Pressure-Induced Release of Basic 7S Globulin from Cotyledon Dermal Tissue of Soybean Seeds

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Protein release by high-pressure treatment of soybean seeds was investigated. When soybean seeds were immersed in distilled water and pressurized at 100−700 MPa, a large amount of proteins, accounting for 0.5−2.5% of the total seed protein, were released from the seeds. By sodium dodecyl sulfate−polyacrylamide gel electrophoresis, immunoblot, and amino acid sequencing analyses, the major protein released was identified as basic 7S globulin (Bg), consisting of 16 and 27 kDa subunits. Bg was shown immunohistochemically to be localized in the dermal tissue of seed cotyledons, presumably in epidermal cells. There were almost no structural changes in seed inner cells, as determined by scanning electron microscopy, but considerably large changes in structures of cotyledon surface and epidermal cells were observed in the pressurized seeds. These results suggest that Bg localized in seed cotyledon dermal tissues is released from soybean seeds as a result of structural changes in cotyledon surface and epidermal cells that are caused by high-pressure treatment.

Keywords: *High pressure; basic 7S globulin; soybean seed*

INTRODUCTION

Legume seeds are one of the most important foodstuffs and contain a large number of proteins of known and unknown physiological functions. Some of the seed proteins are degraded during germination and serve as the nitrogen source for the various nitrogenous compounds synthesized by the developing seedlings. These proteins are "storage proteins" for future use during germination and are found in protein bodies of cotyledon. In general, the storage proteins are dominant in quantity compared with the other proteins with enzymatic and membranous roles. Earlier studies showed that much of the content of soybean seed proteins are salt-soluble proteins, globulins, which could be separated into two major fractions, 11S globulin and 7S globulin (Hill and Breidenback, 1974a,b). The globulin fraction of soybean seeds also contains another group of components that are proteins with higher isoelectric points that are called basic 7S globulin (Bg; Yamauchi *et al*., 1984). Bg has recently been shown to structurally resemble a carrot *e*xtracellular *d*ermal *g*lyco*p*rotein (EDGP), which is induced in response to wounding (Satoh *et al*., 1992).

Asano *et al*. (1989) reported that a large amount of protein was released from mature soybean seeds into the surrounding water when the seeds were immersed in hot water at 50−60 °C for 1 h (heat treatment). The mechanism for such a heat-induced protein release is still uncertain. The major component of the released proteins was later identified as Bg (Hirano *et al*., 1992). Bg is a cysteine-rich glycoprotein that consists of 27- and 16-kDa subunits linked by disulfide bonding, and accounts for ∼3% of the total protein in mature soybean seeds (Yamauchi *et al*., 1984; Sato *et al*., 1986, 1987; Hirano *et al*., 1987; Kagawa and Hirano, 1989). Bg or Bg-like proteins are distributed widely in seeds of various legume species, such as azuki bean, mung bean, pea and winged bean, and have similar properties to Bg (Kagawa *et al*., 1987; Hirano *et al*., 1992). Recently, it has been reported that Bg and Bg-like proteins have binding activity for insulin and insulin-like growth factor (Komatsu and Hirano, 1991), and Bg shows protein kinase activity (Komatsu *et al*., 1994). These facts suggest that Bg or Bg-like proteins are proteins with important functions and not simply seed storage proteins.

It is well-known that high-pressure treatment, hydrostatically pressurizing at several hundred MPa, induces physical, chemical, and biological changes in various substances (Hayashi, 1993). Many studies on effects of high pressure on microorganisms, animal tissues, enzymes, and proteins have been reported (Ohmori, 1993; Zobell and Kim, 1972; Gross and Jaenicke, 1994). On the other hand, few studies concerning plant tissues have been reported.

We found a novel physical and/or biochemical phenomenon, specific protein release from several legume seeds during high-pressure treatment. This report describes the specific protein release from soybean seeds induced by high-pressure treatment. The major components of the released proteins are isolated and identified as basic 7S globulin. Possible mechanisms for the pressure-induced protein release are discussed on the basis of the results obtained on the Bg localization in seed dermal tissue and pressure-induced structural changes of the dermal tissue.

MATERIALS AND METHODS

High-Pressure Treatment. Twenty grams of soybean seeds (*Glycine max*, cropped in China, yellow, ∼6 × 7 mm) were immersed in 80 mL of distilled water in a polyethylene film bag at 5 °C for 17 h, and then pressurized at 300 MPa and 20 °C for 25 min with a hydrostatic pressure system (MFP-7000;

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Mitsubishi Heavy Industry Company). After the pressurization, the surrounding solution was used for the following analysis.

Protein Content. Protein content was assayed by a protein assay kit (Bio-Rad), with bovine serum albumin (BSA; Sigma) as a standard protein.

Electrophoresis and N-Terminal Amino Acids Sequencing. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970), with 15%, and 10−20% gradient polyacrylamide slab gels. A molecular weight marker kit (RPN-755, Amersham, England) was used as standard proteins. After electrophoresis, some of the gels were stained with Coomassie brilliant blue (CBB) R-250. The others were electroblotted onto a PVDF membrane (Bio-Rad) at 15 V for 30 min in Towbin buffer (Towbin *et al*., 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 preparation was determined with a gas-phase sequencer (Applied Biosystems, model 477A).

Immunoblotting. After blotting, the PVDF membrane was rinsed in Tris-buffered saline (TBS; 20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 3% BSA at 5 °C overnight, and then incubated with primary antibody (antibasic 7S globulin, described later) diluted 1:1000 with TBS containing 1% BSA at 37 °C for 2 h. As a control, a preimmune serum was also used. The membrane was washed three times with TBS containing 0.05% Tween-20 (TBST), incubated with peroxidase-coupled anti-mouse IgG antibody (goat, Gibco BRL), diluted 1:1000 with TBS containing 1% BSA at 37 °C for 2 h, and washed with TBST three times and once with TBS. The protein bands were visualized with 4-chloro-1-naphthol as the peroxidase substrate.

Mouse Antiserum to Soybean Basic 7S Globulin. Soybean basic 7S globulin (Bg) was purified by the method of Asano *et al*. (1993). One hundred micrograms of Bg were dissolved in phosphate-buffered saline (PBS; 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2), emulsified with the same volume of aluminum adjuvant gel [130 mM $Al(OH)_3$, 150 mM NaCl], and injected in 2-week-old female mice. Two booster injections of the same antigen were administered 2 and 4 weeks later. Bleeding was performed 7 days after the last booster injection and the serum was separated by centrifugation and stored at −18 °C until use.

Bg Content. Bg content was measured by competitive enzyme-linked immunosorbent assay (ELISA; Engvall and Perlmann, 1971). An appropriate concentration of purified Bg (1.0×10^{-1}−10^{-4} μg/mL) and sample solution were mixed with the antiserum to Bg (diluted 1:5000). After incubation at 5 °C overnight, 100 μL of the Bg-antiserum mixture were added to flat-bottom microtiter plates, which had previously been coated with 100 μL of Bg (1 μg/mL). The microtiter plates were incubated at 37 °C for 2 h. Then, antibody that reacted with plate-bound Bg was determined, using peroxidase-coupled antimouse IgG with *o*-phenylenediamine as the enzyme substrate. Bg content was calculated from a standard curve using purified Bg.

Scanning Electron Microscopy. Cotyledons of soybean seeds, before and after pressurization at 300 MPa and 20 °C, were cut into 3-mm cubes, 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 Company) operating at an accelerating voltage of 15 kV.

RESULTS AND DISCUSSION

When soybean seeds immersed in distilled water were pressurized at 300 MPa and 20 °C for 30 min, the surrounding solution became markedly turbid. The dried precipitate, prepared from the solution by centrifugation (6000*g*, 20 min) and lyophilization, contained 80% (w/w) of protein. These facts indicated that proteins were released from soybean seeds by pressurization.

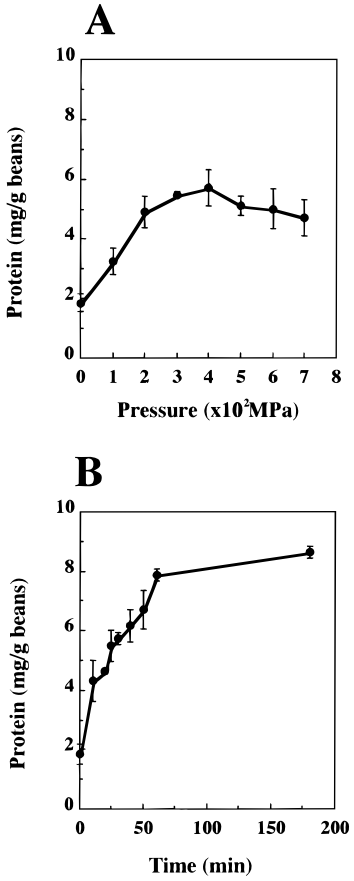

Figure 1. Effect of high-pressure treatment on the release of proteins from soybean seeds. Water-immersed soybean seeds were pressurized at (A) 0−700 MPa and 20 °C for 25 min and at (B) 300 MPa and 20 °C for 0−180 min.

