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Partial Purification of Antioxidative Peptides from Gelatin Hydrolysates of Alaska Pollock Surimi Refiner Discharge

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This study is conducted to partially purify an antioxidative peptide in a two-step gelatin hydrolysate from Alaska pollock surimi refiner discharge, which was obtained by sequential treatment with Pronase E and Flavourzyme. The two-step gelatin hydrolysate was fractionated using chromatographic methods. Based on the same protein concentration of each fraction, the antioxidative activities (85.1-95.4%) of positive fractions fractionated by ion-exchange chromatography were higher than those (27.2-87.8%) from gel filtration. Then, further purification of the positive fractions was performed. Among them, the partially purified A1C1L2G1 and A1C1L2G2 fractions showed 96.2% and 85.1% inhibition, respectively, of linoleic acid peroxidation. The A1C1L2G1 fraction was composed of 15 kinds of amino acids and the predominant amino acids were proline, glycine and alanine. The results obtained in this study suggested that the fraction partially purified through chromatographic methods from the two-step gelatin hydrolysate of Alaska pollock surimi refiner discharge could be useful as a supplementary source for improving health functionality.

Key words: Gelatin hydrolysate, Seafood by-products, Surimi refiner discharge, Surimi, Fish gelatin

Introduction

The refiner discharge from surimi processing accounts for 4-8% 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 human food source has not been extensively studied. Most surimi refiner discharge components have been used conventionally to produce fish meal and fertilizer, or directly discharged into estuaries, resulting in environmental pollution (Ciarlo et al., 1997). Therefore, new challanges must be attempted to find a way to upgrade the processing of waste to food-grade ingredients such as collagen and gelatin.

Oxidation is generally considered as the most frequent form of lipid deterioration, which leads to the development of rancidity, off-flavor compounds, polymerization, reversion, and other reactions causing a reduction in the shelf-life and nutritive value of the food products. In this sense, food processors increasingly use antioxidants [butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol] to enhance the shelf-life and improve the stability of food products. Antioxidant are classified into two large groups, the synthetic antioxidants and natural antioxidants, but they are of limited use in food due to some safety concerns and high cost, respectively (Kim et al., 2001). Thus, a need exists to find cheaper, natural antioxidants with high activity from various sources.

In recent years, several studies have described the antioxidative activity of protein hydrolysates such as milk casein (Yamaguchi et al., 1980), soy protein

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(Pratt, 1972), bovine serum albumin (Yukami, 1972), oilseed protein (Rhee et al., 1979), egg yolk protein (Park et al., 2001), fish frame protein (Jeon et al., 1999; Jun et al., 2004; Je et al., 2005, 2007), pork muscle protein (Carlsen et al., 2003), fish muscle protein (Wu et al., 2003; Klompong et al., 2007), shellfish muscle protein (Rajapaske et al., 2005), and fish skin gelatin (Kim et al., 2001; Mendis et al., 2005). However, little research has evaluated antioxidative peptides from the refiner discharge generated from surimi processing.

The enzymatic digestion of gelatin is an efficient method for producing antioxidative peptides (Mendis et al., 2005; Kim et al., 2001). The facts suggest that the gelatin from refiner discharge can be used as a resource for producing antioxidative peptides.

The objective of this study was to partially purify an antioxidative fraction from an enzymatic gelatin hydrolysate of Alaska pollock surimi refiner discharge using preparative column chromatography.

Materials and Methods

Materials

Refiner discharge of Alaska pollock, a surimi processing by-product, was provided from 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 for the measurement of 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), and Flavourzyme (from *Asperigillus oryzae*, optimum temperature, 50°C; optimum pH, 5.0-7.0) was obtained from Novo Nordisk (Bagsvaerd, Denmark).

Various resins for the fractionation and partial purification of antioxidative fractions from hydrolysates included Sephadex G-50 and Sephadex G-15 (Sigma-Aldrich Co., St. Louis, MO, USA), Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) and Toyopearl SP-650S and Toyopearl Super Q-650 (Tosoh Bioscience LLC, Tokyo, Japan). All other reagents used were of analytical grade.

Preparation of gelatin hydrolysate

The gelatin hydrolysate from surimi refiner discharge was prepared using a sequential two-step protease treatment. To prepare the first hydrolysate, gelatin was dissolved in distilled water and hydrolyzed with Pronase E (a ratio of protein to enzyme of 100:2, *w/w*) at 40°C for 2 hr, and then heated at 98°C for 10 min to inactivate the protease used. To prepare the second gelatin hydrolysate, the first hydrolysate was further hydrolyzed with Flavourzyme (a ratio of protein to enzyme 100:2, *w/w*) at 50°C for 2 hr, and then heated at 98°C for 10 min to inactivate the protease. The resultant second gelatin hydrolysate was lyophilized and then stored at -20°C until use.

