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A seed protein induced by heat treatment in soybean (*Glycine max* (L.))

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When soybean seeds were immersed in water at 50–60°C, a large amount of protein was released. The major protein secreted was the basic 7*S* globulin, a glycoprotein found in the cotyledon of dry mature seeds. Northern blot analysis revealed that the heat treatment markedly increased the amount of the basic 7*S* globulin mRNA transcribed in the seeds. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the proteins from the cotyledon, hypocotyl, and seed coat indicated that the released basic 7*S* globulin was specifically synthesized de novo in the cotyledon tissue. This protein is considered to be a secretory heat-shock protein.

INTRODUCTION

Mature soybean seeds contain approximately 40% protein, of which about 75% consists of the globulin seed-storage protein. This globulin can be separated into two major fractions with different sedimentation coefficients, the 11*S* and 7*S* globulin fractions, containing glycinin and β-conglycinin, respectively, as the major components.

Recently, a novel basic protein with an isoelectric point (pH 9·05–9·26) has been found in the 7*S* globulin fraction and designated as basic 7*S* globulin (Yamauchi *et al.*, 1984). This protein accounts for approximately 3% of the total protein content of mature seeds. It has a molecular mass of approximately 168 kDa and is composed of four high- and four low-kDa sub-units designated as H and L sub-units respectively. Each H sub-unit is liked to one L sub-unit by disulfide bond(s), forming a paired complex. The high- and low-kDa sub-units are each known to have two isoforms, named HI and HII (approximately 26 kDa), and LI and LII (approximately 16 kDa) (Sato *et al.*, 1987).

The basic 7*S* globulin does not cross-react immunologically with the other globulin species of soybean (Kagawa *et al.*, 1987). In addition, the N-terminal amino-acid sequences of the HI/HII sub-unit and the LII sub-unit show no homology with those of other globulin species (Hirano *et al.*, 1987). The basic 7*S* globulin therefore seems to be a completely different protein from the other proteins so far identified from soybean seeds (Hirano *et al.*, 1984, 1985, 1987; Fukazawa *et al.*, 1985; Momma *et al.*, 1985; Kagawa *et al.*, 1987, 1988*a*).

Subsequently, the amino-acid sequence of the internal portions of the basic 7*S* globulin LII sub-unit was determined by protein sequencing; this sequence is highly homologous to that of the conglutin γ, one of the lupin-seed-storage proteins (Kagawa *et al.*, 1987). Furthermore, several seed proteins from other legume species cross-reacted with the antiserum prepared against the basic 7*S* globulin (Kagawa *et al.*, 1987), suggesting that the basic soybean 7*S* globulin-like protein is widely distributed in legumes (Kagawa *et al.*, 1987). Two other types of major storage proteins, legumin-and vicilin-like proteins, occur in these plants. The basic 7*S* globulin is considered to be the third common type of seed-storage protein in many legume species (Kagawa *et al.*, 1987).

It was recently found that, when the dry mature soybean seeds were immersed in water at 50–60°C, a large amount of protein was specifically released into the water. The protein was provisionally identified as the basic 7*S* globulin based on the basis of its electrophoretic mobility (Kagawa *et al.*, 1992). The basic 7*S* globulin may not be simply a 'seed-storage protein' for germination, but could also have some important function.

In this study, we demonstrate that the basic 7*S* globulin is synthesized *de novo* in the soybean cotyledon in hot water and secreted outside the soybean seeds.

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MATERIALS AND METHODS

Materials

The dry mature seeds of soybean (*Glycine max* L. cv. Miyagishirome), harvested in the previous year, were used for this experiment.

Heat treatment of the seeds

The seeds were put into a beaker, and deionized water (seed(s):water = 1:10 (w/v)) was poured into the beaker. The beaker was heated in the water bath.

