エゴマタンパク質の乳化特性 ―低分子グロブリンの特性―

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Emulsifying Properties of Perilla (*Perilla Frutescens var. Frutescens*) Proteins: Characteristics of the Low-molecular-weight Globulin

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Abstract

エゴマは油糧種子として利用されているが、その脱脂粕はタンパク質含量が高いにも関わらず有効利用されていない。この未利用食品資源であるエゴマタンパク質の有効利用を目指し、エゴマタンパク質の乳化活性を測定した。植物タンパク質として広く食品工業に用いられる分離大豆タンパク質と比較して、エゴマタンパク質は 2.9 倍も強い乳化活性を示した。エゴマタンパク質を溶解度の違いをもとに、アルブミン画分、低分子グロブリン画分、高分子グロブリン画分に分け、それぞれの乳化活性を測定したところ、低分子グロブリン画分が最も強い乳化活性を示した。ゲル濾過クロマトグラフィーおよび SDS 電気泳動による分析の結果、低分子グロブリン画分の主要タンパク質は、分子量約 12000 の単量体であり、分子量約 6000 の 2 つのポリペプチド鎖がジスルフィド結合していると推測できた。

Perilla seeds are widely used as a source of edible vegetable oil; however, other ingredients are scarcely utilized in spite of containing abundant protein. To effectively utilize perilla seed proteins, we measured their emulsifying activity. Perilla seed proteins had 2.9-times higher emulsifying activity than that of soy protein isolates (SPIs), which are widely used in the food industry. Perilla seed proteins were divided into albumin, low-molecular-weight globulin, and high-molecular-weight globulin fractions based on differences in their water solubility. The emulsifying activity of each fraction was measured. The low-molecular-weight globulin fraction was found to have the strongest emulsifying activity. By gel filtration chromatography and SDS-PAGE analysis, it is suggested that the low-molecular-weight globulin fraction comprised a monomer of approximately 12 kDa, and that

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this molecule has two polypeptide chains of approximately 6 kDa each, connected by disulfide-bond/s. These findings might provide a reference for the utilization of perilla proteins in the manufacture of processed food.

キーワード：エゴマタンパク質、加工特性
Key Words: perilla protein; processing characteristic

Introduction

The use of perilla (*Perilla frutescens* var. *frutescens*) has a long history in Japan. Perilla seeds have been found in ruins from the Jomon period and are considered to have been used in food; traditional perilla dishes are still prepared in Japan. Perilla oil prepared by compression of perilla seeds contains approximately 43% lipids, and α-linolenic acid accounts for 61% of the fatty acids according to the Standard Tables of Food Composition in Japan (eighth revised version, 2020). Therefore, perilla seeds are attracting attention as a source of edible fat and oil that has several health benefits (Peiretti et al., 2011; Kurowska et al., 2003; Narisawa et al., 1991). This has led to yearly increases in the demand for perilla seeds. Furthermore, the protein content in perilla seeds is high (approximately 18%) and its amino acid score is 92, indicating that it is a high-quality protein among plant proteins. However, the use of perilla seed proteins as a food material has not been studied, and defatted perilla seeds are mainly used only as fertilizer and feed.

We previously found that perilla proteins consist of approximately 11% albumin and 84% globulin. Further, one-third of the globulin fraction is of low-molecular-weight proteins and two-thirds is of high-molecular-weight proteins (Takenaka et al., 2010). We also clarified that the most abundant high-molecular-weight globulin is a single protein with a molecular weight of 340 kDa and subunit structure similar to that of soybean glycinin (Takenaka et al., 2010). It forms a heat-induced gel with a good water retention capacity, and the disulfide bond largely contributes to this gel formation (Takenaka et al., 2011).

Emulsifying properties are an important processing characteristic for the utilization of perilla proteins as a food material. Proteins derived from seeds such as soybean, mungbean, azuki bean, kidney bean, sesame seed, and rapeseed, are reported to have emulsifying activities (Aoki et al., 1980; Garcia et al., 2006; Fukuda et al., 2007; Sathe et al., 1981; Yuno et al., 1988; Mohamed et al., 2002). Especially, the emulsifying activities of soybean proteins enable them to be widely used in meat paste products and mayonnaise-like dressings. In this study, the emulsifying activity of perilla proteins was evaluated and the proteins contributing to it were analyzed.