Fractionation and partial purification of antioxidative peptides

Fractionation and partial purification of antioxidative peptides from the second gelatin hydrolysate of surimi refiner discharge was conducted using two kinds of charge.

For fractionation, the lyophilized hydrolysate (0.1 g) was dissolved in 10 mL of 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl and separated on a Sephadex G-50 (1.0×60 cm) column using the same buffer. This was followed by separation on a Toyopearl SP-650S column (cation-exchange resin, 2.6×20 cm) and Toyopearl Super Q-650 column (anion-exchange resin, 2.6×20 cm) using 0.01 M sodium phosphate buffer (pH 7.0) with a linear gradient of 0-1 M NaCl, respectively.

For partial purification, the lyophilized hydrolysate (0.1 g) was fractionated using a Toyopearl Super Q-650 anion-exchange column (2.6×20 cm, A1 fraction) and subsequently loaded onto a Toyopearl SP-650S column (2.6×20 cm) previously equilibrated with deionized distilled water. Elution was performed with a linear gradient of 0-0.5 M NaCl at a flow rate of 30 mL/hr. The fractions collected were 5 mL each. The strongest positive fraction (A1C1) was concentrated and then loaded onto a Sephadex LH-20 column (1.6×100 cm) as the next purification step. The separation was performed with 30% (*v/v*) methanol at a flow rate of 10.7 mL/hr and collected as 2.5 mL fractions. Fractions that exhibited antioxidative activity were further purified by Sephadex G-15 column (1.6×60 cm) chromatography at a flow rate of 12 mL/hr with 2.5 mL fractions. The positive fractions were pooled and lyophilized.

The elution profiles were monitored at 215 nm for peptides and 280 nm for proteins using a spectrophotometer. Protein content was determined according to the method of Lowry et al. (1951).

Antioxidative activity

The lyophilized gelatin hydrolysate from surimi refiner discharge was dissolved in 1.5 mL of 0.1 M sodium phosphate buffer (pH 7.0) and the resultant

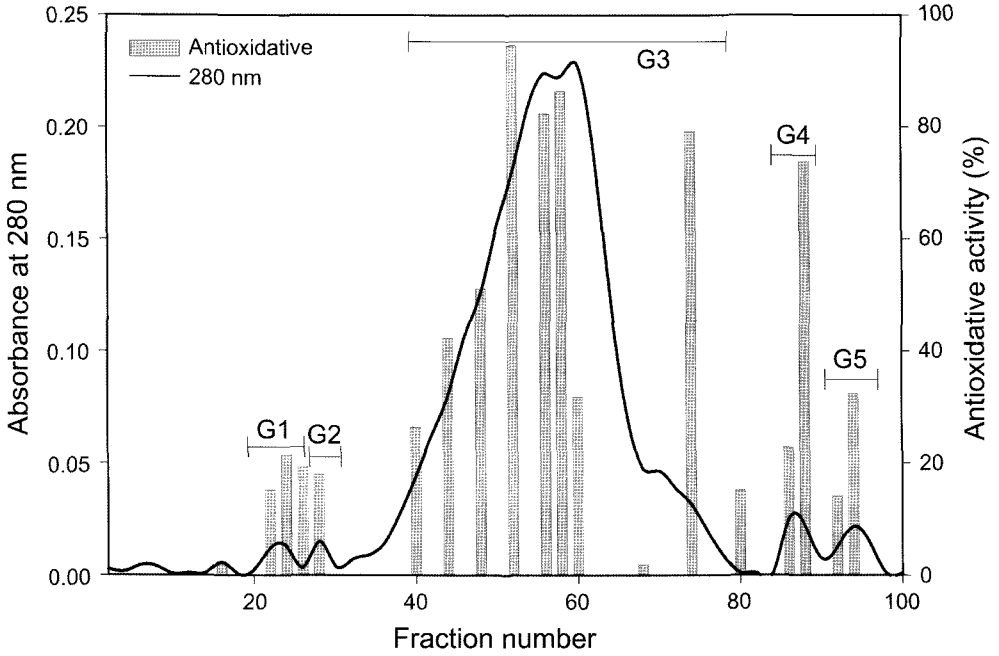

Fig. 1. Gel filtration chromatogram of the second gelatin hydrolysate on the Sephadex G-50 column (ø 1.0×60 cm) for measuring antioxidative activity. Separation was performed at a flow rate of 16 mL/hr and collected at a fraction volume of 0.8 mL. Aliquots, drawn from each fraction, were used to measure antioxidative activity (solid bar).

solution was mixed with 1.0 mL of 50 mM linoleic acid in ethanol (99.5%, *w/v*) in test tubes. Each tube was sealed tightly with a slicone rubber cap and kept at 60°C in the dark. At regular intervals, aliquots of the reaction mixture were withdrawn with a microsyringe for measuring the oxidation using the ferric thio-cyanate method (Mitsuda et al., 1966; Chen et al., 1995) with a slight modification. Next, 2.35 mL of 75% (*v/v*) ethanol, 50 μL of 30% ammonium thio-cyanate, and 50 μL of 20 mM ferrous chloride solution in 3.5% (*v/v*) HCl were added to 50 μL of reaction mixture. After 3 min, the absorbance of the colored solution was measured at 500 nm with a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan).

Amino acid composition

The amino acid composition of the antioxidative peptide was determined using an amino acid analyzer (Biochrom 30, Pharmacia-Biotech, Uppsala, Sweden). The fraction was hydrolyzed in concentrated HCl in evacuated/ sealed tubes at 110°C for 16 hr. The acid-hydrolysate was evaporated to dry in a vacuum evaporator at 40°C, diluted with sodium citrate buffer (pH 2.2), and then used as the sample for amino acid analysis.

Results and Discussion

Fractionation of antioxidative fractions by various chromatography methods

The two-step gelatin hydrolysate (13 mg) was fractionated by size-exclusion chromatography on a Sephadex G-50 column to obtain five pooled antioxidative fractions; G1 (fraction no. 20-26), G2 (fraction no. 27-30), G3 (fraction no. 40-80), G4 (fraction no. 84-90), and G5 (fraction no. 91-98) (Fig. 1). The antioxidative activities ranged from 15% to 70% in fraction G1, 17% to 33% in fraction G2, 25% to 95% in fraction G3 (fraction no. 40-60), 25% to 65% in fraction G4, and 15% to 45% in fraction G5. The five pooled fractions were concentrated and their antioxidative activity measured at the same protein concentration (1 μg/mL, then diluted 100 folds). The strongest antioxidative activity was observed from fraction G3 (87.8%), followed by G4 (76.6%), G5 (50.3%), and G2 (27.2%) (Table 1). The results suggested that gel filtration is an efficient method for obtaining antioxidative fractions from the gelatin hydrolysate.

The hydrolysate (84 mg) was further separated by cation-exchange chromatography on a Toyopearl SP-650S column with a linear gradient of 0-1 M NaCl solution. Four pooled fractions were C1 (fraction no.

Table 1. Antioxidative activity of active fraction of gelatin hydrolysate from Alaska pollack surimi refiner discharge obtained from each chromatographic purification

Gel filtration			Cation exchange			Anion exchange		
Fr.	Fr. no	Antioxidative activity (%)	Fr.	Fr. no	Antioxidative activity (%)	Fr.	Fr. no	Antioxidative activity (%)
G1	20-26	5.8	C1	12-22	93.5	A1	10-19	95.4
G2	27-30	27.2	C2	28-52	87.2	A2	21-26	3.2
G3	40-80	87.8	C3	58-68	90.8	A3	28-40	4.0
G4	84-90	76.6	C4	76-90	85.1	A4	49-58	92.1
G5	91-98	50.3	-	-	-	A5	60-70	90.8

Antioxidative activity was determined with 250 μL of diluted fractions (×100) containing 0.25 μg protein.

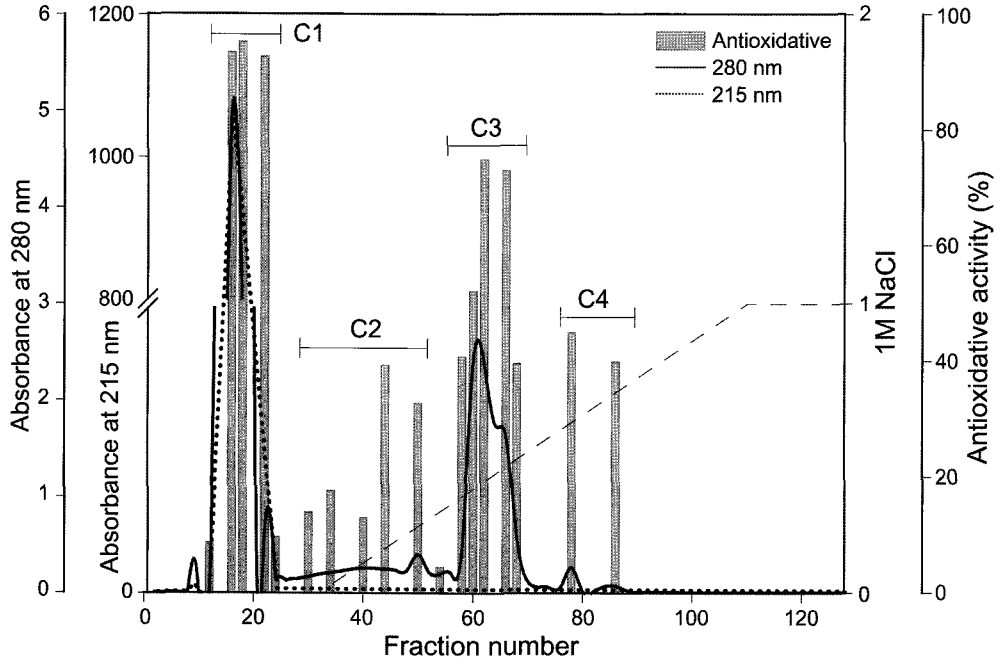

Fig. 2. Cation exchange chromatogram of the second gelatin hydrolysate on the Toyopearl SP 650S column (ø 2.6×20 cm) for measuring antioxidative activity. Its bound peptides were eluted with a linear gradient from 0 to 1.0 M NaCl. Separation was performed at a flow rate of 0.5 mL/min and collected at a fraction volume of 5 mL. Aliquots, drawn from each fraction, were used to measure antioxidative activity (solid bar).

12-22), C2 (fraction no. 28-52), C3 (fraction no. 58-68), and C4 (fraction no. 76-90) (Fig. 2). The antioxidative activity was more than 90% in C1 (flow-through), 35-80% in C2 and C3 (eluted with a linear gradient of 0-0.5 M NaCl), and about 50% in fraction C4 (eluted with a linear gradient of 0.6-0.7 M NaCl). Each pooled fraction was adjusted to a protein concentration of 1 μg/mL. The strongest antioxidative activity was observed from fraction C1, which exhibited 93.5% inhibition of linoleic acid peroxidation (Table 1), followed by C3 (90.8%), C2 (87.2%), and C4 (85.1%). These results suggested that cation-exchange chromatography is an efficient fractionation method of purifying antioxidative peptides from the second gelatin hydrolysate as an early step.

The hydrolysate (42.5 mg) was separated by anion-exchange chromatography on a Toyopearl Super Q-650S column and fractionated with a linear gradient of 0-0.7 M NaCl solution to obtain five pooled fractions; A1 (fraction no. 10-19), A2 (fraction no. 21-26), A3 (fraction no. 28-40), A4 (fraction no. 49-58), and A5 (fraction no. 60-70) (Fig. 3). The antioxidative activity of individual fractions (diluted 100 folds) ranged from 30% to 90% in A1, 10% to 60% in A4, 10% to 40% in A5, and less than 5% in the other fractions. Among the five pooled fractions (adjusted to a protein concentration of 1 μg/mL), fractions A1, A4, and A5 showed high antioxidative activities of 95.4%, 92.1%, and 90.8%, respectively

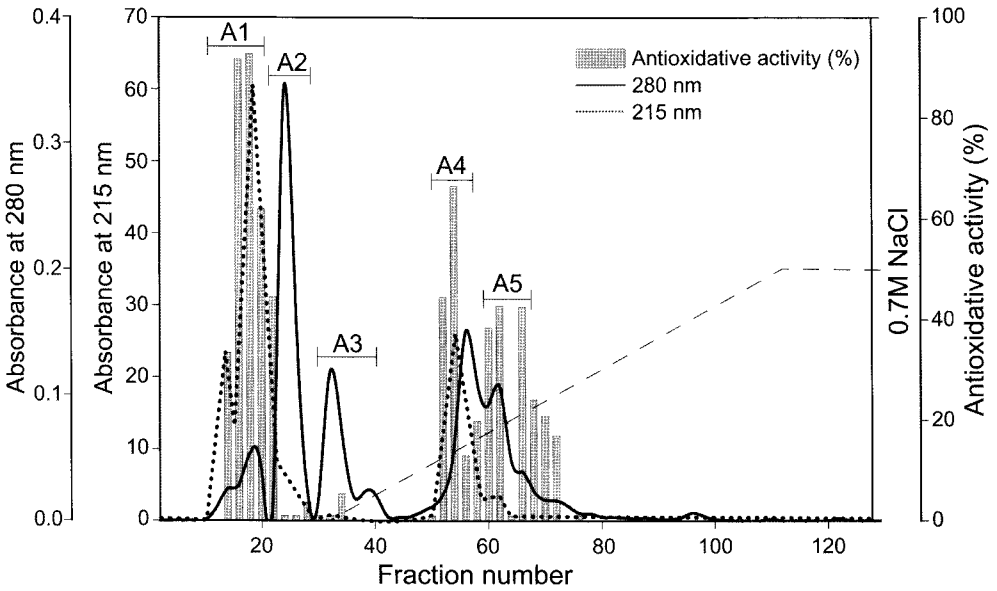

Fig. 3. Anion exchange chromatogram of the second gelatin hydrolysates on the Super Q-650S column (ø 2.6×20 cm) for measuring antioxidative activity. Its bound peptides were eluted with a linear gradient from 0 to 0.7 M NaCl. Separation was performed at a flow rate of 0.5 mL/min and collected at a fraction volume of 5 mL. Aliquots, drawn from each fraction, were used to measure antioxidative activity (solid bar).

Table 2. Antioxidative activity of active fraction of gelatin hydrolysate from Alaska pollack surimi refiner discharge obtained by two-step chromatographic purification

Gel filtration-anion exchange			Cation-anion exchanges or -hydrophobic exchanges			Anion-cation exchanges		
Fr.	Fr. no	Antioxidative activity (%)	Fr.	Fr. no	Antioxidative activity (%)	Fr.	Fr. no	Antioxidative activity (%)
G3A1	10-15	22.1	C1A1	14-19	58.1	A1C1	10-18	85.5
G3A2	17-20	1.3	C1A2	21-26	60.3	A1C2	46-56	81.5
G3A3	21-26	30.5	C3L1	12-15	12.3			
			C3L2	17-22	70.5			
			C3L3	27-38	96.2			
			C3L4	48-76	2.5			

Antioxidative activity was determined with 250 μL of diluted fractions (×300 in gel filtration and anion exchange, and cation-anion exchanges; ×1,000 in anion-cation exchanges) containing 0.025 μg protein.

(Table 1). However, fractions A2 and A3 showed low antioxidative activities of 3.2% and 4.0%, respectively.

Considering each column chromatography step from the above results, ion-exchange chromatography was a more efficient fractionation method than gel filtration. Therefore, we carried out further separations using chromatographic resins on fraction G3 from gel filtration, fractions C1 and C3 from cation-exchange chromatography, and fraction A1 from anion-exchange chromatography.

Further separation of antioxidative fractions

Fraction G3 was further separated by anion-exchange chromatography on a Toyopearl Super Q-650 column and fractionated into three fractions (profile not shown). Fractions G3A1 and G3A3, which were unabsorbed fractions from anion-exchange chromatography before elution with a linear NaCl gradient, showed antioxidative activity of 22.1% and 30.5%, respectively (Table 2), while that from fraction G3A2 exhibited less than 5%. The results suggested that further separation by anion-exchange chromatography on a Toyopearl Super Q-650 column after gel filtration on a Sephadex G-50 column is not an appropriate procedure for purifying antioxidative peptides from fraction G3.

Fractions C1 and C3 were further separated to obtain fractions with high antioxidative activity. Fraction C1, which was an unabsorbed fraction on the Toyopearl SP-650S column, was further separated by

anion-exchange chromatography on a Toyopearl Super Q-650 column and fractionated with a linear gradient of 0-1.0 M NaCl solution (profile not shown) to obtain two pooled fractions, C1A1 (fraction no. 14-19) and C1A2 (fraction no. 21-26), which were unabsorbed fractions before elution with a linear NaCl gradient. The results suggested that fractions C1A1 and C1A2 contained large amounts of hydrophobic peptides unabsorbed to both anion- and cation-exchange resins. Fractions C1A1 and C1A2 (30 μg/mL, diluted 300 folds) displayed antioxidative activity of about 58% and 60%, respectively (Table 2).

Fraction C3, which eluted with a linear gradient of a 0.1-0.5 M NaCl solution, was further separated by hydrophobic chromatography on a Sephadex LH-20 column using 30% methanol and then pooled into four fractions (profile not shown); C3L1 (fraction no. 12-15), C3L2 (fraction no. 17-22), C3L3 (fraction no. 27-38), and C3L4 (fraction no. 48-76). When these four fractions were adjusted to a protein concentration of 0.33 μg/mL (diluted 300 folds) and tested for antioxidative activity, the fractions showed 12.3%, 70.5%, 96.2%, and 2.5% inhibition of linoleic acid peroxidation, respectively (Table 2). The results suggested that the serial chromatography on a Toyopearl SP -650S column and a Sephadex LH-20 column is an effective procedure for purifying antioxidative fractions from the second gelatin hydrolysate.

Fraction A1, which was unabsorbed on anion-exchange resin, was further separated by cation-exchange chromatography on a Toyopearl SP-650S column with a linear NaCl gradient to obtain two pooled fractions; A1C1 (fraction no. 10-18) and A1C2 (fraction no. 46-56) (Fig. 4). Fraction A1C1, which contained a high concentration of protein and peptide, was obtained before elution, while fraction A1C2 contained a relatively low concentration of protein and peptide and was eluted with a linear gradient of 0-0.1 M NaCl. When the fractions were adjusted to a protein concentration of 0.1 μg/mL (diluted 1,000 folds) and tested for antioxidative activity, A1C1 and A1C2 showed 85.5% and 81.5% activity, respectively (Table 2). Fraction A1C1 was higher in both antioxidative activity and protein/peptide content than the fraction A1C2. Thus, the A1C1 fraction was used further.

Partial purification of the antioxidative fraction A1C1

Fraction A1C1 was separated by hydrophobic column chromatography on a Sephadex LH-20 column and fractionated with 30% (*v/v*) methanol into three pooled fractions; A1C1L1 (fraction no. 22-32), A1C1L2 (fraction no. 34-43), and A1C1L3

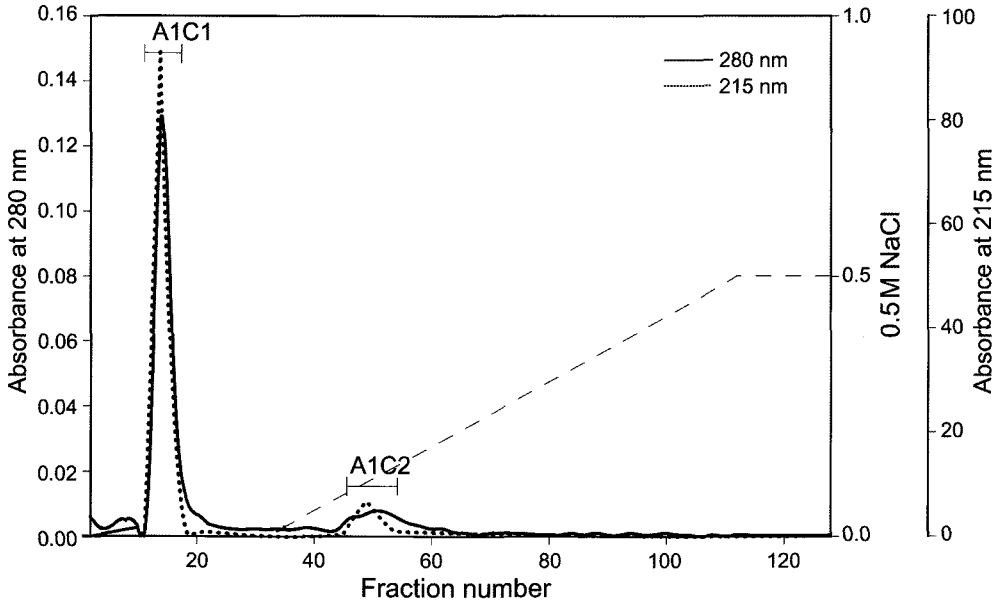

Fig. 4. Ion exchange chromatogram of Toyopearl SP 650S (ø 2.6×20 cm) column of A1 fraction pooled from Super Q-650S chromatography for measuring antioxidative activity. Its bound peptides were eluted with a linear gradient from 0 to 0.5 M NaCl. Separation was performed at a flow rate of 0.5 mL/min and collected at a fraction volume of 5 mL.

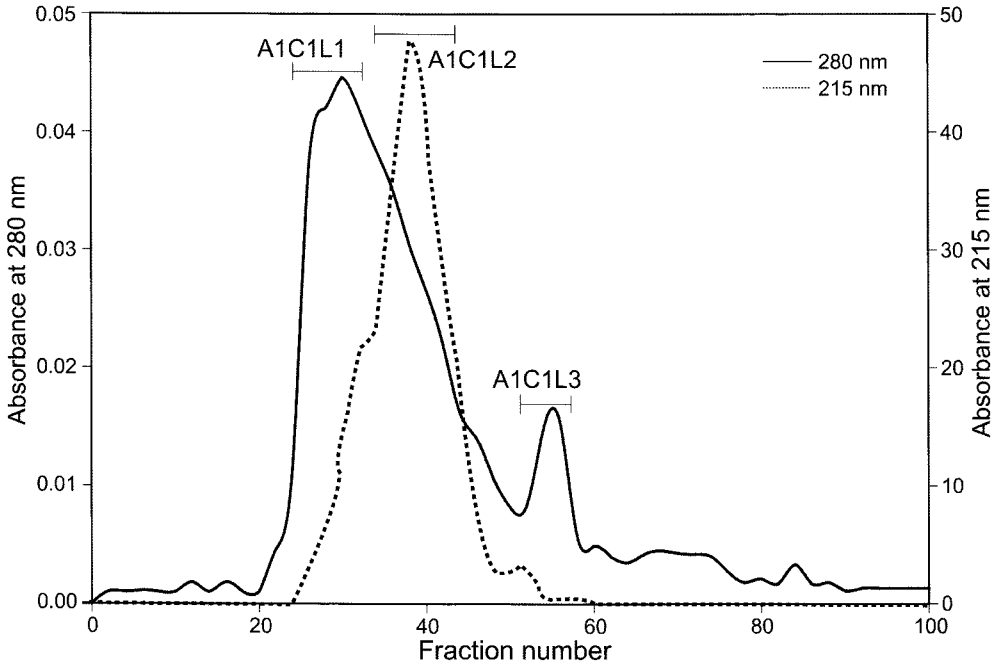

Fig. 5. Chromatogram of Sephadex LH-20 (ø 1.6×100 cm) column of A1C1 fraction pooled from Toyopearl SP 650S chromatography for measuring antioxidative activity. Separation was performed at a flow rate of 10.7 mL/hr and collected at a fraction volume of 2.5 mL.

(fraction no. 52-58) (Fig. 5). When the fractions were diluted 1,000 folds and adjusted to a protein concentration of 0.1 μg/mL, the strongest antioxidative activity was obtained from fraction A1C1L2, which exhibited 92.3% inhibition of linoleic acid peroxidation (Table 3), and fractions A1C1L1 and A1C1L3 showed 80.7% and 81.2% activity, respectively.

Table 3. Antioxidative activity of active fractions from the Sephadex LH-20 (ø1.6×100 cm) column of A1C1 fraction pooled from Toyopearl SP 650S chromatography

Fr.	Fr. no	Antioxidative activity (%)
A1C1L1	22-32	80.7
A1C1L2	34-43	92.3
A1C1L3	52-58	81.2

Antioxidative activity was determined with 250 μL of diluted fractions (×1000) containing 0.025 μg protein.

The lyophilized fraction A1C1L2 was further purified by gel filtration chromatography on a Sephadex G-15 column to obtain two pooled fractions; A1C1L2G1 (fraction no. 24-29) and A1C1L2G2 (fraction no. 31-34) (Fig. 6). When the fractions were diluted 2,000 folds and adjusted to a protein concentration of 0.05 μg/mL, the antioxidative activity of A1C1L2G1 and A1C1L2G2 showed 95.7% and 85.1%, respectively (Table 4). Following the partial purification through four steps of preparative column chromatography, fractions A1C1L2G1 and A1C1L2G2 were enriched by 100 to 2,000 folds compared to the second gelatin hydrolysate.

Table 4. Antioxidative activity of active fractions from Sephadex G-15 column of A1C1L2 fraction pooled from Sephadex LH-20 chromatography for measuring antioxidative activity

Fr.	Fr. no	Antioxidative activity (%)
A1C1L2G1	24-29	95.7
A1C1L2G2	31-34	85.1

Antioxidative activity was determined with 250 μL of diluted fractions (×1000) containing 0.025 μg protein.

