Detection and Cryoprotective Activity of Dehydrin Proteins from Rice Bran and Soybean Whey

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Summary Dehydrin proteins, group2 LEA proteins in rice bran and soybean whey were analyzed by two-dimensional (2D) electrophoresis, and the cryoprotective activity on freeze/thaw inactivation of lactate dehydrogenase was examined as a criterion of its function. Dehydrins in rice bran were detected by immunoblotting using antibody raised against a conserved lysine-rich motif sequence. In the water-soluble fraction of rice bran, 10 spots of 44 kDa and 23 kDa dehydrin-like polypeptides were detected on the immunoblotted membrane. Isoelectric points of the polypeptides were between 6.6 and 7.4. The 23 kDa dehydrin polypeptide was partially purified by ammonium sulfate fractionation and ion exchange column chromatography. CP₅₀ value, protein amount necessary to keep 50% of enzyme activity, of the 23 kDa dehydrin was 0.78 μ M (15.6 μ g/mL), slightly lower than that of bovine serum albumin. Heat-soluble soybean whey proteins were analyzed by SDS-PAGE and 2D-electrophoresis. Dehydrin appeared to be the most abundant protein in the fraction. CP₅₀ value for heat-soluble whey protein was estimated to be 15.8 μ g/mL, while that of total whey was 355 μ g/mL. The result indicated that simple heat fractionation is efficient to concentrate cryoprotective protein from soybean whey.

Key Words dehydrin, rice bran, soybean whey, cryoprotective activity, two-dimensional electrophoresis

Dehydrin is known to be a group2 late embryogenesis abundant (LEA) protein which accumulate in maturing seed. It is suggested that dehydrin has functions to protect protein structure or membrane in plant tissue under abiotic stress like desiccation or low temperature (1). Beside the biological function, its application to preservation of foods is implied similarly to anti freeze proteins in fish (2).

To characterize rice dehydrin, protein in rice bran was analyzed by 2D-electrophoresis. Dehydrins in the protein fraction were detected by immunoblotting using antibody against conserved motif structure, which is known to be specific sequence of dehydrin. Then, cryoprotective activity of fractions and dehydrin protein from rice bran was investigated to assess the function.

Soybean, one of the major protein resources in Japanese food, also contains substantial amount of dehydrin. We have characterized 26/27 kDa dehydrin in soybean varieties in our previous studies (3, 4). It is desirable to establish an efficient method for isolation in order to utilize these proteins. In the latter part of this report, dehydrin fraction was separated by simple heating process from soybean whey. Soybean seed contains numerous enzymes and functional proteins such as amylase, lipoxigenase, trypsin inhibitor and lectin other than the major seed storage proteins, glycinin and β -conglycinin. These proteins are concentrated into whey fraction, when soy protein is extracted by acidic buffer which precipitates storage proteins. Dehydrin-rich fraction was isolated from soybean whey by its heat-tolerance from

the whey.

Materials and Methods

Fractionation and partial purification of proteins

Rice bran was obtained by milling 1 kg of rice seeds (cv. Koshihikari) to 90% by weight. Fifty grams of the rice bran was defatted with 500 mL of hexane for three times and kept at -20°C before use.

The defatted rice bran (20 g) was mixed with 20 mM HEPES-NaOH, pH 7.0 for 3 min in a hiscotron mixer (NS-50, Nichi-On), centrifuged at $8,000 \times g$ for 20 min. Supernatant was centrifuged again at $10,000 \times g$ for 20 min, then the supernatant was collected as water-soluble fraction. A part of the water-soluble fraction was heated in boiling water for 10 min and centrifuged at $12,000 \times g$ for 20 min, and the supernatant was collected as rice bran heat-soluble fraction.

Rice dehydrin was partially purified by modifying the method of purification of soybean dehydrin (5), as shown in Fig. 1. From 20 g of defatted rice bran, 10–50% saturated ammonium sulfate fraction was isolated, dialyzed, heated in boiling water, then centrifuged. Supernatant was put onto an S Sepharose Fast Flow column (2×35 cm) and eluted with a linear gradient of 0–300 mM NaCl. The dehydrin fraction was collected based on detection by SDS-PAGE and immunoblotting with antiserum against conserved lysine-motif of dehydrin. Water-soluble fraction, heat-soluble fraction, and the partially purified dehydrin were dialysed against distilled water and freeze-dried and used for assessment of cryoprotective activity.

Ten grams of soybean seeds (cv. Nattosoryu) were

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20g of defatted rice bran
 ↓ homogenized in 500ml of 20mM HEPES-NaOH, pH7.0
 ↓ filtrated and centrifuged at 8,000xg, 12,000xg for 20 min
 10-50% saturated (NH₄)₂SO₄
 ↓ dialyzed against 20mM MES-NaOH, pH6.5
 ↓ heated at 100°C for 5 min
 ↓ centrifuged at 3,000rpm for 20 min.
 Sup.
 ↓
 S Sepharose Fast Flow chromatography

Fig. 1. Purification procedure of dehydrin protein from rice bran.

ground on a mill (Sicer Type I) and sieved through 60-mesh sieve. Sample powder (1 g) was mixed with 10 mL of 100 mM HEPES-NaOH, pH 7.0 or 200 mM Na-Acetate, pH 5.2 in mortar and pestle. Extracts were collected after centrifugation at 10,000 ×g for 15 min as water-soluble fraction and whey fraction, respectively. Whey fraction was heated in boiling water for 10 min and centrifuged at 10,000 ×g for 15 min. Supernatant was collected as heat-soluble whey fraction. Water-soluble fraction and heat-soluble whey fraction were dialyzed against distilled water and freeze-dried and used for assessment of cryoprotective activity.

SDS-PAGE and Two-dimensional (2D) electrophoresis

SDS-PAGE was carried out on 5–20% polyacrylamide gradient gel (PAGEL, NPG-520L, ATTO) by the method of Laemmli. Gels were stained with Coomassie Brilliant Blue (CBB) R-250.

Proteins fractions isolated from soybean whey and rice bran was analyzed by 2D-electrophoresis using Multiphore IISystem (Amersham Pharmacia Biotech) following the supplier's instruction. IPG strips (pH 3–10 NL, 7 cm) were incubated overnight in a protein fraction mixed with sample buffer (3–10 NL). Then the IPG strips were washed with distilled water and put onto a Multiphore system. Then electrophoresis was carried out at 200–3,500 V for 90 min and at 3,500 V for 65 min. After equilibration in 50 mM Tris-HCl, pH 8.8 containing 6 M urea, 30%(w/v) glycerol, 2% SDS, 10 mg/mL dithiothreitol, and trace of bromophenol blue, SDS-PAGE was carried out on a 5–20% polyacrylamide gradient gel and stained with CBB R-250.

Immunoblot analysis

SDS-PAGE gel was incubated in a blotting buffer (25 mM Tris-HCl, pH 9.5 containing 40 mM ε-amino-caproic acid, 20% methanol, 0.05% SDS) for 20 min, then protein on the gel was blotted onto a PVDF membrane (Immun-Blot, Biorad) at 2.5 mA for 30 min. The PVDF membrane was kept overnight in 3% BSA-TBS (Tris-Buffered Saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 3% bovine serum albumin). Dehydrin protein was detected by polyclonal antiserum raised against conserved lysine-motif sequence (DQNEKK-GIMDKIKEKLPGGH) conjugated with ovalubumin. The

Fig. 2. SDS-PAGE and immunoblotting of rice bran protein. A, SDS-PAGE. B, Immunoblot against conserved motif of dehydrin. left, water soluble fraction; right, heat-soluble fraction *, 44 kDa; **, 23 kDa.

PVDF membrane was incubated for 60 min in the anti-serum of rabbits diluted to 1:200 with 1% BSA-TBS, then washed with TBS-t (Tris-Buffered Saline containing 0.05% tween 20) for 20 min 3 times, and with TBS for 20 min. Washed membrane was incubated with peroxidase conjugated anti-rabbit goat antibody diluted to 1:2000 with 1% BSA-TBS, washed with TBS-t (Tris-Buffered Saline containing 0.05% tween 20) for 20 min 3 times, and with TBS for 20 min, and detected by a peroxidase detection kit (Immunostain HRP1000 Konica).
Cryoprotective activity on lactate dehydrogenase

Cryoprotective activities of dehydrins in comparison with bovine serum albumin (BSA, A4378, Sigma) on freeze/thaw inactivation of lactate dehydrogenase were assayed following the method of Lin et al. (6). Lactate dehydrogenase from rabbit muscle (L5132, Sigma) was dissolved in 10 mM sodium phosphate buffer, pH 7.5, at a concentration of 2.5 μg/mL. The enzyme solution (0.1 mL) was mixed with equal amounts of test compounds dissolved in the same buffer at the concentration of 2×10²–2×10⁴ μM. The solutions were frozen at –20°C for 24 h and thawed at room temperature for 5 min. Enzyme activity was measured with the absorbance change at 340 nm at 25°C using a commercial assay kit (DG1340K, Sigma). All samples were assayed twice in triplicate. Residual activity was shown as the percentage of the control activity assayed immediately after mixing of enzyme and protein solution.

