研究ノート

Purification of Dehydrin Protein from Buckwheat Seed (*Fagopyrum esculentum* cv. Kitawase)

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Abstract

The presence of a dehydrin-like protein in buckwheat seeds had been revealed using an antibody against a Lys-rich conservative motif sequence for dehydrin. In this study, the dehydrin protein was purified from a Japanese variety of buckwheat seed (Fagopyrum esculentum cv. Kitawase) through four purification steps: heat treatment, addition of 10–45% saturated ammonium sulfate, ion-exchange chromatography on a HiTrap Q Fast Flow column, and gel filtration on a HiPrep Sephacryl S-200 column. Results of peptide mapping with trypsin indicated that the purified protein contained conservative motif sequences and Gly-rich sequences, which are characteristic to dehydrin proteins. The results of the pepsin digestion experiment showed that the purified dehydrin was promptly cleaved by simulated gastric fluid, and that a 16-kDa polypeptide that is highly resistant to pepsin digestion was produced.

Key dehydrin; group 2 LEA protein; buckwheat; 16-kDa pepsin-resistant protein

Abbreviations: LEA protein, late embryogenesis abundant protein; PAGE, polyacrylamide gel electrophoresis; SGF, simulated gastric fluid

Introduction

Buckwheat (*Fagopyrum esculentum*), a member of the Polygonaceae family, has a long history of cultivation in Japan as a substitute crop of rice, owing to its tolerance to low temperatures and adaptability to sterile soil. It has been widely used as a food material for noodles, dumplings, and confectionaries in East-Asian countries, and is also used in batter for cakes, pancakes, or crepes in Western countries. Although interest in buckwheat as a health food is growing, allergic reactions caused by its ingestion, including severe symptoms called anaphylaxis, are a serious issue.

Dehydrin is a class of late embryogenesis abundant (LEA) proteins that are expressed in the late stage of seed maturation in response to water stress and/or formation of abscisic acid (Kleinwächter et al., 2014, Radwan et al., 2014). Based on the conserved motif of amino acid sequences across species, and the protein's extreme hydrophilicity, heat stability, and induction by dehydration, dehydrin is thought to be involved in the desiccation tolerance of plants (Close, 1997, Hanin et al., 2011). However, the function of dehydrin protein is not clear (Allagulova et al., 2003). We have studied the dehydrin

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proteins in soybean and rice seeds (Momma, 2002, Momma et al., 1997, 2003) and extended the study to functional aspects, such as the protective activity against the freeze/thaw denaturation of enzyme proteins (Momma, 2003a, 2003b).

In a previous paper, the presence of dehydrin-like proteins in buckwheat and its products were shown for the first time, by immunoblotting assay using an antibody against a conservative motif sequence for dehydrin (Momma, 2009). The possibility that this dehydrin-like protein in buckwheat seeds might be associated with pepsin resistance and buckwheat allergy was also implied. In this current study, the 20-kDa dehydrin-like protein was purified and subjected to pepsin digestion analysis.

Materials and Methods

Purification of dehydrin protein

Whole grain flour of buckwheat (cv. Kitawase, from Japan) was supplied by Dr. Horigane at the National Food Research Institute. Thirty grams of the flour was mixed with 300 mL of 20 mM Tris-HCl buffer (pH 8.0), containing 0.02% sodium azide and 1 mM polymethanesulfonyl fluoride. The suspension was homogenized in a Hiscotron apparatus (NS50; Nichi-On, Tokyo) at 10,000 rpm for 10 min and then centrifuged at 10,000 ×*g* for 20 min. The supernatant was heated in boiling water for 10 min and centrifuged at the same condition in order to remove major seed proteins of buckwheat. After adding ammonium sulfate (10% saturation), the supernatant was stirred on ice for 10 min and centrifuged at 10,000 ×*g* for 20 min. Ammonium sulfate was added to the supernatant to a final concentration of 45% saturation and the mixture was stirred on ice for 10 min. The precipitate that developed was collected by centrifugation at l0,000 ×*g* for 20 min and dialyzed against 20 mM Tris-HCl buffer (pH 8.0). The dialyzate was centrifuged and filtrated through a 0.45-mm filter membrane.

