Release of Allergenic Proteins from Rice Grains Induced by High Hydrostatic Pressure

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Protein release from rice grains during high-pressure treatment was investigated. When polished rice grains were immersed in distilled water and pressurized at 100−400 MPa, a considerable amount of proteins (0.2−0.5 mg per gram of grains) was released. By sodium dodecyl sulfate−polyacrylamide gel electrophoresis and immunoblot analyses, the major proteins released were identified as 16 kDa albumin, α-globulin, and 33 kDa globulin, which were known as major rice allergens. By scanning electron microscopic observation of rice grains pressurized at 300 MPa, partial morphological changes in endosperm cells but no apparent structural changes in protein bodies were detected. The content of these allergenic proteins decreased by pressurization and almost completely disappeared from rice grains by the pressurization in the presence of proteolytic enzyme. These results suggest that partial destruction of endosperm cells caused by pressurization enhances permeability of a surrounding solution into rice grains and that a part of the proteins are solubilized and subsequently released into a surrounding solution.

Keywords: *High pressure; rice grains; allergenic proteins*

INTRODUCTION

Rice is the important cereal as both energy and protein sources for a large population in Asia. Rice grains contain proteins accounting for 8% (w/w) of the dried starchy endosperms, and most of them are accumulated in protein bodies. Ogawa et al. (1987) estimated that endosperm storage proteins comprised 60−65% of glutelin in protein body-II, 20−25% of prolamin in protein body-I, and 10−15% of albumin and globulin in cytoplasm. Cereal grains are known to be a cause of allergic disorders, such as asthma and dermatitis (Baldo and Wrigley, 1984). Shibasaki et al. (1979) first reported on rice allergy and showed that a high degree of allergenicity was found in a globulin fraction of rice seed proteins. Many studies on rice allergenic proteins have been done, and 16 kDa albumin (Matsuda et al., 1988) and α-globulin (26 kDa, Limas et al., 1990) are known as major rice allergens. Furthermore, Urisu et al. (1991) reported a highly allergenic 33 kDa globulin, which has recently been identified as a novel type of plant glyoxalase-I (Usui and Matsuda, unpublished results). It is difficult to strictly remove rice or rice proteins from diets, since rice is not only a staple food but also a major material for many kinds of processed foods, fermented seasonings, and fermented beverages. Therefore, rice allergy would be a serious problem for Asian patients.

Numerous studies on effects of high hydrostatic pressure on microbial physiology and sterilization, control of enzymatic reaction, biochemical effects on animal tissues, and food processing have been done (Hayashi, 1993; Hashizume et al., 1995; Gross and Jaenicke, 1994; Ohmori et al., 1992; Kimura et al., 1994). Now, the high-pressure technology has become important for both basic and applied research works (Hayashi, 1993). In a previous study (Omi et al., 1996), we reported specific protein release from several legume seeds during pressurization at 300 MPa, and the protein released from soybean seeds was identified as basic 7S-globulin.

We applied the pressurization to seeds of some other plant species and found the protein release from grains of several cereals. In the present study, we show the protein release from rice grains during pressurization and identify the released proteins as 16 kDa albumin, α-globulin, and 33 kDa globulin, which are known as major rice allergens. We also show pressure-induced morphological changes in rice grains and discuss a possible mechanism of pressure-induced protein release from rice grains.

MATERIALS AND METHODS

Rice Grains. Commercially available polished rice, *Oryza sativa* L. *Japonica* cv Akitakomachi, newly harvested, was used in this study. In some experiments, polished rice such as *Indica*, waxy, and rice for sake brewing were also used.

High-Pressure Treatment. Twenty grams of rice grains were gently washed under tap water for 30 min. Then, rice grains were packed in a polyethylene film bag together with 40 mL of distilled water, and pressurized at 300 MPa at 20 °C for 30 min with a hydrostatic high-pressure system (MFP-7000, Mitsubishi Heavy Industry). The pressurized sample was filtered through four layers of gauze to separate the rice grains and surrounding solution. Before the analyses described below, the rice grains were washed with distilled water and freeze-dried, while the surrounding solution was centrifuged at 450*g*

