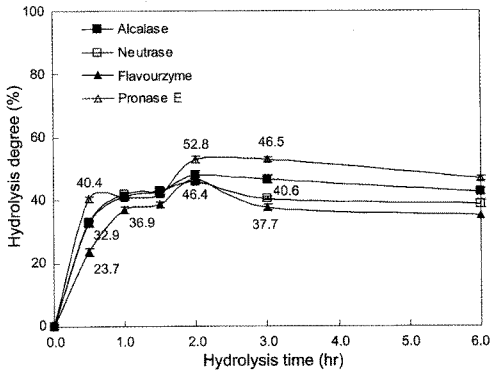

Fig. 1. Hydrolysis degree of the first gelatin hydrolysates from refiner discharge of Alaska pollock incubated for different times with various enzymes.

Table 1. ACE inhibitory activity (IC_{50}) of the first gelatin hydrolysates from refiner discharge of Alaska pollock incubated with various enzymes for different times (mg/mL)

Enzyme	Hydrolysis time (hr)					
	0.5	1.0	1.5	2.0	3.0	6.0
Alcalase	0.934	1.014	0.976	0.904	0.863	0.893
Flavourzyme	11.425	4.366	3.799	3.677	3.301	4.098
Neutrase	3.640	3.635	2.923	1.606	2.242	1.599
Pronase	0.782	0.693	0.656	0.614	0.662	0.638

ACE inhibition was determined with 15 uL of each hydrolysate. IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE inhibitory activity.

Neutrase, and Flavourzyme. These results were likely due to differences in substrate specificity for each of the enzymes. The ACE-inhibitory activity of the hydrolysates, expressed as IC_{50} (mg/mL), increased with proteolytic digestion but was independent of hydrolysis time (Table 1). Among the hydrolysates produced with the same enzyme, the ACE-inhibitory activity was highest in gelatin hydrolysates formed by digestion with Alcalase for 3.0 hr, Flavourzyme for 3.0 hr, Neutrase for 6.0 hr, and Pronase E for 2.0 hr, which exhibited IC_{50} values of 0.863 mg/mL, 3.301 mg/mL, 1.599 mg/mL, and 0.614 mg/mL, respectively. Ukeda et al. (1992) reported an IC_{50} value of 0.62 mg/mL, which is similar that obtained herein for Pronase E with peptic hydrolysates of sardine. In that study, pepsin was the most efficient protease among trypsin, chymotrypsin, and Denazyme AP. These results indicate that the ACE-inhibitory activity of the first hydrolysates was independent of the hydrolysis enzyme and the hydrolysis time. Furthermore, among the proteolytic enzymes evaluated, Pronase E has

been identified as the most effective in the preparation of hydrolysates with high ACE-inhibiting activity from refiner-discharge gelatin of Alaska pollock.

Characterization of the second hydrolysates

The degree of hydrolysis observed with the second gelatin hydrolysates was as high as 72.7-80.2% (Fig. 2), much greater than the 52.8% obtained in the first hydrolysis reaction with Pronase E. The highest degree of hydrolysis in this second reaction was observed with Alcalase (80.2%), followed by Neutrase (74.3%) and Flavourzyme (72.7%). The ACE-inhibitory activity was as high as 24.4% following serial treatment with Pronase E and Flavourzyme (Fig. 3). However, this behavior was not observed with Pronase E/Neutrase-treated hydrolysates or Pronase E/Alcalase-treated hydrolysates. Matsui et al. (1993) reported that with respect to alkaline protease hydrolysates derived from sardine muscle, ACE-inhibitory activity increased markedly with increasing proteolysis. Cheung et al. (1980) reported on a series of peptides that act against ACE-inhibitory activity and concluded that aromatic amino acids at the N-terminus allowed the peptides to bind ACE as a competitive inhibitor. However, other studies have found that inhibitory peptides posses an aliphatic