The effect of pressure on the protein release from soybean seeds is shown in Figure 1. Protein amounts released into the surrounding water increased with an increase in pressure value, reached a maximum at 400 MPa, and slightly decreased at higher pressure (Figure 1A). When pressurized at 300 MPa for 0−180 min, 1.9−8.6 mg of proteins were released from 1 g of soybean seeds (Figure 1B). These values of released proteins accounted for 0.5−2.5% of the total seed protein. No apparent changes in a shape, size, and color were observed between control and pressurized soybean seeds.

The SDS-PAGE patterns of the released proteins are shown in Figure 2. Whereas trace protein bands, such as glycinin and β-conglycinin, and major soybean storage proteins were present in control, several new protein bands, especially 27 and 16 kDa bands, emerged after pressurization. The staining intensity of these bands increased with an increase in pressure value up to 400 MPa. By densitometoric analysis, two bands comprised more than 40% of the total density. At 700 MPa pressurization, several proteins, estimated to be glycinin or β-conglycinin, also increased their staining intensity.

To identify the major components in the released proteins, N-terminal amino acid sequences of 27- and 16-kDa proteins were determined to be NH_2-VTPTK-

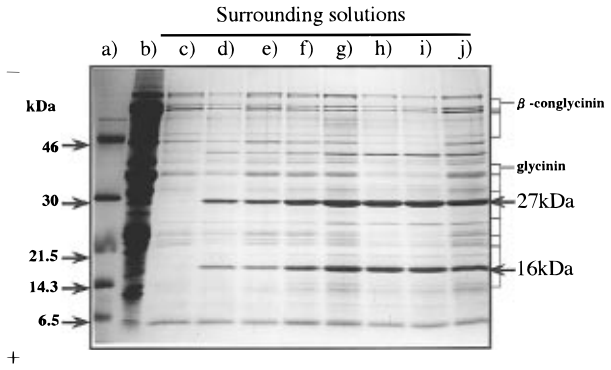

Figure 2. SDS-PAGE patterns of the released proteins (10−20% polyacrylamide slab gel was used): (a) MW marker, (b) soybean flour (20 μg, Sigma); (c~j) Ten microliter of surrounding solution of each sample treated at 0, 100, 200, 300, 400, 500, 600, or 700 MPa.

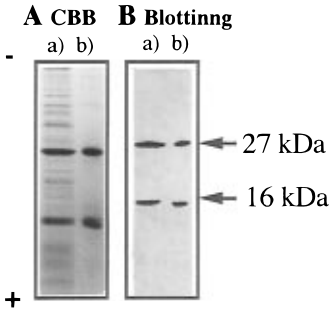

Figure 3. Immunoblotting of the released proteins: (A) SDS-PAGE (15% of polyacrylamide slab gel); (B) immunoblotting; (a) 300 MPa treatment; (b) basic 7S globulin. About 2 μg of proteins was applied for SDS-PAGE.

PINLVVLPVQ and NH_2-STIVGSTSGGTMIST, respectively. These proteins are in complete agreement with those of the high molecular subunit (27 kDa) and the low molecular subunit (16 kDa) of soybean basic 7S globulin (Bg; Kagawa and Hirano, 1989), respectively.

To confirm the identity of the released proteins with Bg, immunochemical reacting of the released proteins was analyzed by immunoblot with antiserum raised against the purified Bg. As shown in Figure 3, these two proteins were clearly recognized by the anti-Bg antibodies. On the basis of these results, it was concluded that 27 and 16 kDa proteins, which were preferentially released during high-pressure treatment, originated from soybean basic 7S globulin. This new finding is very interesting because mature soybean seeds contained Bg at only ~3% of the total protein (Sato *et al.*, 1986). A somewhat specific manner for Bg release might be present. At first, to elucidate where Bg is present, several parts of dry soybean seeds were immunochemically assayed.