Electrophoresis

Proteins released from the seeds into water were lyophilized and dissolved in sodium dodecyl sulfate (SDS)-sample buffer (0·0625M Tris-HCl buffer at pH 6·8, containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol) and heated at 90°C for 5 min. The protein solution was subjected to SDS–polyacrylamide-gel electrophoresis as described by Kagawa *et al.* (1988*b*).

Quantitative analysis of proteins

Protein concentration was determined spectrophotometrically by using bicinchoninic acid (Smith *et al.*, 1985).

Detection of glycoproteins

Proteins released from the soybean seeds were separated by SDS–polyacrylamide-gel electrophoresis, electroblotted onto the polyvinylidene difluoride membrane (Immobilon, Millipore), and made to react with peroxidase-coupled concanavalin A, lentil lectin, castor-bean lectin, common-bean lectin, peanut lectin, and wheat-germ lectin (Honen Oil) according to the procedure described by Kijimoto-Ochiai *et al.* (1985). The glycoproteins having sugar chains were detected as colored bands on the polyvinylidene difluoride membrane.

Northern-blotting analysis

RNA was extracted by using the SDS–phenol method of Watanabe and Price (1982). Total RNA was prepared from soybean seeds immersed in water at 60°C for 2 h. Poly (A) + RNA was prepared by standard oligo(dT)-cellulose affinity chromatography. Poly(A) + RNA was separated on 1·2% agarose Mops–formaldehyde gel and transferred to a nylon membrane (Gene Screen Plus, Du Pont) in 10 × SSC (1 × SSC = 0·15M NaCl–0·015M sodium citrate). The blot was probed with a 32P-dCTP random-primer-labeling (Feinberg & Vogelstein, 1983) soybean basic 7*S* globulin cDNA (Kagawa & Hirano, 1989) probe with constant agitation in 10 ml of hybridization solution containing 10% dextran sulfate, 50% deionized formamide, 1M NaCl, 1% SDS, and denaturated salmon sperm DNA (100 μg/ml). Hybridization was performed at 42°C for 20 h. The hybridized membrane was washed twice with 2 × SSC plus 0·1% SDS at room temperature for 10 min, then twice for 30 min in 1 × SSC plus 0·1% SDS at 65°C, and finally with 0·1 × SSC at room temperature for 30 min with constant agitation. After drying, the membrane was exposed to X-ray film in a cassette with intensifying screens at −80°C and developed after one or two days.

RESULTS AND DISCUSSION

When immersed in water at 60°C, the dry mature soybean seeds swelled rapidly. After that, the seeds apparently started to release some proteins into the surrounding water (Fig. 1). The SDS–polyacrylamide-gel electrophoresis revealed that the water contained three major polypeptides, designated as S1, S2, and S4, and two minor polypeptides, S3 and S5. All these polypeptides were analyzed by amino-acid sequencing. The amino-acid sequences determined for the major polypeptides S1, S2 and S4, are homologous with those of the basic 7*S* globulin H and L sub-units determined previously by Hirano *et al.* (1987) (Kagawa *et al.*, 1992). On the other hand, the amino-acid sequence of the S5 polypeptide was found to be consistent with the sequence of the previously reported hydrophobic protein of soybean (Odani *et al.*, 1989). Although we compared the N-terminal sequence of the S3 polypeptide with the sequences of the amino-acid-sequence database, no proteins showed a clear homology with the S3 polypeptide (Kagawa *et al.*, 1992). Among these polypeptides, the S3 was released even when the seeds were soaked in water at 20°C. The release of this minor polypeptide is therefore considered to be independent of the heat treatment.