Results

1. Dehydrins in rice bran

1) Electrophoresis of rice bran proteins

As shown in Fig. 2, two bands of dehydrin-like protein were detected in water-soluble and heat-soluble fractions of rice bran by immunoblotting using antiserum for conserved dehydrin motif. The 44 kDa and 23 kDa bands had similar intensity in this experiment. Still et al. had investigated accumulation of dehydrin in developing rice seeds in relation to seed maturation using

Fig. 3. Two-dimensional electrophoresis and immunoblotting. A, Two-dimensional electrophoresis of water-soluble fraction; B, Immunoblot of water-soluble fraction; C, Two-dimensional electrophoresis of heat-soluble fraction. Spots reacted with dehydrin peptide antibody are circled. Arrows show concentrated in heat soluble fraction.

antisera for lysine motif of dehydrin. They detected a 21 kDa polypeptide as a major dehydrin-like protein in the seeds of 15–39 DAF along with small amount of 38 kDa polypeptide which also crossreacted with the antibody. It had been known that about 20 kDa dehydrin accumulate in rice seedling under desiccation stress. Still et al. concluded in their report that the 21 kDa polypeptide detected was a dehydrin protein. As for the dehydrin-like proteins with higher molecular weight, beside the 38 kDa polypeptide in developing seed, Jayaprakash et al. found trace amount of 60 kDa and 45 kDa polypeptides in desiccated rice seedling (7). In pea and wheat, dehydrin cognates with higher molecular weight than major dehydrin were detected. Some of them lacked N-terminal motif sequence containing DEYGNP (8, 9). Based on these findings, we supposed that the 23 kDa polypeptide detected by the immunoblot analysis was the major dehydrin in rice seed and seedling, and that the 45 kDa polypeptide would be the dehydrin-cognate with higher molecular weight.

Figure 3 shows two-dimensional electrophoresis patterns of water-soluble and heat-soluble fractions of rice bran. Though the difference was not clear in the SDS-PAGE pattern between these fractions, their 2D-electrophoresis patterns were considerably different. In general, number of the detected spots decreased by the heat treatment, but some of the spots in acidic side seemed to be concentrated in heated sample. On the immunoblotted membrane for dehydrin motif, 3 spots around 44 kDa and 5 spots for 23 kDa (circled in Fig. 3A) were detected. In heat-soluble fraction, crossreacted spots (circled in Fig. 3C) were shifted to acidic side and the separation of the spots became obscure, implying that

Fig. 4. Cryoprotective activity of 23 kDa dehydrin and protein fractions from rice bran. ●, Water-soluble fraction; ◆, Heat-soluble fraction; ■, 23 kDa dehydrin.

they might interact with other components in rice bran.

2) Cryoprotective activity of rice proteins

Lin et al. showed that in vitro translated COR15 from Arabidopsis had cryoprotective activity on freeze/thaw inactivation of lactate dehydrogenase. It was the first report indicating a function of LEA related proteins (6). This cryoprotective activity was found in Chrollera HIC6, a group3 LEA protein (10), a protein from an ice-nucleating bacterium (11) and others. It is presumed that hydrophilicity of LEA proteins relates to protective function to proteins. The cryoprotective activity is considered as an index of function of protein induced by stresses such as low temperature or desiccation. Breton et al. suggested the possibility that the cryoprotective activity of LEA protein would be applicable to preserve the quality of frozen food or prevention of frostbite (2).

Figure 4 shows the cryoprotective activity of fractions from rice bran on the freeze/thaw inactivation of lactate dehydrogenase. The CP₅₀ values, protein amounts necessary to keep 50% activity of lactate dehydrogenase, of water-soluble fraction, heat-soluble fraction, and 23 kDa dehydrin were 43 μg/mL, 29 μg/mL, and 15.6 μg/mL (0.78 μM), respectively. In our previous study, we assayed the activities of purified soy dehydrins and several commercially available albumins (4). Compared with the results, rice 23 kDa dehydrin had similar level of cryoprotective activity to ovalbumin and lactoalbumin, and about one-third activity of 26 kDa soybean dehydrin, whose CP₅₀ was 5.2 μg/mL (0.2 μM).