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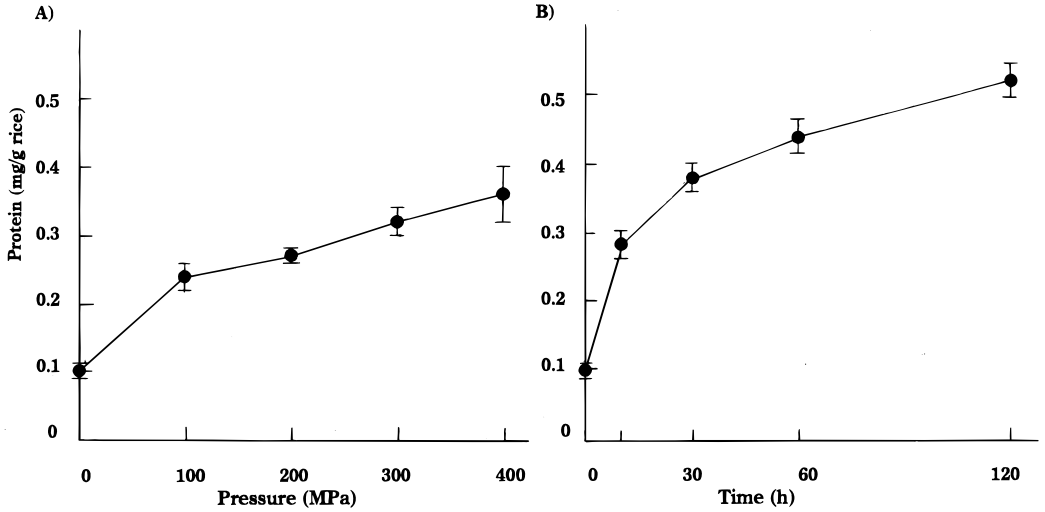

Figure 1. Effect of high-pressure treatment on the release of proteins from rice grains. Water-immersed rice grains were pressurized at 0−400 MPa for 30 min (A) and at 300 MPa for 0−120 min (B). Each value represents the means of five independent experiments with standard deviation.

for 5 min to remove precipitates. In some experiments, washed rice grains were immersed and packed in a film bag together with 1 M NaCl, 70% ethanol, and 0.1 N NaOH, respectively, and pressurized at 300 MPa at 20 °C for 30 min.

High-Pressure Treatment in the Presence of a Protease. Twenty grams of rice grains were washed, immersed in distilled water, packed in a film bag together with 40 mL of protease solution, pressurized at 300 MPa at 20 °C for 30 min, and incubated at 30 °C for 18 h. Protease-N (for food use, from *Bacillus subtilis*, Amano) was dissolved in 50 mM phosphate buffer (pH 7.0), aseptically filtered, and used as the protease solution (140 U/mL) as described above. After the treatment, rice grains were washed with tap water for 1 h, rinsed with distilled water, and freeze-dried.

Electrophoresis and N-Terminal Amino Acid Sequencing. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was done as described by Laemmli (1970), using 15% polyacrylamide slab gels. A molecular weight marker kit (Bio-Rad and Pharmacia) was used as standard proteins. After electrophoresis, a part of the gels were stained with Coomassie Brilliant Blue R-250 (CBB). The others were used for electroblotting onto a poly(vinylidene difluoride) membrane (PVDF, Bio-Rad) at 15 V for 30 min in Towbin buffer (1979). After blotting, the membrane was stained with CBB. The protein band was excised and washed in methanol. The N-terminal amino acid sequence of the protein on the membrane was determined with a peptide sequencer (Applied Biosystems, model 477A).

Immunoblotting. After electroblotting, the PVDF membrane was soaked in Tris-HCl-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 3% bovine serum albumin (BSA, Sigma) at 5 °C overnight, and then incubated with the primary antibodies. Mouse antiserum to rice 16 kDa albumin (Alb16), mouse antiserum to rice α-globulin (α-Glb), and mouse antiserum to 33 kDa globulin (Glb33) were diluted at 1:1000 with TBS containing 1% BSA and were incubated at 37 °C for 2 h. A preimmune serum was also used as a control. Then, the membrane was washed three times with TBS containing 0.05% Tween-20 (TBST) and incubated with goat anti-mouse IgG gold conjugate (Bio-Rad) overnight.