The total amount of protein released from the seeds in water at 60°C was measured. A large amount of protein was released after from two to three hours of soaking (Fig. 2). The rate of protein secretion decreased after three hours. After eight hours, the total amount of protein released from the seed was approximately 5·9 mg per grain, which corresponds to about 4% of the total proteins of unheated seed. About 90% of the

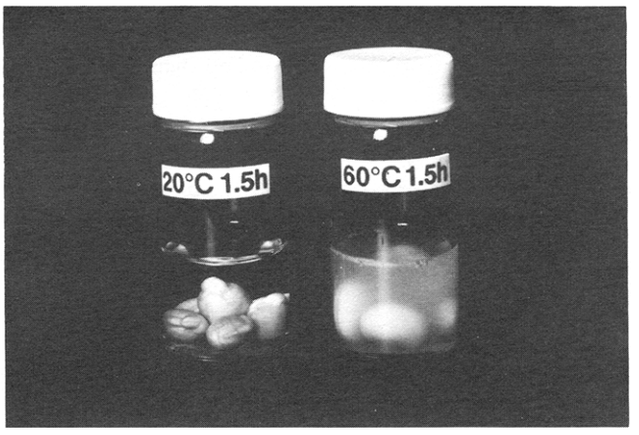

Fig. 1. Examples of the large amount of protein released from soybean seeds by hot-water treatment.

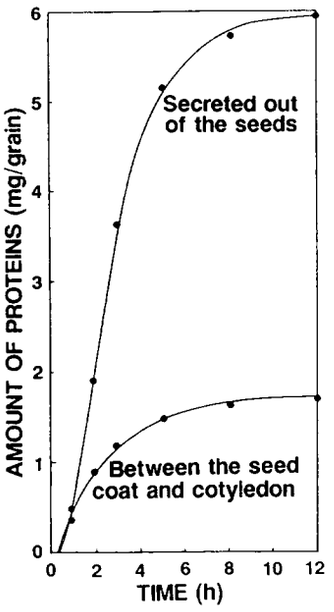

Fig. 2. Amounts of proteins released and accumulated between the seed coat and the cotyledon in one soybean seed. Twenty seeds were incubated in water at 60°C (seeds : water = 1 : 10 (w/v)), and an aliquot (100 μl) of the water was removed at the respective time to analyze the protein content. Values are means of two replicates.

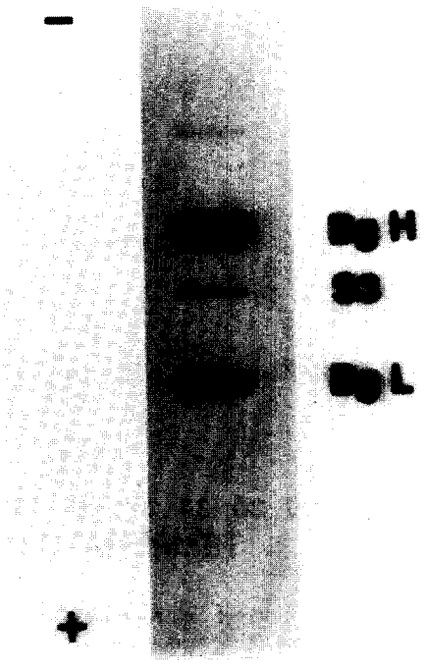

Fig. 3. Detection of the glycoprotein with N-linked oligosaccharide chains on the protein-blotted poly vinylidene difluoride membrane by using concanavalin A-peroxidase reagent. The basic 7*S* globulin H and L sub-units and the S3 polypeptide reacted strongly with concanavalin A.

proteins released were evaluated by SDS–polyacrylamide-gel electrophoresis to consist of the basic 7*S*-globulin H subunit and L subunit.

When immersed in water at 60°C for two hours, the seed absorbed, on average, 0·5 ml of water. Some of the water (approximately 85 μl) accumulated between the seed coat and the cotyledon, and contained as major components the same proteins as were released by the seeds and also some cotyledon proteins as minor components. The amount of protein accumulated was approximately 1·8 mg per grain after eight hours. This corresponds to approximately 1·2% of total protein of unheated seed. Among these proteins, only the basic 7*S*-globulin H, L sub-units, S3, and S5 selectively passed through the seed coat to be released into the surrounding water.