Dry soybean seeds were divided into seed coats, hypocotyls, and cotyledons with a forcep. The center parts, containing storage cells, were collected from cotyledons by sectioning with a razor. The surface parts, containing dermal tissues, were collected from flat and round side of cotyledons, respectively, by grinding with a razor under microscopic observation. These samples were homogenized with PBS, and their Bg contents were measured by competitive ELISA (Figure 4). Whereas the seed coats, hypocotyls, and center parts of cotyledons contained almost no Bg, both the flat and round surface parts of cotyledons specifically contained Bg, which accounted for about ~10% of total protein. Almost the same result was obtained in the samples

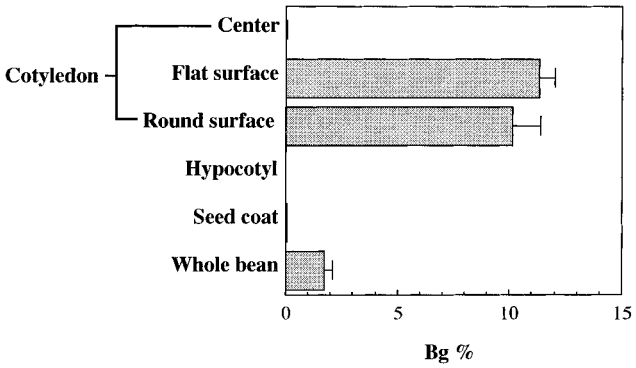

Figure 4. Basic 7S globulin (Bg) content of the dry soybean seeds. Bg content was measured by competitive-ELISA. Values are shown as percent of Bg per total protein.

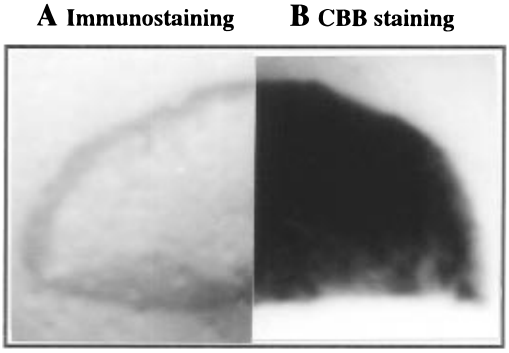

Figure 5. Localization of basic 7S globulin (Bg) in soybean seeds.

prepared from the water-immersed soybean seeds (data not shown). By the immunoblot analysis, Bg was also detected only in the proteins extracted from surface parts of cotyledons (data not shown). To confirm the localization of Bg in the surface part of cotyledon, a water-immersed cotyledon was transversely fractured with a razor, and a fractured face was stumped on a PVDF membrane that had previously been rinsed in TBS. After thoroughly washing in TBS, a half of PVDF membrane was stained with CBB, and the other half was immunostained as just described. Whereas the whole area stumped was strongly stained with CBB, the dermal part of cotyledon was clearly stained with the antiserum raised against Bg (Figure 5). These results suggest that a large amounts of Bg were present in the dermal tissue of soybean seed cotyledon.

Structural changes in soybean seeds after high-pressure treatment were observed by SEM. An epidermal cell in the intact cotyledon appeared to form a single monolayer of dermal tissue and to be a cubelike structure of ~10−15 μm (Figure 6a). The surface of the flat side of cotyledon consisted of smooth tissue which formed a highly wrinkled structure (Figure 6b). The surface of the round side of cotyledon was also a smooth and wrinkled structure (data not shown). No aperture was observed in both surfaces. When pressurized at 300 MPa for 25 min, the epidermal cells considerably deformed, and the space between epidermal cells and storage cells became appreciably large (Figure 6c). The surface of cotyledon, especially at the flat side lost their smooth tissue and became markedly rough as if forming fine convex or concave. The wrinkled structure also almost disappeared by the pressurization (Figure 6d). Structural changes caused by pressurization were scarcely observed in the center of cotyledon (data not shown). By SEM, we could not observe the destruction,

