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Fractionation of Gelatin Hydrolysates with Antioxidative Activity from Alaska Pollock Surimi Refiner Discharge

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This study was conducted to obtain the gelatin fraction with a high antioxidative activity from Alaska pollock surimi by-products using a two-step enzymatic hydrolysis and ultrafiltration. Among gelatin hydrolysates from refiner discharge of Alaska Pollock surimi, the highest antioxidative activity (81.5%) resulted from gelatin hydrolysate sequentially treated with Pronase E and Flavourzyme each for 2 hr. However, no difference was seen in the antioxidative activity of the second hydrolysate (Pronase E-/Flavourzyme-treated hydrolysate) when compared to the permeate fractionated through a 10-kDa membrane. The results suggest that the Pronase E-/Flavourzyme-treated hydrolysate from refiner discharge gelatin of Alaska pollock surimi can be used as a supplementary raw material for improving health functionality.

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

Introduction

Antioxidants are increasingly used to enhance shelf-life and improve the stability of lipids and lipid-containing foods. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used by the food industry because they are effective and inexpensive when compared to natural antioxidants. However, synthetic antioxidants are suspected of causing some safety concerns and have been restricted in their use as food additives. Natural antioxidants, such as α-tocopherol, have also been the focus of considerable interest, but α-tocopherol has limitations for food usage because of its high cost. Thus, a need exists to find inexpensive and natural antioxidants with high activity from various sources (Kim et al., 2001a).

Refiner discharge from surimi processing is about 4% to 8% of the whole fish (Wendel, 1999; Park et al., 2007) and contains a significant amount of collagen (Morrissey et al., 2000). However, the use of surimi refiner discharge as a food for humans has not been widely studied. Most surimi refiner discharge is conventionally used to produce fish meal and fertilizer or directly discharged into estuaries, resulting in environmental pollution (Ciarlo et al., 1997). Thus, new challenges exist to identify methods to upgrade the processing of waste to food-grade ingredients such as collagen and gelatin.

Enzymatic digestion of gelatin is an efficient method of producing peptides containing improved angiotensin-I converting enzyme (ACE) inhibitory and antioxidative activities (Byun and Kim, 2001; Kim et al., 2001a). The facts suggest that gelatin usage can be extended further to health-functional foods not requiring strong physical properties, as well as various ordinary foods, and to pharmaceutical applications, and other industrial applications that do

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require strong physical properties (Cho et al., 2004). Most commercial gelatin (95%) is made from porcine and bovine hides (Cho et al., 2005), but today's health-conscious consumers are reluctant to consume gelatin extracted from land animals due to recent outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease, and avian flu.

Fish gelatin must have rheological properties similar to land animal gelatin for it to be applied to various industrial products, such as ordinary foods, cosmetics, and pharmaceutics. However, satisfying the similar rheological properties of land animal gelatin is difficult with a gelatin from a cold water fish; Alaska pollock, cod, haddock, or Pacific whiting because of lower imino acid content (proline and hydroxyproline; Park, 2000). For these reasons, if fish gelatin is offered as a material for ordinary foods, or cosmetic, pharmaceutical, and other products requiring high rheological properties, it must be modified using chemicals or expensive enzymes; transglutaminase, which have problems with safety or cost. However, if the functionality of a cold water fish gelatin can be improved with cheap commercial enzymes; Alcalase, Flavourzyme, Neutrase, or Pronase E (Kim et al., 1994), it may receive 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 resource for health-functional foods, no research utilizing gelatin from surimi refiner discharge has been conducted.

The objective of this study was to obtain gelatin fractions with a high antioxidative activity derived from enzymatic gelatin hydrolysates from refiner discharge of Alaska pollock surimi.

Materials and Methods

Materials

Refiner discharge, a surimi processing by-product of Alaska pollock, was provided by a commercial surimi processing plant (Trident Seafood Co., Warrenton, Oregon, USA) in January 2006. Refiner discharge gelatin was extracted with hot water according to the method of Park (2006).