The electroblotted proteins were examined by the lectin-peroxidase method (Kijimoto-Ochiai *et al.*, 1985) to see whether they were glycoproteins. Among the proteins released, the basic 7*S*-globulin H and L subunits and S3 polypeptide reacted strongly with the jack-bean concanavalin A (Fig. 3) and weakly with the lentil lectin, castor-bean lectin (ricin), common-bean lectin (E4), and peanut lectin (anti-T agglutinin). This agrees with the results of our preliminary experiments (Hirano & Kagawa, 1988). However, the basic 7*S* globulin did not react with the wheat-germ lectin. On the other hand, the S5 polypeptide did not react with any of these lectins. Accordingly, the basic 7*S* globulin and S3 polypeptide among the proteins released are considered to be glycoproteins with mainly high-mannose-type sugar chains.

The dry unheated seeds contain approximately 3% basic 7*S* globulin as a storage protein (Yamauchi *et al.*, 1984). The amount of the basic 7*S* globulin released by heat treatment is nearly 5%, as described above. This discrepancy suggests that the basic 7*S* globulin released originated not only from the storage-protein pool but should be at least partly, if not entirely, synthesized *de novo*. After the seeds were immersed in hot water, the proteins from the cotyledon, hypocotyl and seed coat were separated by SDS-polyacrylamide gel electrophoresis. In the cotyledon only, the amounts of the basic 7*S*-globulin H and L sub-units increased markedly (Fig. 4). When immersed in water at 50 and 60°C for 1·5 h, basic 7*S* globulin was accumulated drastically in the cotyledon. At 60°C, the accumulation of basic 7*S* globulin was seen in the cotyledon after 90 minutes. This suggests that the basic 7*S* globulin was produced in the cotyledon.

We examined the basic 7*S*-globulin mRNA transcription by Northern hybridization. The pBG108 cDNA encoding the basic 7*S* globulin (Kagawa & Hirano, 1989) strongly hybridized with a particular mRNA in the extracts of the seeds incubated in water at 60°C (Fig. 5). These results indicate that a large proportion of the basic 7*S* globulin released into the water is synthesized *de novo* by the basic 7*S*-globulin mRNA transcribed in response to the heat shock. The basic 7*S* globulin is thus concluded to be a 'heat-shock' protein.

Before release from the cotyledon, the basic 7*S*-globulin precursor is found to be cleaved at the site between the H and L sub-units, which are linked together via disulfide bond(s) and glycosylated as will be described in detail elsewhere (Kagawa, H. & Hirano, H., in preparation).