Materials and Methods

Materials.　Perilla seeds harvested in Shimane, Japan were purchased from Wadaman Corp. (Osaka, Japan) and soybeans harvested in Hokkaido, Japan were obtained from Takada Seed Co., Ltd.

(Osaka, Japan). The perilla seeds and soybeans were milled and defatted by three rounds of extraction with 10X volume of n-hexane. HiPrep 16/60 Sephacryl S-100 HR was from GE Healthcare Japan (Tokyo, Japan), SDS-PAGE gels were from ATTO Corporation (Tokyo, Japan), corn oil was from J-OIL MILLS, Inc. (Tokyo, Japan).

Preparation of whole perilla proteins. Ninety-five percent of the perilla proteins was recovered by extraction with 10% NaCl solution. Perilla proteins were prepared using a slightly modified version of a method we previously reported (Takenaka et al., 2010). Defatted perilla seeds (5 g) were extracted with 10X 10% NaCl by stirring for 1 h at room temperature followed by centrifugation at 14,000×g for 15 min. The supernatant was dialyzed overnight against distilled water at 4℃ using a 3.5-kDa molecular weight cutoff membrane (Standard RC tubing, Spectrum Japan, Siga, Japan), and then lyophilized. In total, 2.04 g of protein was obtained.

Preparation of Soy protein isolates. Soy protein isolates (SPIs) were prepared based on their isoelectric points according to the standard procedure. Defatted soybeans (5 g) were dispersed in 10X H_2O, adjusted to pH 9 with 1.0 N NaOH with stirring for 1 h at room temperature, and then centrifuged at 14,000×g for 15 min. The supernatant was separated and the pH was adjusted to 4.5 with 1 N HCl. The sample was then stirred for 1 h and centrifuged at 14,000×g for 15 min. The pH of the precipitate was adjusted to 7, following which it was lyophilized. In total, 1.54 g of protein was obtained.

Extraction and separation of the perilla proteins based on their solubility. Perilla proteins were fractionated using a previously described method (Takenaka et al., 2010). Defatted perilla seeds (5 g) were extracted by stirring for 1 h at room temperature with 10X sequential amounts of H_2O, 1.5% NaCl, and 10% NaCl. The water-soluble fraction was lyophilized. The 1.5% NaCl- and 10% NaCl-soluble fractions were dialyzed overnight against distilled water at 4℃. The dialysate of the 1.5% NaCl-soluble fraction was lyophilized. The dialysate of the 10% NaCl-soluble fraction was centrifuged at 14,000 × g for 15 min at 4℃, and the precipitate was lyophilized. The proteins extracted with H_2O, 1.5% NaCl, or 10% NaCl were regarded as albumin, low-molecular globulin, and high-molecular globulin, respectively.

Protein concentration. The protein concentration of each sample was measured using the Protein Assay Rapid kit Wako II (Wako Pure Chemical Industries, Osaka, Japan), with bovine serum albumin as the standard.

Estimation of emulsifying activity. Emulsifying activity was measured according to the method of Pearce (Pearce and Kinsella, 1978). Pure corn oil (2 mL) and 0.1% protein solution (6 mL, pH 8.0) were homogenized in a mechanical homogenizer (ULTRA-TURRAX T25 basic, IKA-Werke, Staufen,

German) at 24,000 rpm for 1 min. Fifty-microliter aliquots of the emulsions were pipetted from the bottom of the test tube immediately after homogenization. Each aliquot was diluted with 5 mL of 0.1% sodium dodecyl sulfate (SDS) solution. The absorbance of these diluted emulsions was measured at 500 nm, and used to calculate the emulsifying activity index (EAI):

EAI (m^2/g) = 2T (A × dilution factor/C × Φ × 10,000)

where T = 2.303; A= absorbance measured immediately after formation of emulsion, dilution factor = 100, C = weight of protein/unit volume (g/mL) of the aqueous phase before formation of emulsion, and Φ = oil volume fraction of the emulsion.

Sephacryl S-100HR column chromatography. Defatted perilla seeds (4 g) were extracted twice with 10X H_2O and then with 50 mM sodium phosphate buffer (pH 8.0) containing 1.5% NaCl and 0.1 mM (*p*-amidinophenyl) methanesulfonyl fluoride (*p*-APMSF). The globulin sample (2 mL) extracted with the phosphate buffer was subjected to gel filtration chromatography in the same buffer in a Sephacryl S-100HR column (16 mm × 60 cm) at a flow rate of 1 mL/min. A 2.5-μL amount of each fraction was applied to SDS-PAGE.

SDS-PAGE analysis. The perilla proteins and the fractions produced by gel filtration were analyzed by SDS-PAGE using a 10%–20% gradient gel and 15% homogenous gel with or without 50 mM dithiothreitol (DTT), according to the methods of Laemmli (Laemmli, 1978) and Schaegger, (Schaegger and Jagow, 1987), respectively. For molecular weight markers, Low Range (Wako Pure Chemical Industries, Osaka, Japan) and Precision Plus ProteinTM Standards (BIO-RAD Laboratories, Hercules, CA, US) were used.

Amino acid analysis. The amino acid analysis was carried out in an amino acid analyzer according to the standard procedure for protein hydrolysis in 6N HCl (110℃, 24 h). To measure cysteine and methionine, the samples were oxidized using performic acid before hydrolysis. To measure tryptophan, the samples were hydrolyzed in barium hydroxide and then analyzed by HPLC using an XBridge TMC18 column (4.6 × 150 mm, Waters, Milford, MA, US). The column was eluted with 10 mM perchloric acid:methanol (95:5, v/v) at a flow rate of 1 mL/min.

Results and Discussion

SPIs are widely used in the food industry because they are a source of high-quality protein with an amino acid score of 100; further, they have numerous beneficial properties such as water-retaining, gelling, and emulsifying properties (Yamauchi, 1979; Aoki et al., 1980; Nagano et al., 1996). Of note, the emulsifying activity of perilla proteins was 2.9-times higher than that of SPI (Fig. 1). This result suggests that perilla proteins might be suitable for a broad range of industrial food applications.

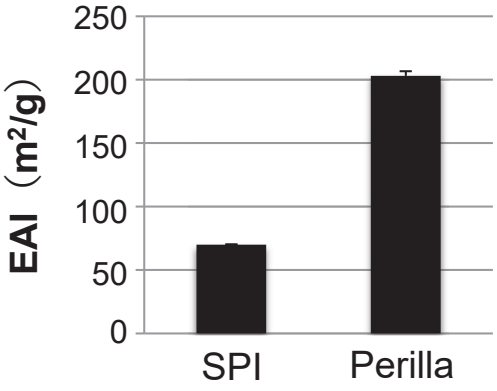

Fig. 1. Emulsifying Properties of SPI and Perilla Proteins.
Each value is the mean ± SEM (n = 5).

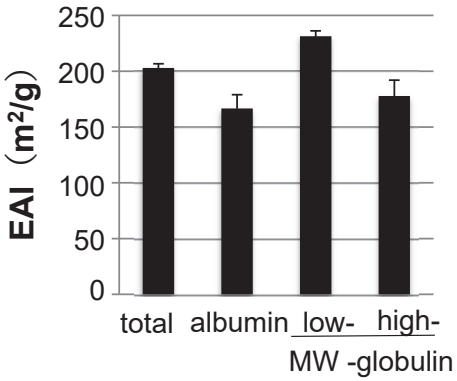

Fig. 2. Emulsifying Properties of Perilla Proteins Based on Their Solubility.
Each value is the mean ± SEM (n = 5).

Albumin and globulin accounted for 95% of the proteins present in perilla seeds, and the main proteins were easily separated based on solubility into albumin (water-soluble), low-molecular-weight globulin (extracted with 1.5% NaCl), and high-molecular-weight globulin (extracted with 1.5-10% NaCl) fractions. The amounts of protein recovered from 5 g of defatted perilla seeds were as follows: albumin fraction, 0.22 g; low-molecular-weight globulin fraction, 1.11 g; and high-molecular-weight globulin, 2.35 g. We previously reported that high-molecular-weight globulin forms a heat-induced gel with a good water retention capacity (Takenaka et al., 2010). Interestingly, the emulsifying activity was the highest in the low-molecular-weight globulin fraction (Fig. 2).

Proteins contained in each fraction were analyzed by SDS-PAGE without a reducing agent. A band was detected at a position corresponding to a molecular weight of 12 kDa as the main component in the low-molecular-weight globulin fraction. The albumin fraction consisted of mainly low-molecular-weight proteins similar to low-molecular-weight globulin, and 2 main bands with molecular weights of approximately 11 and 12 kDa were detected (Fig. 3, lanes 2 and 3). The approximately 12 kDa molecular weight bands detected in the albumin and low-molecular-weight globulin fractions were considered to be different proteins because of differences in their solubility; however, further investigation is necessary to confirm this.

The low-molecular-weight globulin was purified by gel filtration chromatography; its molecular weight was estimated to be 12 kDa from the elution volume of gel filtration chromatography, consistent with the estimated molecular weight on SDS-PAGE without a reducing agent (Fig. 4). This suggests that the low-molecular-weight globulin is a monomer. In addition, the low-molecular-weight globulin was applied to SDS-PAGE in the presence of a reducing agent (Fig. 4, lane 3). A single band with a molecular weight of approximately 6 kDa was observed, suggesting that the low-molecular-weight globulin is present as a monomer consisting of two 6-kDa two polypeptide chains connected by

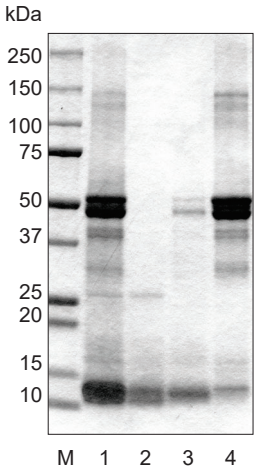

Fig. 3. SDS-PAGE Patterns of Perilla Proteins Based on Their Solubility under Non-Reducing Conditions.

10%–20% gradient gel was used. M, molecular weight markers. lane 1, whole proteins in perilla seeds; lane 2, albumin fraction; lane 3, 1.5% NaCl-soluble globulin fraction; lane 4, 1.5-10% NaCl-soluble fraction.

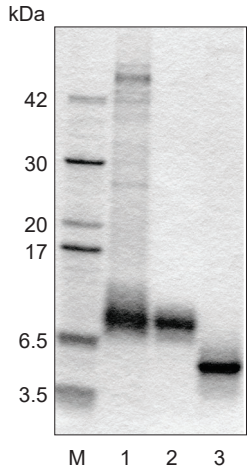

Fig. 4. SDS-PAGE Patterns of Purified Low-molecular-weight Globulin by Gel Filtration Chromatography.

15% homogenous gel was used. M, molecular weight markers. lane 1, 1.5% NaCl-soluble globulin fraction; lanes 2 and 3, purified low-molecular-weight globulin. lane 2, under non-reducing conditions; lane 3, under reducing conditions (with 50 mM DTT).

Table 1. Amino Acid Compositions of Low-molecular-weight Globulin

Asx	244
Thr	101
Ser	231
Glx	1308
Pro	163
Gly	201
Ala	162
Cystine	231
Val	134
Met	130
Ile	116
Leu	254
Tyr	222
Phe	170
Lys	149
His	104
Arg	692
Trp	23

(mg / 1 g of nitrogen)

disulfide-bond/s.

The low-molecular-weight globulin fraction was next subjected to amino acid analysis (Table 1). On comparison with the amino acid scoring pattern (1-2 years old, 2007 WHO/FAO/UNU), amino acids other than sulfur-containing and aromatic amino acids were found to be limiting amino acids. The first limiting amino acid was tryptophan with the amino acid score of 49, for which utilization of the low-molecular-weight globulin as a protein source cannot be expected.

In conclusion, perilla seed proteins were demonstrated to have an emulsifying activity superior to that of soy protein isolates, suggesting their application as a new food material in the food industry. The optimal conditions of the emulsifying activity for application to food processing are currently under investigation. The subunit structure of the low-molecular-weight globulin, which is the largest contributor to the

emulsifying activity, was elucidated. This globulin was estimated to be a single protein with a molecular weight of 12 kDa and constructed from 6 kDa proteins cross-linked by disulfide-bond/s.

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