Linoleic acid and α-tocopherol to measure antioxidative activity and Pronase E (from *Streptomyces griseus*, type XIV, optimum temperature, 37°C; optimum pH, 7.5) 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 amyloliquefacien*; optimum temperature, 45-55°C ; optimum pH, 5.5-7.5) were purchased from Novo Nordisk (Bagsvaerd, Denmark).

The ultrafiltration system (Minitan^{TM} system) and membranes for the fractionation of each hydrolysate were purchased from Millipore (Bedford, MA, USA). All other reagents used in this study were reagent-grade chemicals.

Preparation of the first and second gelatin hydrolysates and the permeates

The permeates from the refiner discharge gelatin hydrolysates of Alaska pollock surimi were made applying a serial three-step process; the first and second gelatin hydrolysates were prepared from surimi refiner discharge using commercial enzymes and fractions with a high antioxidative activity were separated using four kinds of ultrafiltration membranes (molecular weight cutoff: 3, 5, 10, and 30 kDa) according to the following methods. For preparing the first-step gelatin hydrolysate, gelatin was dissolved in a buffer solution and hydrolyzed with either Alcalase, Neutrase, Flavourzyme, or Pronase E at a 100:2 (w/w) ratio of protein substrate to enzyme at an optimal temperature for the enzymes (Alcalase, 60°C; Flavourzyme, 50°C; Neutrase, 45°C; and Pronase E, 40°C) for 0.5-6.0 hr, and then heated at 98°C for 10 min to inactivate the proteases. For preparing the second-step gelatin hydrolysate, the first-step gelatin hydrolysate was further hydrolyzed with one of the other proteases, except Pronase E, at a 100:2 (w/w) ratio of protein substrate to enzyme and optimal enzyme temperature for 2.0 hr and then heated at 98°C for 10 min to inactivate the proteases. For fractionating the ultra-filtrates with high antixidative activity, the resultant second-step gelatin hydrolysate was sequentially fractionated through membranes with a 3, 5, 10, and 30 kDa molecular weight cutoff, lyophilized, and stored at -20°C until use.

Degree of hydrolysis

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

DH (%) = (10% TCA-soluble nitrogen / Total nitrogen) × 100

Antioxidative activity

Surimi refiner discharge gelatin hydrolysate disolved in 1.5 mL of 0.1 M phosphate buffer (pH 7.0) and 1.0 mL of 50 mM linoleic acid in ethanol (99.5%) was mixed in 5-mL test tubes. The tubes were sealed tightly with silicone rubber cap and kept at 60°C in the dark. At regular intervals, aliquots of the reaction mixtures were withdrawn with a microyringe to measure oxidation using the ferric thiocyanate method (Mitsuda et al., 1966; Chen et al., 1995) with a slight modification. Then 2.35 mL of 75% ethanol, 50 μL of 30% ammonium thiocyanate, and 50 μL of 20 mM ferrous chloride solution in 3.5% HCl were added to 50 μL of the reaction mixtures. The antioxidative activity was expressed as an absorbance of the colored solution at 500 nm measured after 3 min.

Molecular weight distribution

The molecular weight distribution of the hydrolysate was determined by gel filtration chromatography using a Sephadex G-50 column (1.6×95 cm). A 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl was used in this experiment. Chromatography was conducted at room temperature at a flow rate of 40 mL/hr, and absorbance was measured at 280 nm. Two milligrams of each hydrolysate was loaded onto the column. A standard distribution was determined by separating aprotinin (6,500 Da), cytochrome (12,400 Da), and carbonic anhydrase (29,000 Da).

Amino acid composition

The amino acid composition was determined using an amino acid analyzer (Biochrom 30; Pharmacia Biotech, Uppsala, Sweden). The sample was 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 then diluted with lithium citrate buffer (pH 2.2) to analyze the amino acids containing hydroxyproline and hydroxylysine.

Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA). Significant differences between means were identified using Systat version 7.5K (SPSS, Inc., Chicago, IL) at $P<0.05$ (Steel and Torrie, 1980).

Results and Discussion

Degree of hydrolysis and antioxidative activity of the first hydrolysate

The degree of hydrolysis of the hydrolysates increased markedly within 2 hr in all enzyme systems, followed by a slight decrease or little difference with further hydrolysis (Fig. 1). The degree of hydrolysis of the four kinds of hydrolysates digested for 2 hr was the highest in the Pronase E-treated hydrolysate (52.8%), followed by the Alcalase-treated hydrolysate (47.9%), Flavourzyme-treated hydrolysate (46.4%), and Neutrase-treated hydrolysate (45.7%). However, no differences were observed in the degree of hydrolysis among the three kinds of hydrolysates, except for the Pronase E-treated hydrolysate. The results were probably due to a difference in enzyme substrate specificities.

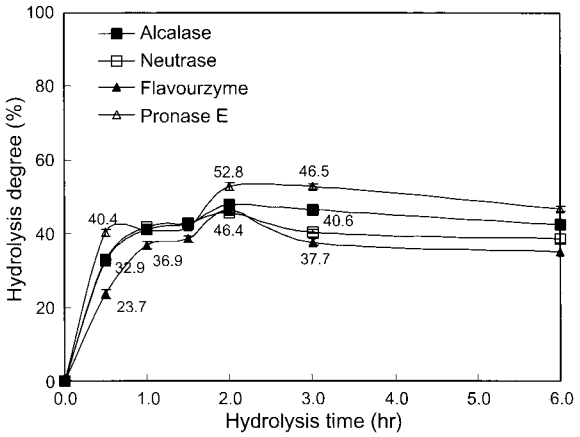

Fig. 1. Degree of hydrolysis of the first gelatin hydrolysates from refiner discharge of Alaska pollock surimi incubated for different times with various enzymes.

The functional properties of bioactive fractions are highly influenced by molecular structure, molecular mass, and processing conditions, and enzymatic hydrolysis has become the most important tool for modifying the functionality of dietary proteins to indentify different bioactivities (Korhonen et al., 1998; Mendis et al., 2005). The antioxidative activity of the first gelatin hydrolysates, which were diluted 300-folds, was measured and compared to that of α-tocopherol (Fig. 2 and Table 1). The antioxidative activity of the hydrolysates was independent of enzyme type and hydrolysis time. The highest antioxidative activity occurred in the hydrolysates digested for 6.0 hr among the Alcalase- or Neutrase-treated hydrolysates, in the hydrolysate digested for 1.0 hr among the Flavourzyme-treated hydrolysates,

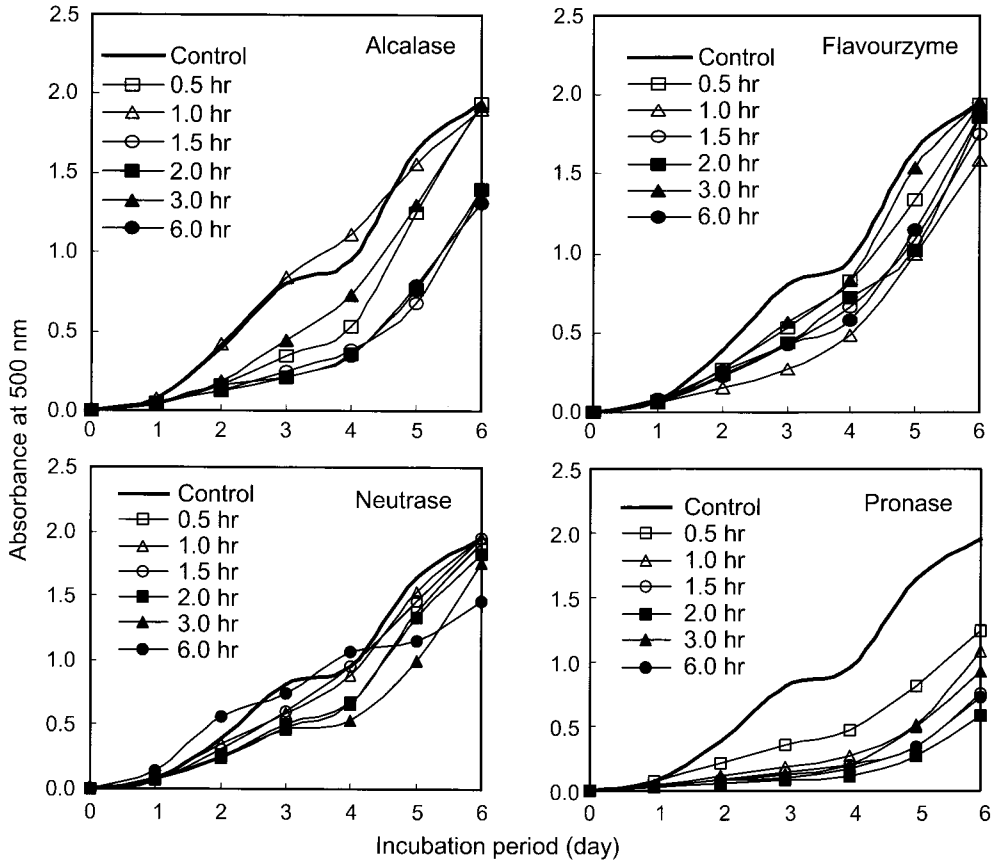

Fig. 2. Antioxidative activity of gelatin hydrolysates from Alaska Pollack surimi refiner discharge (dilution ratio, ×300) digested for different times with various enzymes. The control defined where no gelatin hydrolysate is added in the antioxidative activity test.

Table 1. Antioxidative activities of gelatin hydrolysates from Alaska pollack surimi refiner discharge incubated for 6 days (%)

Enzyme	Hydrolysis time (hr)					
	0.5	1.0	1.5	2.0	3.0	6.0
Alcalase	0.3	2.8	28.4	28.2	1.1	32.6
Flavourzyme	0.1	19.0	10.0	4.4	0.1	3.7
Neutrase	2.3	0.6	0.3	6.8	10.3	25.0
Pronase E	36.4	44.7	62.4	70.1	52.2	62.4

and in the hydrolysate for 2.0 hr among the Pronase hydrolysis or antioxidative activity in the first hydrolysates, probably because their antioxidative activity was affected by the peptide amino acid sequences. Wu et al. (2003) also reported finding no significant difference between the degree of hydrolysis and antioxidative activity of enzymatic hydrolysates from mackerel. Kim et al. (1989) reported that linoleic acid oxidative activity was inhibited to about 40% by E-treated hydrolysates, which showed 32.6%, 25.0%, 19.0%, and 70.1% activity, respectively. No significant differences were observed in the degree of adding hydrolysate from Alaska pollock skin digested with Alcalase.

The results of the first gelatin hydrolysates suggested that Pronase E was the most effective protease to prepare hydrolysates with high antioxidative activity from gelatin of Alaska pollock surimi refiner discharge.

Degree of hydrolysis, antioxidative activity, molecular weight profile, and amino acid composition of the second hydrolysates

The degree of hydrolysis in all of the second gelatin hydrolysates increased compared to that of the first gelatin hydrolysates (Fig. 3). The degree of hydrolysis was highest in the Alcalase-treated hydrolysate (80.2%), followed by the Neutrase-treated hydrolysate (74.3%) and the Flavourzyme-treated hydrolysate (72.7%).