2. Whey fraction of soybean

1) Protein composition of heat-soluble fraction

Figure 5A shows the SDS-PAGE patterns of water-soluble protein, whey, and heated whey fraction from soybean seeds. In the water-soluble fraction, major storage proteins, glycinin and β-conglycinin, were the abundant components. Since these storage proteins precipitate in acidic solution about pH 5, whey fraction extracted by the acidic buffer composed of enzyme proteins like lipoxxygenase, β-amylase, and trypsin inhibitor and (12). Heat-soluble whey fraction obtained by boiling the whey fraction for 10 min contained 26 kDa polypeptides as a major component and several other polypeptides such as 50 kDa and 29 kDa. Figure 5B shows the 2D-electrophoretic pattern of the heat-soluble whey fraction. In the region of 26 kDa where the major polypeptide was observed in the SDS-PAGE, clustered spots around pI 6

Fig. 5. SDS-PAGE and two-dimensional electrophoresis of heat-soluble whey fraction of soybean. A, SDS-PAGE lane 1, water-soluble fraction; lane 2, whey fraction; lane 3, heat-soluble whey fraction. *, dehydrin; g, glycinin; c, β -conglycinin. B, Two-dimensional electrophoresis of heat-soluble whey fraction.

Fig. 6. Cryoprotective activity of soybean fractions on lactate dehydrogenase. ♦, Water-soluble fraction; ●, Heat-soluble fraction; ▲, purified 26 kDa dehydrin; ◇, Bovine serum albumin; ○, Ovalbumin; △, Sucrose.

and spots in acidic side were eminent. Since the pI value of purified dehydrin from soybean seeds was estimated to be 6.2 in our previous paper (3), those spots of pI 6 were assumed to be soybean dehydrins. Similar diversity of dehydrin-like proteins was observed in rice bran fraction as shown above. Lei et al. found a component called “Y” that is concentrated in whey and heat-tolerable fraction in their 2D-electrophoretic analysis of soybean proteins (13). From the isoelectric point and molecular weight, component “Y” is supposed to be derived from soybean dehydrin. Spots in acidic side were presumably those of protease inhibitors. Spots around 50 kDa ranging from pI 5.4 to 7.6 and those of 19 kDa and 21 kDa in basic side were also reported in the 2D-electrophoresis pattern by Lei et al. although none of them were identified. From these results of electrophoresis, it is clarified that heat-soluble whey fraction of soybean seed composes of 26 kDa dehydrin as major protein, protease inhibitors, and other unidentified polypeptides.

2) Cryoprotective activity on lactate dehydrogenase

Cryoprotective activity of the soybean fractions was assayed similarly to the rice proteins. (Fig. 6). The CP_{50} of whey heat-soluble fraction was 15.8 μ g/mL, while that of water-soluble fraction was 335 μ g/mL, indicating that the activity was concentrated by 20-fold. The

activity of whey heat-soluble fraction was estimated to be approximately one-third of purified 26 kDa dehydrin from soybean seed. From this result it is shown that the simple heating process can concentrate the cryoprotective component in soybean seed. The polypeptide composition of heat-soluble whey fraction suggested the cryoprotective activity reflect its dehydrin content, though it would be possible some other unidentified component contribute to the activity.

Discussion

As describe above, dehydrin proteins, a group 2 LEA protein in rice bran and soybean whey were detected using polyclonal antiserum raised against conserved lysine-motif sequence conjugated with ovalbumin. Before the experiment we knew that dehydrin protein in soybean were remarkably concentrated by boiling the whey fraction of soy protein extracts (3). Despite our expectation, dehydrin in rice bran was not concentrated by heating the extract. It is assumed that interaction of dehydrin proteins and other components like lipids might be involved the phenomenon. The cryoprotective activity on lactate dehydrogenase was also examined for the extracts of rice bran. Heat treatment and partial purification process result in the increase of cryoprotective activity on enzyme. But the effect of these treatment seemed to be limited comparing to soybean dehydrin (Figs. 3 and 6).

The cryoprotective activity had been considered as an index of function of protein induced by stresses such as low temperature or desiccation. And it had been suggested that the cryoprotective activity of LEA protein could be applicable to preserve food quality by preventing frozen damage (2). We have characterized some dehydrin proteins in seed crops (14–16). Though the function of dehydrin as food component still to be clarified, applications of dehydrins to preserve the quality of frozen foods are also sought in these years. We expected rice bran could be one of the candidates for dehydrin resources, since it is abundant by product of rice consumption. It might be necessary to isolate dehydrin proteins from rice bran in order to utilize its potential in the field of food application more effectively.

