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Extraction and Digestibility of *Perilla frutescens* Seed Proteins

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Abstract

Proteins of *Perilla frutescens* seeds were step-extracted with 20 mmol/L phosphate buffer, 0.5 mol/L NaCl, 70% ethanol, and 3% acetic acid. The total amount of extracted proteins from 1 g of seeds was 138.6 mg and 96.7% of the amount of proteins was water- and NaCl-soluble. The water- and NaCl-soluble perilla proteins contained large amounts of Glu, Gly, Asp, Val, and Arg, but lower amounts of Met and His. The water- and NaCl-soluble perilla proteins were immediately digested in simulated gastric fluid containing pepsin. The water-soluble perilla proteins had less trypsin-inhibitive activity compared with soybean and the NaCl-soluble perilla protein had no trypsin- inhibitive activity.

Discipline: Food
Additional key words: defatted perilla meal, pepsin, simulated gastric fluid, trypsin inhibitor

Introduction

Perilla frutescens is widely cultivated in East Asia and the seeds contain about 40% oil[10]. Perilla oil contains 60% α-linolenic acid which is an n-3 unsaturated fatty acid[9], but other plant oils, except flax oil, contain lower amounts of α-linolenic acid. Perilla oil suppressed leukotriene B4 production in rats[3], anaphylactic shock in mice[13] and atopic dermatitis in humans[5], and these physiological effects are probably caused by α-linolenic acid. Perilla seeds are also a feed for pigs to raise the contents of n-3 fatty acids in fat and muscle of pigs[12].

Perilla seeds contain 17.7% protein[10] and defatted perilla meal seems to contain over 30% protein, however the perilla meal has not been utilized as food in Japan. Details of perilla seed proteins have not been reported, except for the cDNA of a methionine-rich storage protein[4]. In addition, trypsin-inhibitive activity of perilla seed proteins is unknown, although many plant seeds contain trypsin inhibitors[2,6–8]. To utilize proteins of defatted perilla meal as food, we studied the stepwise extraction of perilla seed proteins using some solutions, amino acid composition, trypsin-inhibitive activity of the proteins, and the digestibility of the proteins in simulated gastric fluid (SGF).

Materials and methods

Perilla frutescens was cultivated in Fukushima Agricultural Technology Centre. Soybean cultivar "Fukuibuki" was cultivated in National Agricultural Research Center for Tohoku Region. Perilla or soybean seeds were ground using a mortar and 1 g of each seed paste was defatted 3 times with 3 mL chloroform for 10 min at 20ºC. Defatted and dried perilla paste was stepwise extracted with 5 mL of 20 mmol/L phosphate buffer (pH 7.5), the phosphate buffer containing 0.5 mol/L NaCl, 70% ethanol-30% distilled water, and 3% acetic acid. Defatted and dried soybean paste was extracted with 5 mL of the phosphate buffer and next with the phosphate buffer containing 0.5 mol/L NaCl. Every extraction was done 2 times for 30 min at 20ºC, and the supernatants were separated after centrifugation (12,000 × g, 5 min). The proteins extracted with the phosphate buffer are referred to as water-soluble proteins.

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Low-molecular-weight contaminants and NaCl were removed from the water- and NaCl-soluble extracts using a disposable column, PD-10 (GE Healthcare Science, Tokyo), and the phosphate buffer. The extracts of 70% ethanol or 3% acetic acid were dried by a centrifuging evaporator and dissolved with 10% dimethylsulfoxide (DMSO). Protein concentration was determined by a DC-protein assay kit (BioRad, CA, USA) and bovine serum albumin (BSA, Sigma, MO, USA, Cat. #A-2153) as a standard protein. Perilla proteins were hydrolyzed by incubation for 24 hr at 110ºC in 6N HCl, and amino acids were measured by amino acid analyzer, L-8500 (Hitachi).

Protein extracts were mixed with equal volume of Tris-SDS-βME sample treatment solution (Daiichi Pure Chemicals, Tokyo) which contained 0.125 mol/L tris (hydroxyl methyl) aminomethane (Tris)-HCl, 4.3% sodium dodecyl sulphate (SDS), 30% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue (pH 6.8). The mixed samples (20 μL) after boiling at 95ºC for 5 min were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 10–20% acrylamide gradient gels (Oriental Instruments, Tokyo, Japan) and Tris-Tricine buffer (Daiichi Pure Chemicals). Proteins in gels were detected by a silver staining kit (Daiichi Pure Chemicals).

Perilla proteins were digested by SGF[11] with slight modification. Pepsin from porcine gastric mucosa was purchased from Sigma (Cat. #P-6887). Perilla protein solutions (0.08 mL, 5 mg/mL) were mixed with 1.52 ml of simulated gastric fluid (SGF; 0.084 N HCl, 35 mmol/L NaCl, pH 2.0, and 4,000U of pepsin, pH 2.0). BSA was used as a control protein. Portions of 0.1 mL were removed after 0.5, 2, 5, and 10 min of incubation, mixed with 20 μL of 0.2 mol/L Tris and 80 μL Tris-SDS-βME sample treatment solution, and immediately heated at 95ºC for 5 min. Proteins were detected by SDS-PAGE and silver staining.