Amino acid composition of A1C1L2G1

The amino acid composition, expressed as residues/100 residues, of the antioxidative fraction A1C1L2G1 partially purified from the second gelatin hydrolysate by ion-exchange and gel filtration chromatography is shown in Table 5. Fraction A1C1L2G1 was composed of 15 kinds of amino acids. Proline, accounting for 24 residues/100 residues, was the most abundant amino acid, and glycine (21 residues/100 residues), alanine (12 residues/100 residues), glutamic acid (9 residues/100

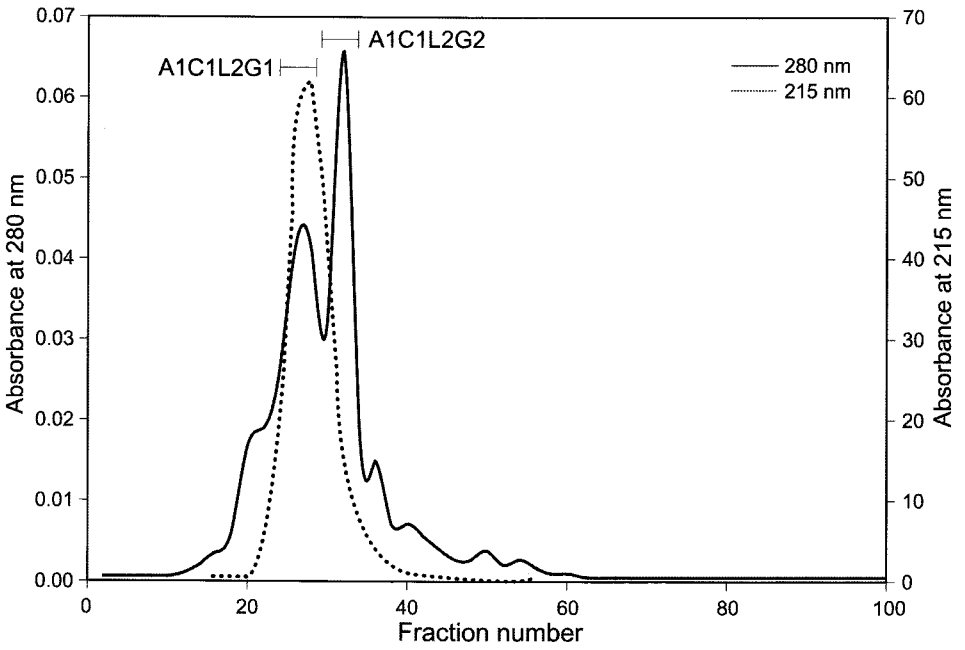

Fig. 6. Chromatogram of Sephadex G-15 (ø1.0×60 cm) column of A1C1L2 fraction pooled from Sephadex LH-20 chromatography for measuring antioxidative activity. Separation was performed at a flow rate of 0.2 mL/min and collected at a fraction volume of 1 mL.

Table 5. Amino acid composition of fraction A1C1L2G1 partially purified through four steps of preparative column chromatography

(residue/100 residues)

Amino acids	A1C1L2G1	Amino acids	A1C1L2G1
Aspartic acid	5	Methionine	2
Threonine	4	Isoleucine	1
Serine	7	Leucine	2
Glutamic acid	9	Phenylalanine	2
Proline	24	Lysine	3
Glycine	21	Histidine	1
Alanine	12	Arginine	5
Valine	2	Total	100

residues), and serine (7 residues/100 residues) were also rich in the fraction. These five amino acids accounted for about 73% of the total amino acids. In contrast, valine, methionine, isoleucine, leucine, phenylalanine, and histidine were much less abundant, at less than 3 residues, compared to the other amino acids. The combined composition of these six low-abundance residues accounted for only 10% of the total amino acids. These observations are probably a result of the various chromatographic methods used to obtain fraction A1C1L2G1 from the second fish gelatin hydrolysate (Kim and Park, 2004). Kim et al. (2001) reported that P2, a peptide isolated from gelatin hydrolysate of Alaska pollock skin using consecutive column chromatographic methods (Sephadex G-25 gel filtration, SP-Sephadex C-25 ion exchange, and HPLC with an ODS column), showed strong antioxidative activity; its amino acid sequence was –Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly. Several amino acids like tyrosine, methionine, histidine, lysine, and tryptophan, are generally accepted as antioxidants despite their pro-oxidative effects in some cases (Marcuse, 1962; Karel et al., 1966; Yamaguchi, 1971). Moreover, many antioxidative peptides include a hydrophobic amino acid residue, valine or leucine at the N-terminus (Uchida and Kawakishi, 1992; Chen et al., 1995). The fraction isolated in this study mainly constituted glycine and proline. These results indicated that the antioxidative activity of the fraction obtained from the second gelatin hydrolysate of Alaska pollock surimi refiner discharge depends on their amino acid sequences. According to the results above, we concluded that the antioxidative fraction (A1C1L2G1) can be used as a supplementary source for improving health functionality. However, further study is needed to obtain more information on the chemical properties of the antioxidative peptide from fish gelatin hydrolysate.

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