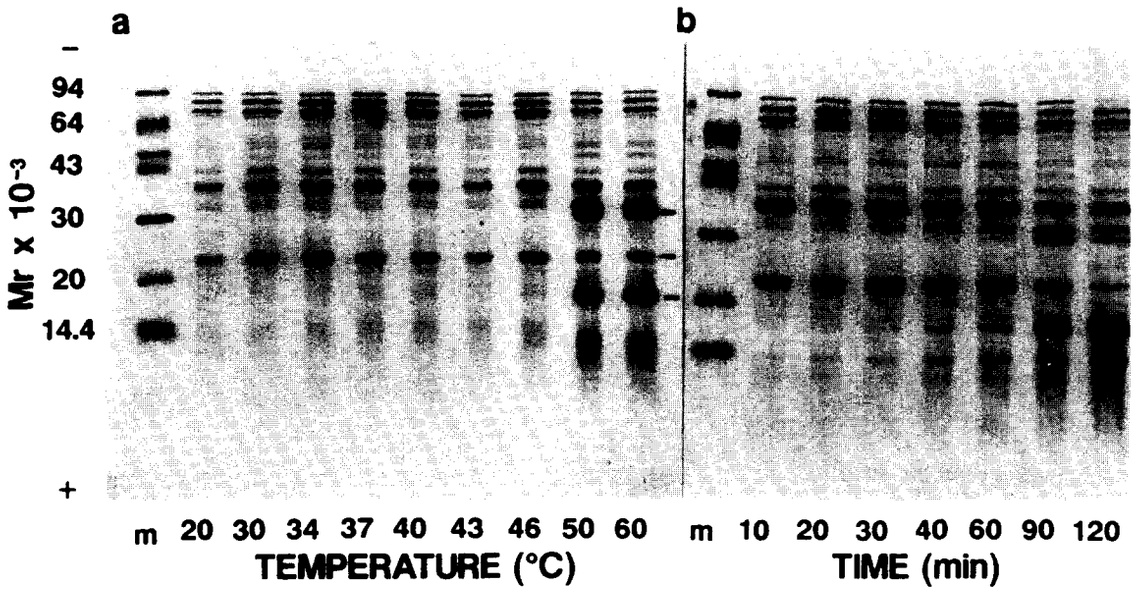

Fig. 4. SDS–polyacrylamide-gel electrophoresis of the cotyledon proteins from the soybean seeds incubated at different temperatures for 1·5 h (a) and in 60°C water for different times (b). The incubated cotyledon (10 mg) was homogenized with 300 μl of SDS–sample buffer and heated at 90°C for 5 min. The homogenate was centrifuged at 15000g for 2 min, and the supernatant (3 μl) was applied to the gel. m; Mr marker proteins. Bg: basic 7*S* globulin.

In soybean, the 17- and 8-kDa heat-shock proteins expressed in young seedlings have been identified and the genes encoding these proteins have been cloned (Schoffl *et al.*, 1984; Czarnecka *et al.*, 1985). Their deduced amino-acid sequences are different from that of the basic 7*S* globulin. To date, heat-shock proteins structurally homologous to the basic 7*S* globulin have not been identified in soybean. We compared the sequence of the basic 7*S* globulin with the sequences of the heat-shock proteins from many other organisms compiled in the amino-acid sequence database, but no significant homologies were found.

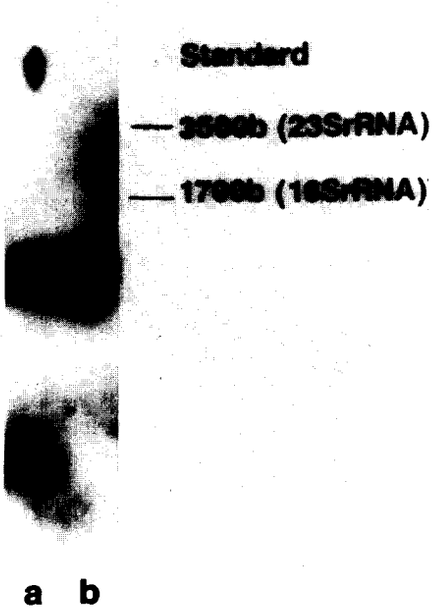

Fig. 5. Northern-blot analysis of poly(A)+RNA was isolated from the soybean seeds immersed in 20°C water (a) or in water at 60°C (b) for 1·5 h. The poly(A)+RNA (5 μl) was electrophoresed in an agarose Mops–formaldehyde gel, transferred to nylon membrane, and hybridized to the 1·4-kb basic 7*S*-globulin cDNA probe (Kagawa & Hirano, 1989).

As described above, the basic 7*S* globulin is secreted from the seeds. Previously, no heat-shock proteins have been known to be secreted out from the plant. The basic 7*S* globulin could be a novel type of heat-shock protein. Other similar experiments suggested that proteins similar to the soybean basic 7*S* globulin were widely distributed in legume species (Kagawa *et al.*, 1992).

The sulfur-containing amino acids in legume-seed proteins are present only in low concentrations; these amino acids are considered to be a nutritionally limiting factor for animals and humans. However, if the seeds of the legume species studied here are heated in water at 50–60°C for a few hours, the seeds synthesize a large amount of the basic 7*S* globulin or basic 7*S*-globulin-like proteins. In the case of soybean, the amount of the synthesized basic 7*S* globulin corresponds to nearly 5% of the total proteins of an unheated seed. The mature soybean seeds originally contained approximately 3% basic 7*S* globulin. