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Note

Antioxidative Activity of Peptides Prepared from Okara Protein

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Protease hydrolyses of an Okara protein yielded antioxidative activity against the peroxidation of linoleic acid in an aqueous system at pH 7.0. Four antioxidative peptides were isolated from the hydrolysate prepared with Protease N by size exclusion chromatography and reversed-phase HPLC. The peptides were composed of two and three amino acid residues, including aromatic amino acid at the C terminal end. Their amino acid sequences were determined to be Ala-Tyr, Gly-Tyr-Tyr, Ala-Asp-Phe, and Ser-Asp-Phe, respectively. The antioxidative activity of Gly-Tyr-Tyr is nearly equal to that of carnosine.

Keywords: antioxidative activity, amino acid, peptide, hydrolysate, Okara

In the manufacturing of soymilk and tofu, the soymilk residue, Okara, is produced as a by-product with little market value. However, Okara still contains about 25% protein (dry basis) with high nutritive quality. Studies on the utility of Okara (Takenaka *et al*., 1996; Miyamura *et al*., 1998; Tamura & Takenaka, 1999) have been done, but utilization of the substance is still in the developmental stage. Antioxidative activity has recently been detected in peptides from the proteolytic hydrolysis of many food proteins (Suetsuna *et al*., 2000; Chen *et al*., 1995). In this study, we examined the antioxidative effects of enzymatic hydrolysates of Okara protein with seven different proteases.

Materials and Methods

Materials The Okara protein was prepared using the method of Yamauchi *et al*. (1984). Proteases were purchased from Amano Enzyme Co., Ltd (Nagoya, Japan): Protease A from *Aspergillus oryzae*, 10,000 u/g, pH 7.0; Protease M from *Aspergillus oryzae*, 150,000 u/g, pH 7.0; Protease N from *Bacillus subtilus*, 30,000 u/g, pH 7.0; Protease P from *Aspergillus melleus*, 30,000 u/g, pH 8.0; Protease S from *Bacillus* sp. 10,000 u/g, pH 7.0; Prolezer from *Bacillus subtilus* 10,000 u/g, pH 9.0; and Papain W-40 from *Carica pancreas*, 400 u/g, pH 8.0.

Preparation of the Okara protein hydrolysate Okara protein (10 g) was dissolved in 200 ml of distilled water, and heated at 100°C for 10 min. Protease (0.2 g) was added to the protein solution after the pH was properly adjusted. Enzymatic hydrolysis was performed under optimum pH conditions as the manufacturer recommended. After digestion, hydrolysates were heated in boiling water for 5 min to inactivate proteases, then neutralized and centrifuged (20,000×*g* for 10 min). The supernatants were lyophilized and stored in 4°C until use.

Measurement of antioxidative activity Linoleic acid (10 mg) in 0.5 ml of ethanol, samples in 4.0 ml of 0.2 M phosphate buffer (pH 7.0), and 0.15 ml of 20 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were placed in a vial, which was then sealed tightly and kept at 50°C in the dark. At regular intervals, an aliquot of the reaction mixture was withdrawn for measurement of the oxidation using the ferric thiocyanate method (Mitsuda *et al*., 1966). The time taken to attain an absorbance of 0.3 was defined as the induction period. The relative antioxidative activity was calculated by dividing the induction period of test samples by that of the control.

Isolation of antioxidative peptides The hydrolysate (0.2 g) was dissolved in 1 ml of 50 mM acetic acid and fractionated by size exclusion chromatography on a Sephadex G-25 column (16×900 mm, Pharmacia Co.) equilibrated and eluted with the same solvent. Fractions of 4 ml were collected at a flow rate of 0.8 ml/min. The absorbance at 280 nm and antioxidative activity of all fractions were measured. Tyrosin (Mw, 181), maltohexaose (Mw, 990), and bacitracin (Mw, 1422) were used as the molecular markers. The yield of antioxidative fraction estimated by the *o*-phthalaldehyde method (Church *et al*., 1983) was 13 mg from 1 g of Okara protein. The antioxidative fraction was dissolved in distilled water and separated by reversed-phase HPLC on a TSK-gel ODS 120T column (4.6×250 mm, TOSOH Co., Tokyo) using a linear gradient of acetonitrile (0–50% in 60 min) containing 0.1% trifluoroacetic acid (TFA). Separations were accomplished at 35°C and a flow rate of 1.0 ml/min. The elution peaks were monitored at 230 nm, and their antioxidative activities were measured using 1/50 of each sample. The active peaks were collected and lyophilized.

Amino acid analysis The peptides were hydrolyzed with 6 N HCl at 110°C for 24 h. Amino acid composition was analyzed by an automated amino acid analyzer (JCL-500V; JEOL, Tokyo).

Sequence analysis Amino acid sequences of peptides were analyzed using a Procise 494 cLC protein sequencer (PERKIN ELMER, Applied Biosystems).

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Results and Discussion

The antioxidative activity of the hydrolysates with seven different proteases was measured (Fig. 1); in each experiment, the activity of all hydrolysates increased with hydrolysis. The antioxidative activity of hydrolysates treated with Protease A, N, M and P, reached maximum at 3–6 h of hydrolysis and decreased slightly after 8 h, while the activities of hydrolysates treated with the proteases Protease S, Prorezer, and Papain W increased rapidly for 8 h and reached a plateau.

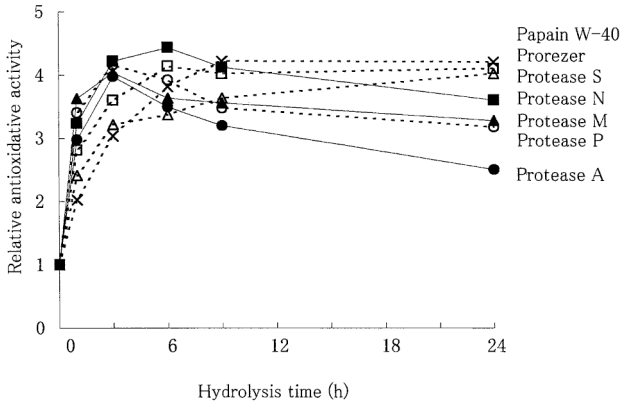

Fig. 1. Antioxidative activity of Okara protein hydrolysated with seven proteases. The antioxidative activity of each hydrolysate was measured by the ferric thiocyanate method. Ten milligrams of sample was used for the assay. (×); Papain W-40, (□); Prorezer, (△); Protease S, (■); Protease N, (▲); Protease M, (○); Protease P, (●); Protease A.

This results indicated that antioxidative activity of the hydrolysates was inherent to their characteristic amino acid sequences of peptides depending on the protease specificities. The hydrolysate with Protease N for 6 h showed the highest antioxidative activity.

To characterize the antioxidative peptides derived from Okara protein hydrolyzed with Protease N for 6 h, the hydrolysate was separated by size exclusion chromatography on Sephadex G-25 (Fig. 2). Fraction II showed the strongest antioxidative activity, and was estimated to be about 1000 Da by molecular weight standard. This fraction was subjected to HPLC to isolate the antioxidative peptide, and fractionated to F-1, F-2, F-3, and F-4 (Fig. 3). From the hydrolysate with Protease N, four antioxidative peptides were finally isolated; their sequences were in good agreement with their amino acid compositions. The amino acid se-

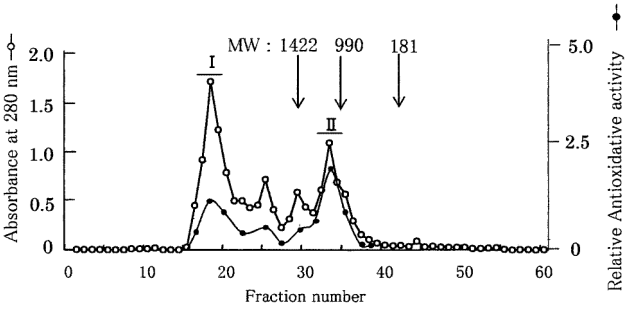

Fig. 2. Elution profile and antioxidative activity of Okara protein hydrolysated with Protease N for 6 h by Sephadex G-25. One milliliter of each fraction (5 ml) was used to determine the the antioxidative Activity. (○); Absorbance at 280 nm, (●); Relative antioxidative activity

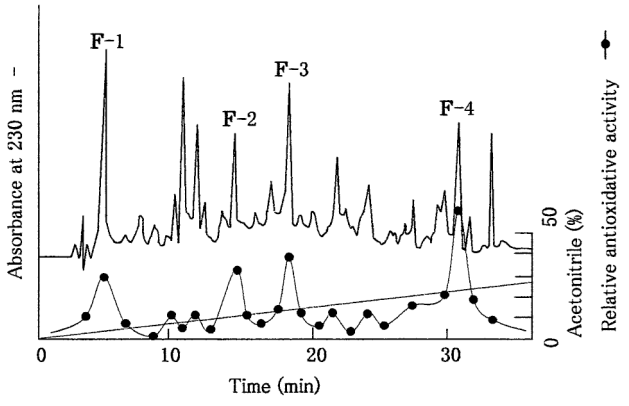

Fig. 3. Elution profile and antioxidative activity of F-II separated by HPLC. One-fiftieth of each fraction was used to determine the antioxidative activity (●).

Table 1. Amino acid sequences of antioxidative peptides isolated from the hydrolysate of Okara protein with Protease N for 6 h.

Peptide	Amino acid sequences
F-1	Ala-Tyr
F-2	Gly-Tyr-Tyr
F-3	Ala-Asp-Phe
F-4	Ser-Asp-Phe

Table 2. Arnino acid composition of antioxidative peptides derived from the hydrolysis of Okara protein.

Amino acid	F-1	F-2	F-3	F-4
	(nmol)			
Asp			1.1 (1)	1.0 (1)
Ser				0.9 (1)
Gly		0.9 (1)		
Ala	1.2 (1)		0.9 (1)	
Tyr	1.1 (1)	2.0 (2)		
Phe			1.0 (1)	1.0 (1)
Total	2	3	3	3

Numbers in parenthesis deduced from the sequences.

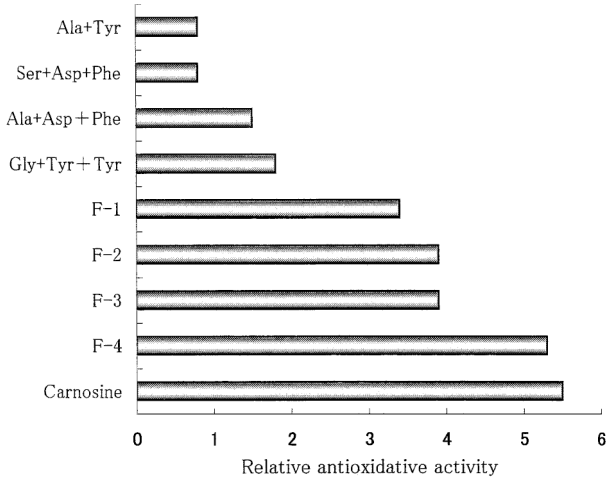

Fig. 4. Antioxidative activities of various amino acids mixtures and peptides. The sample concentration was performed with 10 μM. Data represent the mean of three replications.

quences and amino acid composition of these peptides are shown in Tables 1 and 2. All peptides were di- or tripeptide which were lower in molecular weight than the other antioxidative peptides reported previously (Kudoh *et al*., 2001; Kim *et al*., 2001; Chen *et al*., 1995), and each had an aromatic amino acid at the C-terminal end.

Since the antioxidative effects of some amino acids have been shown (Karel *et al*., 1966), we compared the antioxidative activity of the four peptides with those of each constituent amino acid. When these amino acids were mixed at the same concentration as the peptides, the antioxidative activity of the amino acid mixture of each corresponding peptide was much less than that of each peptide itself (Fig. 4). The antioxidative activity of Gly-Tyr-Tyr was nearly that of carnosine, but those of Ala-Asp-Phe and Ser-Asp-Phe and Ala-Tyr were less than that of carnosine. Thus, it was obvious that characteristic amino acid sequences of a peptide are important for the antioxidative activity. The isolation of other antioxidative peptides from hydrolysates is now being done to determine the relationship between amino acid sequence and activity.

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