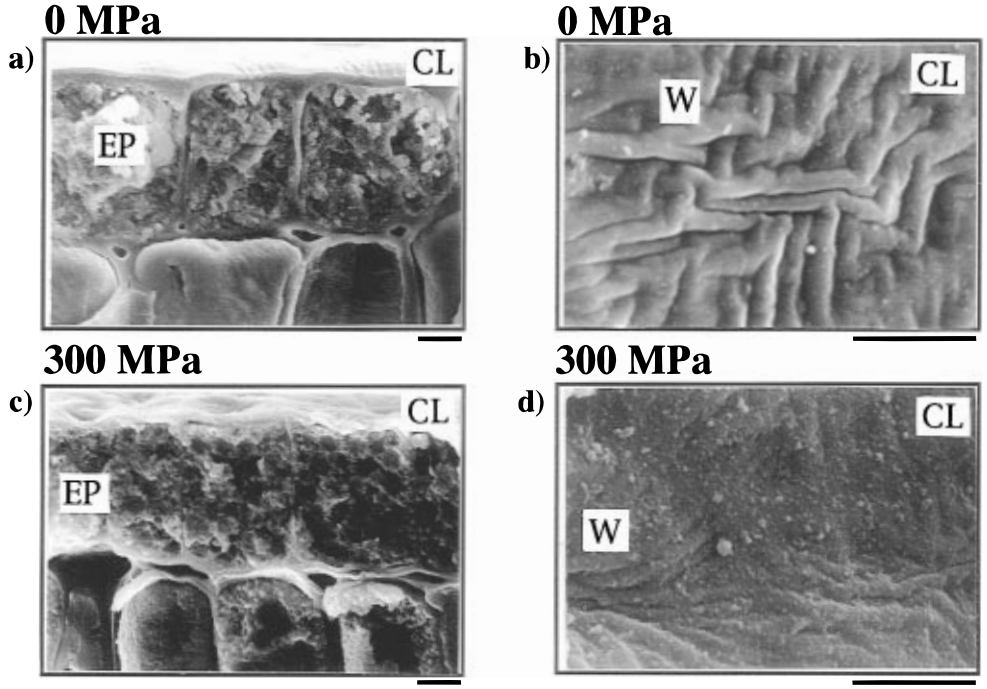

Figure 6. Structural changes in soybean seeds after high-pressure treatment at 300 MPa and 20 °C for 25 min: (a, c) cross sections of the flat side of cotyledon; (b, d) the surface of the flat side of cotyledon. (CL) cuticle layer; (EP) epidermal cell, (W) wrinkled structure (bar indicates 5 μm).

such as bursts or holes, through which intracellular substance could directly leak even though pressurised at 300 MPa for 6 h. Therefore, Bg seemed to pass through the cell membrane and the cuticle layer of cotyledon.

It is well-known that high-pressure treatment at ten to hundreds of MPa exerts a lethal effects on cells, destroying cell membrane and/or various organelles *etc.* (Ohmori, 1993). Cell membranes become leaky, and golgi complex, mitochondoria, and nuclear membrane are disrupted or disappeared, and subsequently result in cell death. In the present study, germination ability was lost from soybean seeds at more than 100 MPa pressurization. The pressurization at 300 MPa is considered to be sufficient to destory the soybean cell function. Changes in the surface structure of cotyledon were observed (Figure 6d). Although not detected by SEM, cell membranes and surface tissues might lose their original ultrastructure and might become leaky. Bg seemed to be located in the dermal tissue of cotyledons (Figures 4 and 5), where Bg is easy to leak. On the other hand, major storage proteins such as glycinin or β-conglycinin are stored as protein bodies inside cotyledon, which appeared to be intact after pressurization. By the partial destruction of the dermal tissue of cotyledon, where Bg seemed to be located, during pressurization Bg might be preferentially released from soybean seeds.

Bg-like proteins were distributed in several legume species (Kagawa *et al.*, 1987), and were released in response to heat treatment (Hirano *et al.*, 1992). We also observed that some proteins were released from several other legume species such as azuki bean and kidney bean by pressurization at >100 MPa. It is very interesting that these two different physical treatments induced specific protein release from legume seeds, suggesting Bg and Bg-like proteins are not simply seed storage proteins. Satoh *et al.* (1992) reported cDNA cloning of EDGP in carrot and its expression in response to wounding. They indicated that EDGP had a role in the response of plants to biotic and/or abiotic stresses. Furthermore, EDGP showed 40% of amino acid sequence identity with Bg, and these two proteins contained a short motif, which was present at the active site of aspartyl proteases. These facts indicate that Bg and Bg-like proteins may possess specific functions like EDGP in the legume seed.

It is still uncertain whether only structural changes by pressurization induce specific Bg release. Since Bg release was induced not only by pressurization but also by heat treatment, and despite higher pressurization at 700 MPa accelerating destruction of the cell, Bg release was slightly depressed (Figure 1), it might be possible that an active releasing system stimulated by pressurization or heat treatment induce specific Bg release. There is a possibility that Bg release is caused by degranulation. Degranulation is well-known as an active releasing system in animal and plant cells. By degranulation, a large amount of substances can rapidly be released through the cell membrane without destruction of the cellular structure. By pressurization, Bg was immediately released from seeds (Figure 1), the surface of cotyledon became rough as if forming fine concaves (Figure 6d), and Bg was considered to localize in dermal tissues (Figure 5). These facts suggest that Bg release was caused by degranulation induced by pressurization. To elucidate the releasing mechanism, ultrastructural research on soybean seed cotyledon is now in progress.

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