The antioxidative activities of the second gelatin hydrolysates, which were diluted 500 folds, are shown in Fig. 4. After further digestion of the first hydrolysate with various proteolytic enzymes, the antioxidative activity improved in the hydrolysates

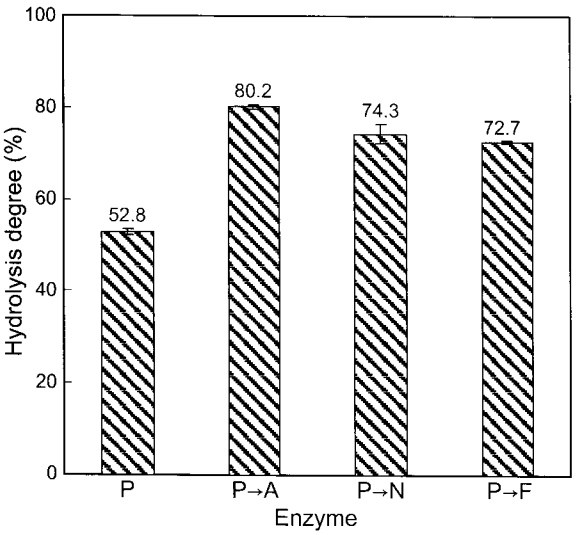

Fig. 3. Degree of hydrolysis of the second gelatin hydrolysates from refiner discharge of Alaska pollock surimi incubated by sequential treatment of two proteases for 2 hr.
P, the first hydrolysate digested with Pronase E; P→A, the second hydrolysate sequentially digested with Pronase E and Alcalase; P→N, the second hydrolysate sequentially digested with Pronase E and Neutrase; P→F, the second hydrolysate sequentially digested with Pronase E and Flavourzyme.

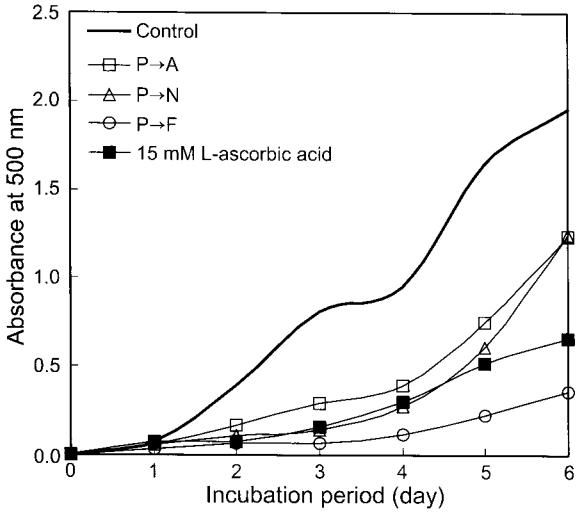

Fig. 4. Antioxidative activity of the second gelatin hydrolysates from Alaska pollock surimi refiner discharge (dilution ratio, ×500) incubated by sequential treatment of two proteases for 2 hr. The control is defined where no gelatin hydrolysate is added in the anti-oxidative activity test.
P→A, the 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.

digested with serial protease-treatments in the order of Pronase E and Flavourzyme; however, those of the Pronase E-/Neutrase -treated hydrolysate and Pronase E-/Alcalase-treated hydrolysate did not improve, suggesting that their antioxidative activity was affected by the peptide amino acid sequences. The antioxidative activity of the Pronase E-/Flavourzyme -treated hydrolysate was superior to that of 15 mM L-ascorbic acid, which has been widely used as a natural antioxidative agent, indicating that the Pronase E-/Flavourzyme-treated hydrolysate seemed to contain some antioxidative peptides. Mendis et al. (2005) reported that the isolated antioxidative peptides from hoki skin gelatin hydrolysate were composed of His-Gly-Pro-Leu-Gly-Pro-Leu. Kim et al. (2001) reported that the isolated P1 and P2 peptides from gelatin hydrolysate of Alaska pollock skin were composed of 13 and 16 amino acid residues, respectively, and that both peptides contained Gly residues at the C-terminus and the repeating motif Gly-Pro-Hyp. Taken together, enzymatic hydrolysis of gelatin from refiner discharge of Alaska pollock by sequential Pronase E and Flavourzyme enhances the antioxidative activity of hydrolytic peptides.