Trypsin and trypsin-like activity was measured by a fluorescent spectrometer using a substrate of benzyloxycarbonyl (Z)-Gly-Gly-Arg-4-methylcoumaryl-7-amide (MCA, Sigma)[14]. Tris-HCl (50 mmol/L, pH 7.5) containing 5% DMSO and 0.1 mmol/L Z-Gly-Gly-Arg-MCA was dispensed in a black 96-well microplate (40 μL per well). Trypsin solution (2.5 mg/mL, Denkaseiken, Tokyo) was diluted 10-fold with 50 mmol/L Tris-HCl (pH 7.5). Perilla and soybean proteins in the Tris-HCl buffer were prepared at concentrations of 4 mg/mL and 1 mg/mL, respectively. The solutions (5 μL each) of trypsin and the seed proteins were added in the wells (total volume 50 μL) and the microplate was incubated at 37ºC for 1 hr. The fluorescence (excitation: 340 nm, emission: 460 mm) was measured by Mithras LB940 (Berthord Japan, Tokyo). One unit (u) of the enzyme activity corresponds to release of 1 nmol MCA per 1 hr at these conditions.

Table 1. Protein extraction per 1 g perilla seeds

Extract	Protein (mg)
20 mmol/L Phosphate buffer	59.4
Phosphate buffer + 0.5 mol/L NaCl	74.6
70% Ethanol	4.1
3% Acetic acid	0.5
Total	138.6

Results and discussion

1. Protein extraction from perilla seeds

The amount of stepwise extracted proteins from perilla seeds is shown in Table 1. The total amount of the extracted proteins was 138.6 mg per 1 g of seeds and 97% of the proteins were water- and NaCl-soluble. Since estimated protein content of perilla seeds by Kjeldahl method is 17.7%[10], some proteins probably remain in the perilla seed paste even after the extraction of 3% acetic acid. The extracted perilla proteins mainly consisted of water- and NaCl-soluble proteins (96.7%). In contrast, wheat flour proteins consisted of water-soluble albumin (15%), 0.5 mol/L NaCl-soluble globulin (3%), 70% ethanol-soluble gliadin (33%), 0.5 mol/L acetic acid-soluble glutenin (16%), and resting protein (33%)[1].

SDS-PAGE of the perilla extracts is shown in Fig. 1. The water- and NaCl-soluble proteins were widely distributed in molecular weight (10–100 kDa), but the 70% ethanol-soluble proteins mainly contained small molecular weight (10–20 kDa) proteins. The acetic acid-extract was not analyzed by SDS-PAGE because of the small amount.

The water- and NaCl-soluble proteins contained large amounts of Glu, Gly, Asp, Val, and Arg, but lower amounts of Met and His (Table 2). In particular, 5.2% and 4.1% of Lys in water- and NaCl-soluble perilla proteins were higher than Lys contents in wheat and maize proteins. Trp and Cys were difficult to determine because these amino acids were decomposed by the hydrolyzing condition.

2. Digestion of perilla proteins in SGF

The water- and NaCl-soluble perilla proteins were well digested in SGF within 0.5 min (Fig. 2, lanes k and p) but the proteins of small molecular weight (< 10 kDa) remained after 10 min of the incubation (Fig. 2, lanes n and s). The 70% ethanol-extracted proteins were not analyzed because of the small amount. BSA was digested

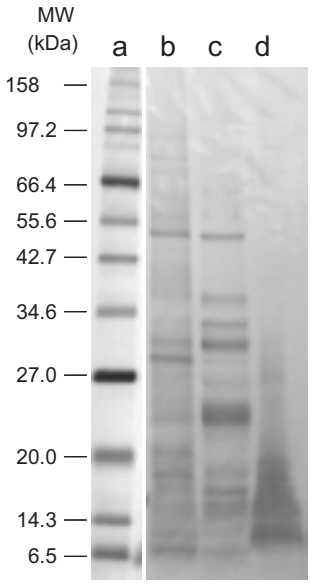

Fig. 1. SDS-PAGE of perilla seed proteins
a: protein markers, b: water-soluble, c: 0.5 mol/L NaCl-soluble, d: 70% ethanol-soluble.

Table 2. Amino acid composition of perilla seed proteins

Amino acid	WS (%)	SS (%)
Asp	10.1	8.8
Thr	4.2	3.5
Ser	7.0	7.0
Glu	16.9	18.9
Gly	13.9	12.4
Ala	8.1	7.9
Val	9.7	9.9
Met	trace	trace
Ile	3.7	3.7
Leu	6.7	6.6
Tyr	2.9	2.9
Phe	3.1	4.1
Lys	5.2	4.1
His	trace	trace
Arg	8.7	10.6

WS: water-soluble proteins.
SS: NaCl-soluble proteins.