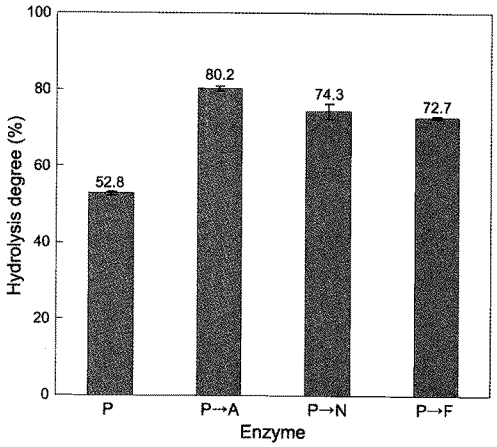

Fig. 2. Hydrolysis degree of the second gelatin hydrolysates from refiner discharge of Alaska pollock incubated by sequential treatment of two proteases for each 2 hr.
P, the first hydrolysate digested with Pronase E; P→A, the second hydrolysate sequentially digested with Pronase E and Alcalase; P→N, the second hydrolysate sequentially digested with Pronase E and Neutrase; P→F, the second hydrolysate sequentially digested with Pronase E and Flavourzyme.

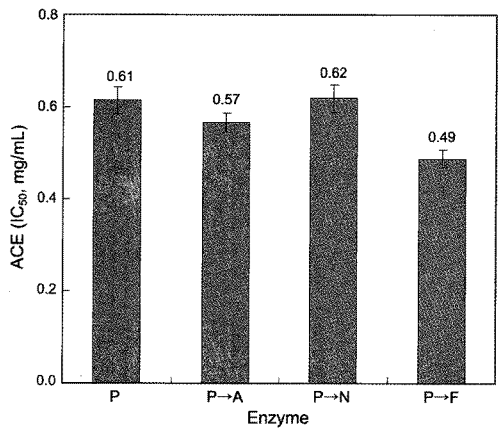

Fig. 3. ACE inhibitory activity (IC_{50}) of the second gelatin hydrolysates from refiner discharge of Alaska pollock incubated by sequential treatment of two proteases for each 2 hr.
P, the first hydrolysate digested with Pronase E; P→A, the second hydrolysate sequentially digested with Pronase E and Alcalase; P→N, the second hydrolysate sequentially digested with Pronase E and Neutrase; P→F, the second hydrolysate sequentially digested with Pronase E and Flavourzyme.

amino acid such as leucine and methionine at the C-terminus (Byun and Kim, 2001). Byun and Kim (2001) and Kim et al. (2001a) also reported that ACE-inhibitory peptides isolated from Alaska pollock skin gelatin hydrolysates and bovine skin gelatin were composed of Gly-Pro-Met and Gly-Pro-Leu, and Gly-Pro-Leu and Gly-Pro-Val, respectively. The current study indicates no significant relationship between ACE-inhibiting activity and the degree of hydrolysis of second gelatin hydrolysates, most likely because the ACE-inhibiting ability depends only on the amino acid sequence of the peptides.

Characterization of hydrolysate ultrafiltrates

The ACE-inhibitory activity, expressed as IC_{50} (mg/mL), of the second hydrolysates after sequential separation through four membrane filters with decreasing MWCOs is shown in Fig. 4. The ACE-inhibiting activity of the second hydrolysates digested by serial treatments of Pronase E and Flavourzyme was 0.49 mg/mL. After fractionation, the ACE-inhibiting activity increased with a decreasing MWCO of the filter. Filtrate containing peptides less than 3 kDa exhibited an IC_{50} of 0.21 mg/mL, which increased approximately two fold in the second gelatin hydrolysates (Pronase E/Flavourzyme-treated hydrolysates). However, no differences in ACE-inhibiting activity were observed among filtrates sequentially

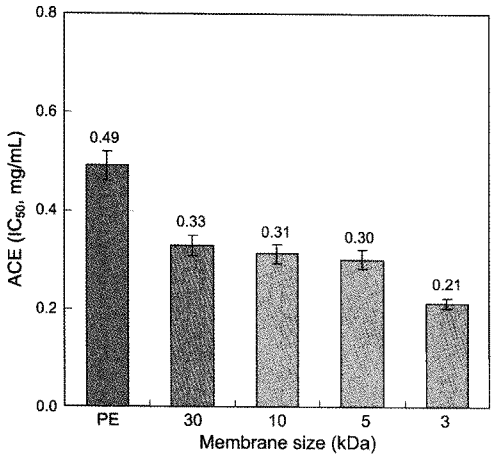

Fig. 4. ACE inhibitory activity (IC_{50}) of the permeates sequentially fractionated through membranes with 3, 5, 10 and 30 kDa molecular weight cut off.
PF, the second gelatin hydrolysate obtained by sequential treatment of Pronase E and Flavourzyme for each 2 hr.

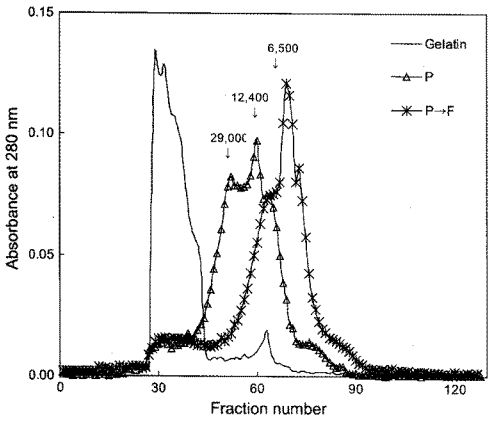

Fig. 5. Molecular weight distribution profile of gelatin, the first gelatin hydrolysates, the second gelatin hydrolysates and the permeate separated through 3 kDa membranes from refiner discharge of Alaska pollock. The gelatin-related materials were chromatographed on Sephadex G-50 column (100×1.6 cm). Standard: carbonic anhydrase (29,000 Da), Cytochrome C (12,400 Da), aprotinin (6,500 Da).
P, the first hydrolysates; P-F, the second hydrolysates; permeates, fraction separated through 3 kDa membranes.

separated through membranes with 5-, 10-, and 30-kDa MWCOs. These results are similar to those of Ariyoshi (1993), whereby the molecular weight of a potent ACE-inhibiting peptide was less than 2 kDa.

Alaska pollock refiner-discharge gelatin, the first and second hydrolysates thereof, and the <3-kDa fraction of the second hydrolysate solution were separated using gel chromatography to determine peptide size. The results are shown in Fig. 5. The gelatin from the refiner discharge was characterized by a high percentage of proteins with molecular weights of about 100 kDa, estimated as an α fraction, and a low percentage of peptides with molecular weights of about 6.5 kDa. Peptide chain length, which depends on the degree of hydrolysis, is of particular interest with regard to organoleptic and health-functional characteristics (Gbogouri et al., 2004). Relative to conventional gelatin derived from land animals, all of the gelatin hydrolysates from the refiner discharge exhibited a lesser fraction of peptides with molecular weights of about 100 kDa, with increased proportions of smaller peptides (<100 kDa). The pattern became even more distinct following the second enzymatic hydrolysis and ultrafiltration. Byun and Kim (2001) reported a correlation between the molecular weight of the hydrolysate and the specificity of ACE-inhibitory activity. The current study corroborates these results and suggests that the

Table 2. Amino acid composition of gelatin hydrolysates from refiner discharge of Alaska pollock incubated with various enzymes for 2 hr
(g/100 g amino acid)

Amino acid	Gelatin	Gelatin hydrolysates		Permeates
		P	P→F	
Aspartic acid	4.4	4.2	4.1	1.7
Hydroxyproline	7.6	7.0	5.7	8.6
Threonine	3.3	3.7	3.3	3.1
Serine	5.3	5.6	5.8	4.9
Glutamic acid	11.3	10.3	10.7	11.3
Proline	12.0	12.2	12.0	11.3
Glycine	21.8	20.3	19.0	22.5
Alanine	11.3	12.4	13.5	9.0
Valine	2.1	2.4	2.2	2.6
Methionine	0.0	0.5	0.7	1.4
Isoleucine	1.3	1.2	2.0	1.6
Leucine	2.2	2.8	3.3	3.7
Tyrosine	0.0	0.3	0.3	-
Phenylalanine	2.2	2.6	2.9	2.6
Hydroxylysine	1.3	0.9	0.8	0.9
Lysine	4.1	4.0	4.1	4.3
Histidine	1.1	1.7	1.6	1.0
Arginine	8.5	7.8	8.1	9.6
Total	100.0	100.0	100.0	100.0

-, not detected; P, the first hydrolysates digested with Pronase E; P-F, the second hydrolysates sequentially digested with Pronase E and Flavourzyme; permeates, fraction sequentially separated through 3 kDa membranes.

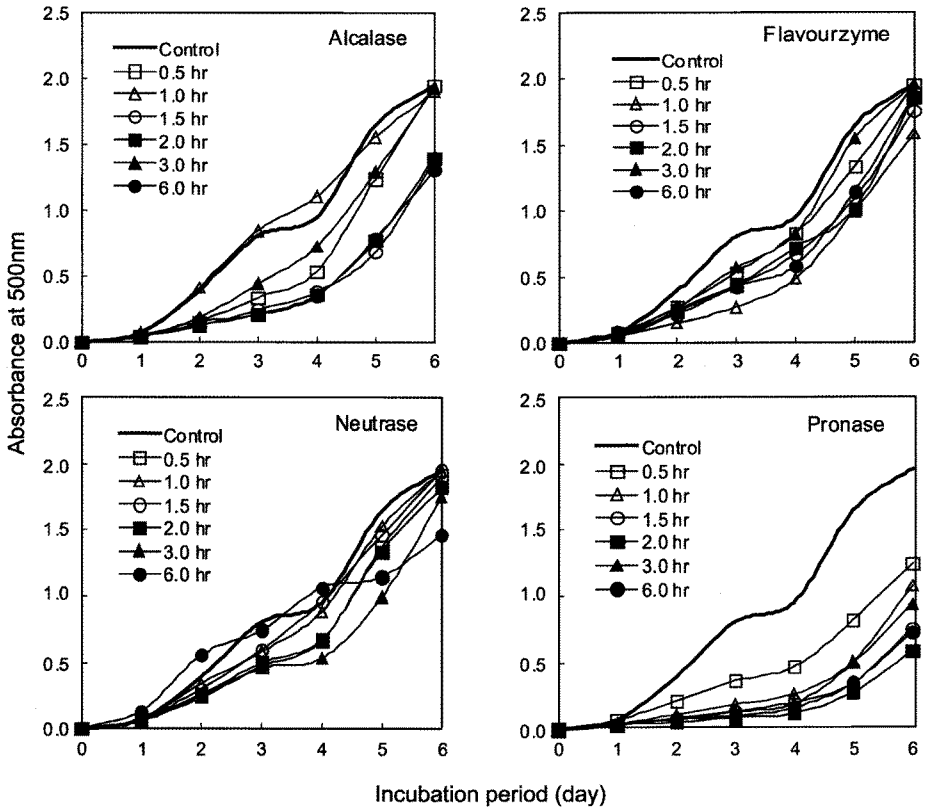

Fig. 6. Antioxidative activities of gelatin hydrolysates from refiner discharge (dilution ratio, ×300) of Alaska pollock incubated for different times with various enzymes. The control is defined where no gelatin hydrolysate is added in the antioxidative activity test.

ACE-inhibiting activity of hydrolysate filtrates <3 kDa was superior to that found for other gelatin-related materials such as refiner-discharge gelatin, the first and second gelatin hydrolysates, and larger filtrates.

The data in Table 2 show that the amino acid compositions (expressed as g/100 g amino acids) of the refiner-discharge gelatin and the first and second gelatin hydrolysates were nearly identical. In these cases, the amino acid profile was rich in glycine (21.8% in gelatin, 19.0-20.3% in gelatin hydrolysates), proline (12.0% in gelatin, 12.0-12.2% in gelatin hydrolysates), and alanine (11.3% in gelatin, 12.4-13.5% in gelatin hydrolysates), while being poor in cysteine (not detected), methionine (not detected in gelatin, 0.5-0.7% in gelatin hydrolysates), tyrosine (not detected in gelatin, 0.3% in gelatin hydrolysates), hydroxylysine (1.3% in gelatin, 0.8-0.9% in gelatin hydrolysates), and histidine (1.1% in gelatin, 1.6-1.7% in gelatin hydrolysates). These amino acid compositions are similar to those of other gelatins and gelatin hydrolysates from various sources (Jamila and Harvinder, 2002; Haug et al., 2004). Relative to the amino acid compositions of the second gelatin hydrolysates, those of the <3-kDa filtrate increased in alanine content (from 9.0% to 13.5%) and decreased in hydroxyproline (from 8.6% to 5.7%), glycine (from 22.5% to 19.0%), and arginine (from 9.6% to 8.1%) content. According to Ondetti et al. (1971), inhibitory peptides of ACE have at least one proline residue. Similar results were observed in this study whereby two isolated ACE-inhibitory peptides were composed of a glycine-proline group at the N-terminus and either a Pro-Leu or Pro-Met group at the C-terminus.

These results suggest that second hydrolysate (Pronase E/Flavourzyme-treated hydrolysates) filtrates that had been fractionated through 3-kDa MWCO membranes, which could be used as a supplementary raw material for improving the health-functional

characteristics of gelatin-containing foods.

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