Mouse Antiserum to 16 kDa Albumin and α-Globulin. Alb16 was purified by the method of Matsuda et al. (1988), and rice α-globulin was purified by the method of Nakase et al. (1996). Purified proteins (100 μg) were dissolved in phosphate-buffered saline (PBS; 8.1 mM $Na_2HPO_4/12H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2), emulsified with the same volume of Freund's complete adjuvant, and injected in 6-week-old female mice (ddY, Japan SLC). Two booster injections of the same antigens (50 μg/mouse) were administered 14 and 28 days after the first immunization. Bleeding was performed 7 days after the last booster injection and the serum was separated by centrifugation.

Protein Determination. The protein concentration was determined by the method of Lowry et al. (1951), using BSA as a standard protein.

Estimation of Albumin and Globulin Content. Pressurized and freeze-dried rice grains were ground into a powder. Fifty miligrams of the powdered rice was suspended in 1 mL of the sample buffer for SDS−PAGE, heated in boiling water for 5 min, and centrifuged. Ten microliters of the supernatants was applied to SDS−PAGE. Purified Alb16 (1.0 μg/μL) and α-Glb (1.0 μg/μL) were also applied as standard proteins. After electrophoresis, the gel was stained with CBB, and the staining intensity of each protein band was estimated by scanning each lane at 565 nm with a densitometer (DM-303, Advantec).

Scanning Electron Microscopy. After being pressurized at 300 MPa for 30 min, rice grains were cut into about 2 mm thickness by a razor, fixed with 1.0% paraformaldehyde/2.0% glutaraldehyde/0.1 M phosphate buffer (pH 7.2) for 3 h, and rinsed in 0.1 M phosphate buffer containing 0.25 M sucrose at 5 °C overnight. Then, the specimen was refixed with 1.0% osmium tetraoxide/Millonig buffer (pH 7.3) for 3 h, dehydrated with a graded series of ethanol, and critical-point-dried in CO_2. Then, the specimen was fractured with a razor, ionspatter-coated with platinum/palladium, and observed with a scanning electron microscope (JSM-820, Japan Electron Optics Laboratory Co.), operating at an accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

When rice grains were pressurized at 100−500 MPa at 20 °C for 30 min, the surrounding solution became turbid. When centrifuged (450*g*, 5 min), the surrounding solution was separated into white precipitate and clean supernatant. The precipitate consisted of starch granules, while the supernatant contained proteins. Figure 1 shows the amounts of protein released into the surrounding solution. The protein amounts increased with an increase in pressure value and reached a maximum at 300 or 400 MPa (Figure 1A). Although almost no apparent changes in shape and size were observed for the rice grains pressurized less than 400 MPa, rice grains appeared to be slightly swelled and made fragile by the pressurization of more than 500 MPa (data not shown). When rice grains were pressurized at 300 MPa for 0−120 min, protein amounts of the surrounding solution increased with pressuring time and consequently reached about 0.5 mg/g rice (Figure

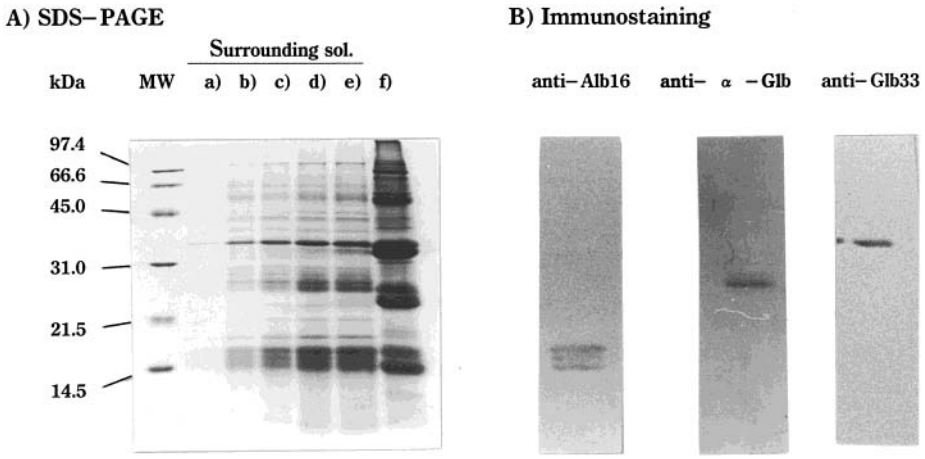

Figure 2. SDS−PAGE patterns and immunoblotting of the released proteins. (A) Lanes a−e, proteins released from rice grains by pressurization at 0, 100, 200, 300, and 400 MPa. Ten microliters of the surrounding solutions, which had been concentrated 10 times, was applied to each lane of SDS−PAGE. Total protein extracted from intact rice grains with the SDS−PAGE sample buffer was also analyzed for comparison (lane f). (B) Immunoblotting of the proteins released by pressurization at 300 MPa for 30 min.