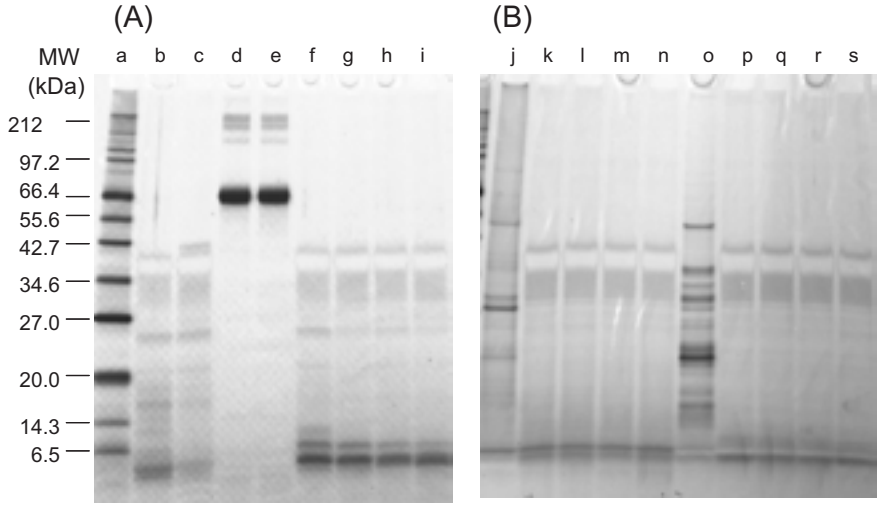

Fig. 2. Digestion of BSA and perilla seed proteins in SGF
(A) a: protein markers, b: SGF (no incubation), c: SGF (60 min), d and e: BSA + SGF without pepsin, d: (no incubation), e: (60 min), f–i: BSA + SGF, f: (0.5 min), g: (2 min), h: (5 min), i: (10 min).
(B) j–n: perilla water-soluble fraction, o–s: perilla 0.5 mol/L NaCl-soluble fraction, j and o: (no incubation), k and p: (0.5 min), l and q: (2 min), m and r: (5 min), n and s: (10 min).

within 0.5 min at the same conditions (Fig. 2, lane f) although the partially digested fragments (<10 kDa) were detected after 10 min of the incubation (Fig. 2, lane i). In contrast, soybean trypsin inhibitor which was difficult to digest by pepsin remained after 60 min of the same incubation[11]. The perilla water- and NaCl-soluble proteins were almost all well digested.

3. Trypsin-like activity and trypsin-inhibitive activity of perilla proteins

Trypsin-like activity and trypsin-inhibitive activity of the perilla and soybean proteins are shown in Table 3. Trypsin-like activities of the water-soluble proteins from perilla and soybean were higher than that of the NaCl-soluble proteins. The water-soluble perilla proteins (20 μg/well) slightly inhibited trypsin activity but NaCl-soluble

Table 3. Z-Gly-Gly-Arg-MCA hydrolyzing activity of perilla and soybean proteins

Proteins and trypsin	Relative activity (%)
Trypsin (0.5 μg/well)	100
Perilla proteins (20 μg/well)	
Water-soluble	27.3
Water-soluble + Trypsin	88.1
NaCl-soluble	6.3
NaCl-soluble + Trypsin	134
Soybean proteins (5 μg/well)	
Water-soluble	35.8
Water-soluble + Trypsin	49.3
NaCl-soluble	16.4
NaCl-soluble + Trypsin	16.0

perilla proteins stimulated the activity. The water- and NaCl-soluble soybean proteins (5 μg/well) strongly inhibited trypsin activity. The amounts of the water- and NaCl-soluble proteins from 1 g of soybean seeds were determined as 157 mg and 46.9 mg. Since the amount of the water-soluble perilla proteins was 59.4 mg per 1 g of seeds (Table 1), perilla seeds had very much lower trypsin-inhibiting activity per seed weight compared with soybean.

Plant trypsin inhibitors unlikely affect intestinal protein digestion because most plants containing trypsin inhibitors are cooked, which inactivates the inhibitors[8]. Wheat amylase/trypsin inhibitors are also reported as allergens for atopic dermatitis[6] and baker's asthma[2]. The perilla water- and NaCl-soluble proteins seem to contain less trypsin inhibitor. Perilla seed proteins can reasonably be expected to be used as food protein because most proteins are extractable with water and NaCl and are readily digestible. Since defatted perilla meal probably contains a reasonable amount of α-linolenic acid which is easily oxidized, addition of antioxidant is necessary to maintain food quality of perilla seed proteins.

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