Ion-exchange chromatography followed by gel filtration was carried out with a chromatography system, AKTa prime (GE Healthcare, Tokyo). The filtrate was put on a HiTrap Q Fast Flow ion-exchange column (5 mL) (GE Healthcare, Tokyo) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). Materials were eluted with a linear gradient of 0–1 M NaCl. Fractions containing the 20-kDa protein were concentrated with an ultrafiltration membrane filter (UltrafreeCL-UFC4, LGC25; Millipore, Bedford, MA, USA) and applied on a HiPrep 16/60 Sephacryl S-200 HR gel filtration column equilibrated with the same buffer. The 20-kDa protein was concentrated with the same membrane filter and kept at -20℃ or freeze-dried. SDS-PAGE was carried out by following the method of Laemmli (1970). Samples were mixed with an equal volume of SDS-PAGE sample buffer of 2× concentration, boiled for 5 min, and then loaded onto a 5–20% polyacrylamide gradient gel (PAGEL, NPG-520L; Atto, Tokyo). Electrophoresis was carried out at 20 mA for 90 min. The resultant gel was stained with Coomassie brilliant blue R-250.

Peptide mapping

Purified dehydrin fractions were separated by SDS-PAGE as described above. Bands of 20-kDa polypeptides on the SDS-PAGE gel were excised and digested by trypsin overnight at 35℃ after washing twice in distilled water. The digested polypeptide was separated by HPLC with a Symmetry C18 column (Waters, Milford, MA, USA). The N-terminus amino acid sequences were analyzed with a Procise 494 HT protein sequencing system. In-gel digestion and subsequent analysis were carried out by Apro Science Co. Ltd., Tokyo.

Stability against pepsin digestion

The *in vitro* pepsin digestibility of the purified dehydrin protein was examined by the method of Astwood et al. (1996) using simulated gastric fluid (SGF) containing 0.32% pepsin (from porcine stomach, 3,300 units/mg; Wako, Tokyo) and 30 mM NaCl. Freeze-dried purified fractions were dissolved in 200 mL of distilled water and then 10 mL of the solution containing 6.8 mg of protein was mixed with 40 μL of SGF (128 mg as pepsin) and incubated at 37℃ for 0, 0.25, 1, 2, 4, 8, 15, or 60 min. SDS-PAGE samples were loaded onto a 5–20% polyacrylamide precast gel (NPG-520; Atto, Tokyo) and subjected to electrophoresis at 20 mA for 90 min. The resultant gel was stained with Coomassie brilliant blue dye as described above.

Results and Discussion

Purification of dehydrin protein

In a previous paper, the presence of a dehydrin-like protein in buckwheat and its products were revealed for the first time, using an antibody against a Lys-rich conservative motif sequence for dehydrin (Momma, 2009). It was also implied that the dehydrin-like protein in buckwheat seeds might be associated with pepsin resistance and buckwheat allergy.

In this study, the dehydrin protein was purified from a Japanese variety of buckwheat seed (*Fagopyrum esculentum* cv. Kitawase) through four purification steps: heat treatment, 10–45% saturated ammonium sulfate, ion-exchange chromatography on a HiTrap Q Fast Flow column, and gel filtration on a HiPrep Sephacryl S-200 column.

As indicated previously, the molecular mass of the dehydrin-like protein (20 kDa) was close to that of legumin (22–23 kDa), the major seed protein of buckwheat (Momma, 2009). These proteins were difficult to distinguish in the SDS-PAGE profile of the extract. Since it is known that dehydrin proteins are highly heat soluble in general, heat treatment in boiling water was applied as the first step of purification. Then, the sample was concentrated and subjected to ion-exchange column chromatography. The 20-kDa polypeptide co-eluted with a 35-kDa polypeptide and other minor components. These polypeptides were separated by gel filtration. Consequently, the 20-kDa polypeptide was purified to one band, as verified by SDS-PAGE analysis (Fig. 1, indicated by the arrow), although a trace amount of a 16-kDa band was often associated with it in the series of purification experiments.

Identification of dehydrin by peptide mapping

In order to confirm that the 20-kDa polypeptide is indeed the dehydrin protein, bands of the 20-kDa polypeptide were excised. The polypeptide was digested in-gel with trypsin, and then the N-terminus sequence of the fragment was analyzed. The generally accepted classification of dehydrins is based on their structural features as Y, S, and K segments (Allagulova et al., 2003). In particular, the lysine-rich motif structure, called the K segment and representing a highly conserved amino acid motif (EKKGIMDKIKEKLPG), has been found in all dehydrins. Results of peptide mapping with trypsin digestion indicated that the 20-kDa protein contained two conservative motif sequences (KIKLPG, DEYGNP) and Gly-rich sequences that are characteristic to dehydrin proteins. From the result, it was suggested that the 20-kDa polypeptide found in buckwheat was one of the dehydrin group of proteins.

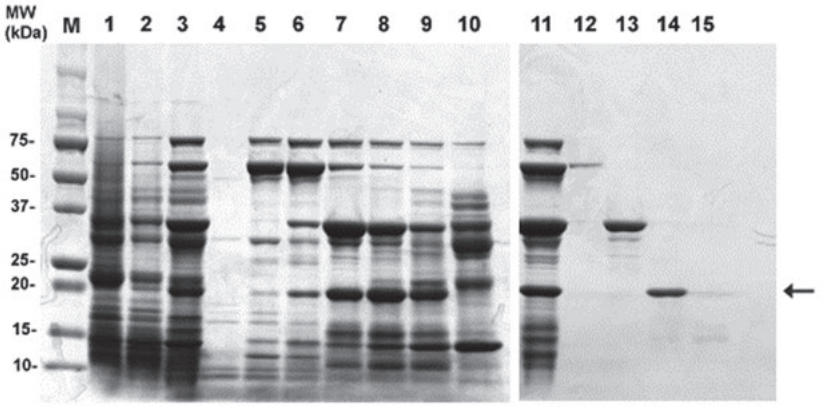

Fig. 1 SDS-PAGE patterns of fractions and purified dehydrin protein from the purification procedure.

M, molecular weight marker; lane 1, extract; lane 2, heated extract; lane 3, sample applied to ion-exchange chromatography; lanes 4–10, fractions 5, 7, 8, 10, 11, 12, and 14 from ion-exchange chromatography; lane 11, sample applied to gel filtration; lanes 12–15, fractions 17, 21, 25, and 28 from gel filtration chromatography.

Pepsin digestibility of purified dehydrin

The pepsin digestion experiment indicated that the purified dehydrin was promptly cleaved enzymatically. The 20-kDa band (marked as * in Fig. 3) almost disappeared within 1 min, producing several intermediate polypeptides of approx. 17 kDa. These polypeptides were also digested within 8 min in the course of the experiment, but the 16-kDa polypeptide (marked as ** in Fig. 3) was found to be highly resistant to pepsin digestion. Based on the above results, it would seem that the trace band of 16 kDa detected in the purified dehydrin (lane 1) was a decomposed fragment of 20-kDa dehydrin.

In a previous paper, minor components of dehydrin-like protein were detected in buckwheat flour and its products by immunoblotting assay using an antibody against the lysine-rich motif structure of dehydrin (Momma, 2009). It is supposed that those minor components are also fragmentation products of the 20-kDa dehydrin.

In general, food allergens are considered to be

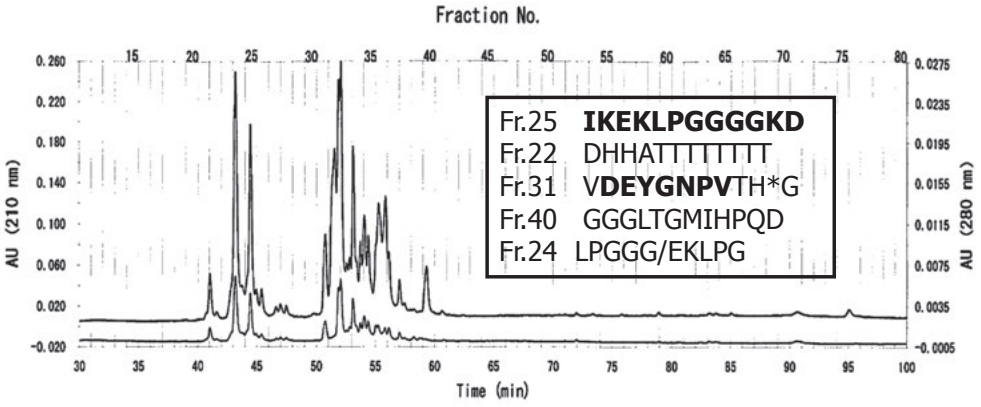

Fig. 2 N-Terminal peptide sequences of fragments from dehydrin proteins.

Profiles of reverse-phase chromatography and N-terminal peptide sequences of fractions 20, 22, 25, 31, and 40 are shown. Conserved motif sequences of dehydrin are shown in bold characters.

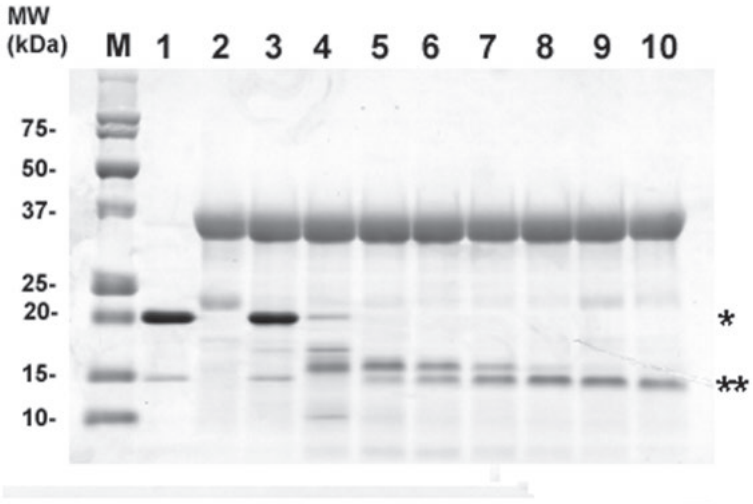

Fig. 3 Pepsin digestion assay of purified dehydrin protein.

M, Molecular weight marker; lane 1, purified dehydrin protein; lane 2, simulated gastric fluid (SGF); lanes 3–10, dehydrin protein incubated in SGF for 0, 0.25, 1, 2, 4, 8, 15, and 60 min, respectively. The asterisk marks on the right indicate the 20-kDa dehydrin and pepsin-resistant polypeptide.

stable against various digestive treatments. Lee et al. (2013) investigated the enzyme-resistant characteristic of buckwheat protein and its allergenic potential. They found a 16-kDa buckwheat protein remaining after 30 min of pepsin treatment on the SDS-PAGE gel, and implied the allergenicity of its hydrolysate. They also indicated the allergenic potentials of fragments produced from buckwheat proteins. In an earlier study, Tanaka et al. (2002) found a pepsin-resistant 16-kDa protein associated with an immediate hypersensitivity reaction to buckwheat allergens. They found that the N-terminal sequence of the 16-kDa protein (RDEGFDLGETQMSSK) had 80% homology with *Oriza sativa* dehydrin (GenBank Accession No. OSU60097). A few researchers have indicated that dehydrin proteins are involved in food allergy reactions. Chung et al. (2003) suggested that the accumulation of dehydrin in the peanut seed increases IgE binding and advances glycation end adducts.

It is of interest how the enzymatic fragmentation affects the protein structure, including its epitope exposure. It is also of importance to clarify the involvement of dehydrin in the allergenicity of crops. Since this protein is induced by maturation drying, there would be a way to control dehydrin levels in the course of harvesting and preservation.

In conclusion, a 20-kDa polypeptide in buckwheat seeds was purified and confirmed to be a dehydrin protein on the basis of its internal amino acid sequences. In an enzymatic digestion assay, a highly pepsin-resistant protein was found to be produced from the 20-kDa dehydrin.

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要約

デハイドリンは、乾燥や低温によって誘導される植物ストレスタンパク質であり、種子にも広く分布することが知られている。前報において、デハイドリン固有のモチーフ配列に対する抗体を用いて、ソバ種子ならびに食品中にデハイドリン様タンパク質が存在することを示した。本研究では日本産ソバ種子（品種キタワセ）から、硫酸アンモニウム分画、イオン交換クロマトグラフィーおよびゲル濾過によって、20kDaデハイドリンを単離精製した。トリプシン消化物のアミノ酸配列分析の結果、デハイドリンのC末端側モチーフ配列と、デハイドリン固有のリジンモチーフ配列を有することが確認された。ペプシン消化性を検討した結果、16kDaペプシン消化耐性ポリペプチドの生成が見られた。

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