Disclosure of State of COI

No conflicts of interest to be declared.

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REFERENCES

- 1) Close TJ. 1997. Dehydrins: A commonality in the response of plants to dehydration and low temperature. *Physiologia Plantarum* **100**: 291–296.
- 2) Breton G, Danyluk J, Ouellet F, Sarhan, F. 2000. Biotechnological applications of plant freezing associated proteins. *Biotechnol Annu Rev* **6**: 59–101.

- 3) Momma M, Haraguchi K, Saito M, Chikuni K, Harada K. 1997. Purification and characterization of the acid soluble 26-kDa polypeptide from soybean seeds. *Biosci Biotechnol Biochem* **61**: 1286–1291.
- 4) Momma M, Kaneko S, Matsukura U. 2002. Characterization of soybean dehydrin (group2 LEA protein): Localization and accumulation in seed, cryoprotective activity on lactate dehydrogenase, and molecular weight diversity among Japanese soy varieties. *Rep Natl Food Res Inst (Shokuhin Sogo Kenkyusho Kenkyu Houkoku)* **66**: 21–28 (in Japanese).
- 5) Momma M, Kaneko S, Haraguchi K, Matsukura U. 2003. Peptide Mapping and Assessment of Cryoprotective Activity of 26/27-kDa Dehydrin from Soybean Seeds. *Biosci Biotechnol Biochem* **67**: 1832–1835.
- 6) Lin C, Thomashow M F. 1992. A cold-regulated Arabidopsis gene encodes a polypeptide having potent cryoprotective activity. *Biochem Biophys Res Commun* **183**: 1103–1108.
- 7) Jayaprakash T L, Ramamohan G, Krishnaprasad, Ganeshkumar, Prasad T G, Mathew M K, Udayakumar M. 1998. Genotypic variability in differential expression of lea2 and lea3 genes and proteins in response to salinity stress in finger millet (*Eleusine coracana* Gaertn) and rice (*Oryza sativa* L.) seedlings. *Ann Bot* **82**: 513–522.
- 8) Robertson M, Chandler P M. 1994. A dehydrin cognate protein from pea (*Pisum sativum* L.) with an atypical pattern of expression. *Plant Mol Biol* **26**: 805–816.
- 9) Danyluk J, Houde M, Rassart E, Sarhan F. 1994. Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant gramineae species. *FEBS Lett* **344**: 20–24.
- 10) Honjoh K, Matsumoto H, Shimizu H, Ooyama K, Tanaka K, Oda Y, Tanaka R, Joh T, Suga K, Miyamoto T, Iio M, Hatano S. 2000. Cryoprotective activities of group 3 late embryogenesis abundant protein from *Chlorella vulgaris* C-27. *Biosci Biotechnol Biochem* **64**: 1656–1663.
- 11) Koda N, Asaeda T, Yamade K, Kawahara H, Obata H. 2001. A novel cryoprotective protein (CRP) with high activity from the ice-nucleating bacterium, *Pantoea agglomerans* IFO12686. *Biosci Biotechnol Biochem* **65**: 888–894.
- 12) Iwabuchi S, Yamauchi F. 1987. Electrophoretic analysis of whey proteins present in soybean globulin fractions. *J Agric Food Chem* **35**: 205–209.
- 13) Lei M G, Tyrell D, Bassette R, Reeck G R. 1983. Two-dimensional electrophoretic analysis of soybean proteins. *J Agric Food Chem* **31**: 963–968.
- 14) Momma M. 2003. Two-dimensional polyacrylamide gel electrophoresis and cryoprotective activity on lactate dehydrogenase of rice dehydrin. *Rep Natl Food Res Inst (Shokuhin Sogo Kenkyusho Kenkyu Houkoku)* **67**: 15–20 (in Japanese).
- 15) Momma M. 2003. Cryoprotective activity on lactate dehydrogenase of heat-soluble proteins in whey fraction of soybean. *Journal of the Japanese Society for Food Science and Technology (Nippon Shokuhin Kagaku Kougaku Kaishi)* **50**: 428–430.
- 16) Momma M. 2015. Purification of dehydrin protein from buckwheat seed (*Fagopyrum esculentum* cv. Kitawase). *Rep Natl Food Res Inst (Shokuhin Sogo Kenkyusho Kenkyu Houkoku)* **79**: 35–39.