1B). Protein release was also observed for some varieties of polished rice grains: 0.20 mg/g from *Indica* rice, 0.33 mg/g from waxy rice, and 0.10 mg/g from rice for sake brewing at 300 MPa for 30 min.

Figure 2A shows SDS−PAGE patterns of the released proteins. Although only trace protein bands were observed in the control sample (lane a), several protein bands emerged with pressurization (lanes b−e). Especially, the bands estimated to be 33, 28, 26, and 14−16 kDa had strong staining intensity. By densitometric analysis, these major protein bands were estimated to comprise about 60% of the total density (lane d). The released proteins did not appear to be major storage proteins such as glutelin (37−39 and 22−23 kDa) and prolamin (13 kDa, lane f). The same results were also obtained in the other batch of polished rice grains tested (data not shown). When the proteins in the surrounding solution were fractionated to albumin, globulin, prolamin, and glutelin based on solubility in distilled water, 1 M NaCl, 70% EtOH, and 0.1 N NaOH, respectively, and assayed by SDS−PAGE, almost all proteins were separated into albumin or globulin fractions, and no gultelin and prolamin were detected (data not shown).

To identify 33, 28, 26, and 16 kDa proteins in Figure 2A, N-terminal amino acid sequence analysis was done. N-Terminal amino acid sequence of 16 kDa protein was determined to be NH_2-DHHQVYSPGE, which was in agreement with those of Alb16 (Izumi et al., 1992). On the other hand, amino acid sequences of 33, 28, and 26 kDa proteins could not be determined, probably due to blocking of the N-terminal ends.

Figure 2B shows the immunoblot analysis of the released proteins using the antisera raised against rice major allergens: Glb33, α-Glb, and Alb16. These three proteins have been considered to be major rice allergens (Matsuda et al., 1988; Limas et al., 1990; Urisu et al., 1991). The 33 kDa protein was clearly recognized by the anti-Glb33 antibody. The 26 kDa protein and 14−16 kDa proteins were also recognized by the anti-α-Glb antibody and anti-Alb16 antibody, respectively. These biochemical and immunochemical results indicated that 33, 26, and 16 kDa proteins, which were released during pressurization, were Glb33, α-Glb, and Alb16, respectively. It was reported that the N-terminal amino acid of α-Glb (Komatsu and Hirano, 1992; Nakase et al., 1996) was pyroglutamic acid, which prevents amino acid sequence analysis. It seems interesting that globulins and albumins, comprising only 4−10% of rice seed proteins, were preferentially released during pressurization. Recently, we found a similar phenomenon for soybean seeds, that is, basic 7S-globulin (Bg) was specifically released from soybean seeds by pressurization (Omi et al., 1996).

Since pressurization induced release of major allergens from rice grains, pressurization might be useful for the reduction of the allergenicity of rice. Figure 3 shows SDS−PAGE patterns of the proteins which remained in pressurized rice grains. Protein band patterns of the pressurized rice grains (lanes b and c) were almost the same as that of control one (lane a). Since protein bands corresponding to α-Glb and Alb16 were clearly observed in the stained gel, the amount of the major allergenic proteins in pressurized rice grains was estimated semiquantitatively by densitometering the CBB-staining intensity of each protein band with each purified protein as a standard.

Table 1 shows Alb16 and α-Glb contents as estimated by the densitometering of the SDS−PAGE gel in Figure 3. Pressurization for 30 min decreased Alb16 from 2.6 to 2.2 mg/g, corresponding to 15% reduction. On the other hand, α-Glb decreased from 1.9 to 1.3 mg/g, corresponding to 33% reduction. When the rice grains were pressurized at 500 or 600 MPa, these proteins still remained in the grains (data not shown), suggesting that the allergenic proteins cannot completely be removed only by pressurization.

Figure 3 (lanes f and g) shows SDS−PAGE patterns of proteins of the protease-treated rice grains with and without pressurization. By the protease-N treatment with pressurization (lane f), several protein bands, indicating Alb16 and α-Glb, disappeared, and some other protein bands markedly decreased their staining intensity compared with those of rice grains with the pressurization or the protease treatment only (lanes b, c, and g). On the other hand, the protein bands of 37−39, 22−23, and 13 kDa mostly remained, which were estimated to be acidic and basic subunits of glutelin, and prolamin, respectively. When the presence of Glb33, α-Glb, and Alb16 were confirmed by immunoblotting using the specific antibodies, only weak reaction origi-

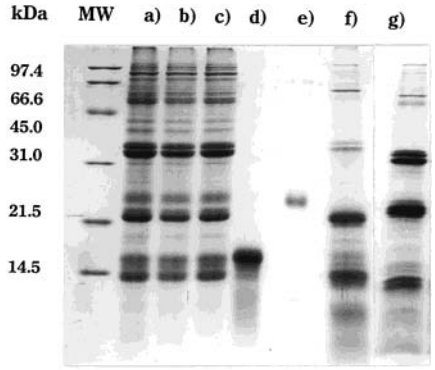

Figure 3. SDS−PAGE patterns of the proteins in pressurized rice grains. Total proteins extracted with the SDS−PAGE sample buffer from intact rice grains (a), rice grains pressurized at 300 MPa for 30 min (b) and 120 min (c), and rice grains pressurized at 300 MPa for 30 min in the presence of protease-N (f) were analyzed in a 15% polyacrylamide gel. The total proteins of rice grains treated with protease-N without pressurization was also analyzed for comparison (g). The purified proteins Alb16 (1.0 μg) and α-Glb (1.0 μg) were applied to lanes d and e as standards for the densitometeric analysis.

Table 1. Estimated Contents[a] of Alb16 and α-Glb in Pressurized Rice Grains

	300 MPa		
	30 min	120 min	control
Alb16	2.16 ± 0.13	2.36 ± 0.27	2.55 ± 0.16
α-Glb	1.30 ± 0.26	1.57 ± 0.21	1.94 ± 0.17

[a] Protein amounts were estimated by the densitometric analysis of the protein bands in the lanes a−e of the SDS−PAGE gel shown in Figure 3. Values shown as mg/g rice represent the mean of estimations for six gels with standard deviation.

nating from Alb16 was detected (data not shown). These results suggested that the combination of pressurization and protease treatment would be effective for the removal of allergenic proteins from rice grains.

When rice grains were pressurized at 300 MPa for 30 min in different surrounding solutions of distilled water, 1 M NaCl, 70% ethanol, and 0.1 N NaOH, respectively, protein amounts of each surrounding solution were 0.38 ± 0.02, 0.76 ± 0.04, 0.26 ± 0.01, and 12.20 ± 0.60 mg/g rice, respectively. Figure 4 shows SDS−PAGE patterns of the released proteins. Pressurization in 1 M NaCl increased considerably α-Glb in the surrounding solution (lane c). Pressurization with 0.1 N NaOH appeared to release most of rice seed proteins, and glutelin and prolamin shown by arrows were clearly seen (lane a). A single protein band of about 13 kDa emerged after pressurization with 70% ethanol (lane b). Because it was insoluble in water or salt solutions and soluble in ethanol solution, this protein was considered to be prolamin. Rice prolamins consist of 10−16 kDa polypeptides and major prolamins have a molecular mass of 13 kDa. These results suggest that, if an appropriate solution was selected, seed proteins with different solubilities, such as glutelin, prolamin, globulin, and albumin, could be extracted individually by pressurization in that solution.

Morphological changes in rice grains before and after pressurization were observed by a scanning electron microscope (Figure 5). Intact endsperm was tightly packed with compound starch granules, each of which consisted of many single starch granules. Protein bodies shown by an arrow were observed in the space among the compound starch granules (Figure 5A). When the

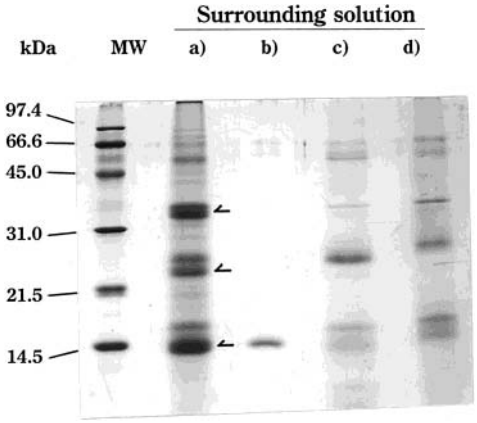

Figure 4. SDS−PAGE patterns of the proteins released from rice grains pressurized at 300 MPa in (a) 0.1 N NaOH, (b) 70% EtOH, (c) 1 M NaCl, and (d) distilled water.

compound starch granules were mechanically removed, a large number of protein bodies (1−5 μm in diameter) became to be clearly seen (Figure 5B). It is known that rice protein bodies consist of two types of particles, such as protein body-I (PB-I) and protein body-II (PB-II) (Yamagata and Tanaka, 1986; Ogawa et al., 1986). Spherical PB-I is about 1 μm in diameter and contains prolamins. Irregularly shaped PB-II is 3−5 μm in diameter and contains glutelin. As shown in Figure 5B, PB-I and PB-II could not be distinguished each other. Protein bodies appeared to be enveloped with membranous substances. After pressurization, it appeared that endosperm lost its tightly packed structure with the compound starch granules, some of which degraded into single starch granules (Figure 5C). Whereas almost no apparent structural changes were observed in protein bodies, the amounts of membranous substances around the protein bodies appeared to decrease (Figure 5D). On the basis of these observations, the pressurization at 300 MPa was suggested to induce morphological changes in the compound starch granules and membranous substances around protein bodies.

In the present and previous studies (Omi et al., 1996), we showed protein release from rice grains and soybeans seeds during pressurization. Some unknown systems for such an active releasing induced by pressurization might be present. Yamazaki and Sasagawa (1998) observed water-immersed and pressurized rice grains with an electron microscope. They reported that endsperm cells began to degrade and water permeated into the internal endsperm cells at 400 MPa, and a part of starch granules began to swell at 600 MPa. We also observed that pressurization above 500 MPa crushed rice grains into small particles, and that pressurization below 300 MPa appeared to partially destroy endsperm structures. The membranous substances around protein bodies shown in Figure 5B,D were considered to be originating from cytoplasmic components and organellas which had compressed into the narrow space as the result of accumulation of starch in amyloplasts. Nakamura and Matsuda (1996) showed that Alb16 was not present in protein bodies. Glb33 was also suggested to be a cytoplasmic protein (Usui and Matsuda, unpublished results). We estimated that Alb16 and Glb33 localized in the space and/or in the membranous substances around protein bodies. On the other hand, Krishnan et al. (1992) immunocytochemically showed that α-Glb was deposited in discrete zones within PB-II during the seed development. However, although