Refiner discharge gelatin, the first gelatin hydrolysates, and the second gelatin hydrolysates were separated using gel chromatography on a Sephadex G 50 to analyze peptide size (Fig. 5). Gelatin from refiner discharge of Alaska pollock surimi was characterized by relatively high proportions of the 100-kDa peak, estimated as the α fraction, and relatively low pro-portions of the 6.5-kDa peak. The peptide chain lengths, which depend on the degree of hydrolysis are of special interest in relation to organoleptic and health-functional characteristics (Gbogouri et al., 2004). As expected, when compared to the gelatin, the relative proportion of all of the gelatin hydro-lysates showed a marked decrease in the 100-kDa peak and an increase in the less than 100-kDa peak. This pattern was distinct as the enzymatic hydrolysis was sequentially advanced. Jeon et al. (1999) re-ported a correlation between hydrolysate molecular weight and specificity of antioxidation activity. According to the molecular weight profile results and the report of Jeon et al. (1999), the Pronase E-/Flavourzyme-treated hydrolysate may show a high antioxidative activity compared to those of the other gelatin-related materials, such as refiner discharge gelatin and the first and second gelatin hydrolysates, except for the Pronase E-/Flavourzyme-treated hydrolysates.

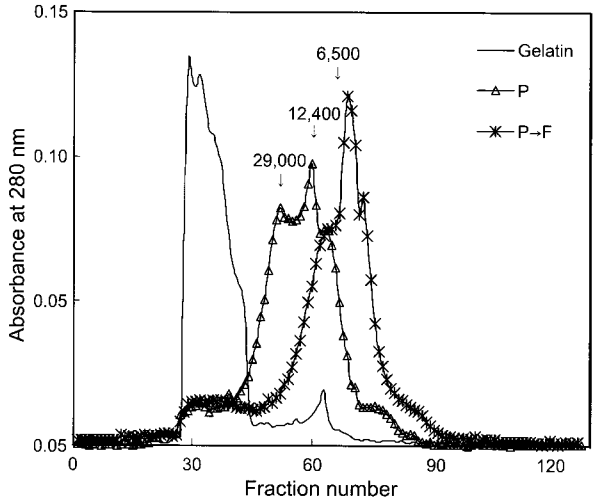

Fig. 5. Molecular weight distribution profiles of gelatin from refiner discharge of Alaska Pollock surimi the first gelatin hydrolysates, the second gelatin hydro-lysates and the permeates sequentially separated through 3 kDa membrane. The gelatin realted materials were chromatographed on a Sephadex G-50 column (100×1.6 cm). standards : carbonic an-hydrase (29,000 Da), cytochrome C (12,400 Da), aprotinin (6,500 Da).
P, the first hydrolysate digested with Pronase E; P→F, the second hydrolysate sequentially digested with Pronase E and Flavourzyme.

The amino acid compositions (expressed as g/100 g amino acids) of the refiner discharge gelatin, the first gelatin hydrolysate (Pronase E-treated hydrolysate), and the second gelatin hydrolysate (Pronase E-/Flavourzyme treated-hydrolysate) are shown in Table 2. The amino acid compositions of the gelatin and the gelatin hydrolysates showed nearly identical values, probably because most of the gelatin used for the hydrolysis was recovered in the gelatin hydrolysate after filtration. The fractions were rich in glycine (21.8% for gelatin and 19.0-20.3% for the hydrolysates), proline (12.0% for gelatin and 12.0-12.2% for the hydrolysates), and alanine (11.3% for gelatin and 12.4-13.5% for the hydrolysates), but poor in cysteine (not detected for gelatin and gelatin hydrolysates), methionine (not detected for gelatin and 0.5-0.7% for the hydrolysates), tyrosine (not detected for gelatin and 0.3% for the hydrolysates), hydroxylysine (1.3% for gelatin and 0.8-0.9% for the hydrolysates), and histidine (1.1% for gelatin and 1.6-1.7% for the hydrolysates). The amino acid compositions were similar to those of other gelatins and gelatin hydrolysates from various sources (Jamilah and Harvinder, 2002; Haug et al., 2004). According to the results of the amino acid composition, the

Table 2. Amino acid composition of gelatin and its hydrolysates from refiner discharge of Alaska pollock surimi (g/100 g amino acid)

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

antioxidative activity of gelatin-related materials is thought to be attributable to Gly, Pro, Ala, Glu, and Arg. Several amino acids, such as Tyr, Met, His, Lys, and Trp, are commonly used as antioxidants (Kim et al., 2001b), despite their pro-oxidative effects in some cases (Marcuse, 1962; Karel et al., 1966; Yamaguchi, 1971).

Antioxidative activity of the permeates by ultrafiltration

The permeates separated through ultrafiltration membranes showed some advantages when compared to the original hydrolysate, including mass production of the desirable fractions and enhancement of some functionalities. Furthermore, the separation process was simplified and the cost of production was good compared to chromatographic processing (Jeon et al., 1999).

For the fractionation of permeates with a high antioxidative activity, the resultant second-step enzymatic gelatin hydrolysate was sequentially fractionated using four kinds of ultrafiltration membranes (30-, 10-, 5-, and 3-kDa molecular weight cutoff) according to molecular size, and four kinds of permeates (30-, 10-, 5-, and 3-K permeates) were obtained.

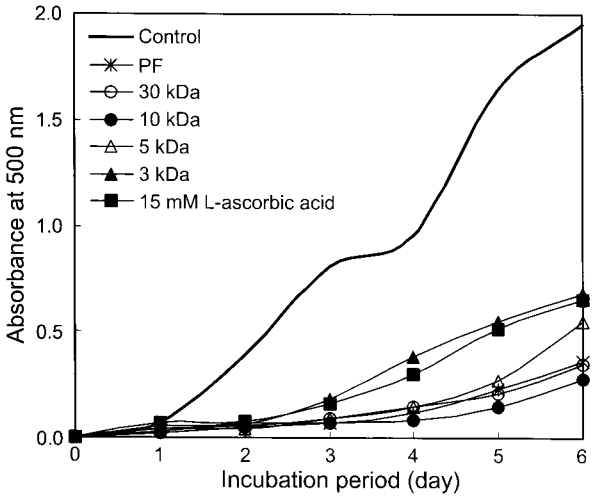

Fig. 6. Antioxidative activity of permeates (dilution times, ×500) separated through four kinds of ultrafiltration membrane using the Pronase E-Flavourzyme treated gelatin hydrolysates from Alaska pollock surimi refiner discharge. The control is defined where no gelatin hydrolysate is added in the antioxidative activity test.
P→F, the second hydrolysate sequentially digested with Pronase E and Flavourzyme; 30, 10, 5 and 3 kDa, permeates separated through various membranes.

The antioxidative activity was the highest in the 10-kDa permeates, followed by the 3-kDa permeates, and the 30-kDa permeates (Fig. 6); however, no difference was detected in antioxidative activity between the second gelatin hydrolysates and the 10-kDa permeates, suggesting that fractionation of the second gelatin hydrolysates through membranes based on molecular weight need not be an intricate process with a high production cost. Jeon et al. (1999) improved the functional properties of cod frame protein hydrolysates using ultrafiltration membranes with molecular weight cutoffs of 30, 10, 5, and 3 kDa and reported that the 10-K and 3-K hydrolysates showed high antioxidative activity and excellent ACE-inhibiting activity, respectively.

The results suggest that the second hydrolysates (Pronase E-/Flavourzyme-treated hydrolysates) from refiner discharge gelatin of Alaska pollock surimi could be used as supplementary raw material for improving health functionality.

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