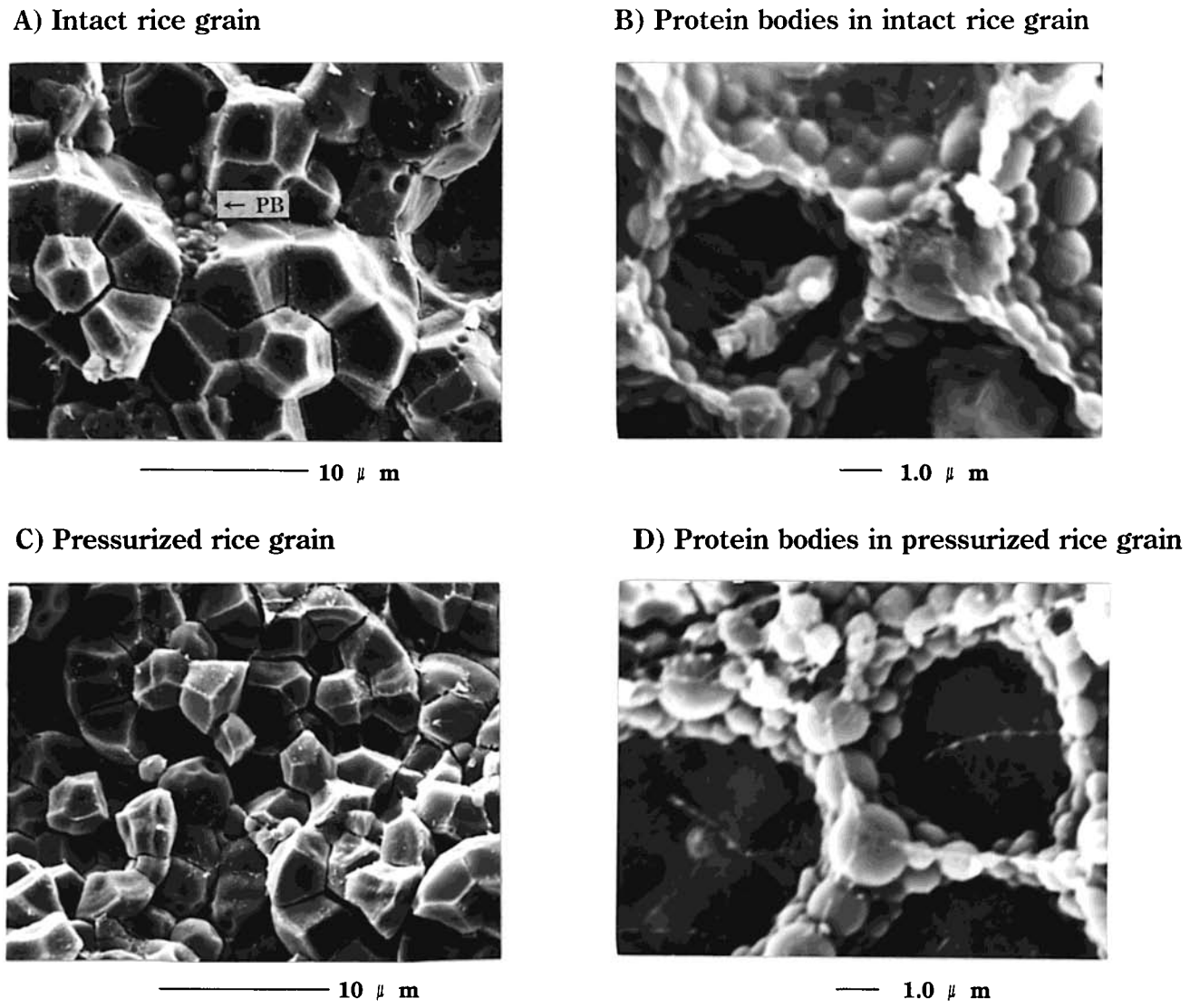

Figure 5. Morphological differences in rice grains before and after pressurization. Rice grains were pressurized at 300 MPa for 30 min and observed by scanning electron microscope.

considerable amounts of α-Glb were released during pressurization, almost no detectable changes were observed in protein bodies (Table 1 and Figure 5). Therefore, α-Glb might be present outside PB-II. The possibility that α-Glb was released from PB-II by pressurization also remains. Taken all together, a possible mechanism for protein release from rice grains induced by pressurization is proposed as follows: (1) partial destruction of endosperm cells, (2) permeation of surrounding solution into endosperm cells, (3) solubilization of proteins, mainly being present outside protein bodies, and (4) diffusion of proteins from endosperm cells to the surrounding solution.

It should be noted that major rice allergenic proteins such as Alb16, α-Glb, and Glb33 were preferentially released during pressurization. This characteristic release gives us an idea that pressurization might be applicable to a processing for hypoallergenic rice. However, as shown in Table 1, about 80% Alb16 and α-Glb still remained in pressurized rice grains, indicating that removal of allergens was insufficient by pressurization only. As shown in Figure 3 (lane f), most allergenic proteins disappeared with pressurization in combination with the protease treatment. These results suggested that the pressurization in the presence of protease was effective for the removal of allergenic proteins from rice grains. Not only absence of allergens but also maintenance of rice qualities are desirable for such a processed hypoallergenic rice. Watanabe et al. (1991) reported that pressurization could improve qualities of cooked rice, such as brightness, flavors, and textures, especially those of aged rice grains. They also succeeded in the development of hypoallergenic rice using proteases and surfactants (Watanabe et al., 1990). The surfactant was believed to enhance permeation of protease solution into endosperm cells through cell walls and membranes. Since pressurization can enhance permeation of the surrounding solution into rice grains, a combination of pressurization and protease treatment would be effective for the production of hypoallergenic rice by a simple processing step. We are now investigating the optimum conditions of the protease and high-pressure treatments for the release and removal of allergenic proteins.

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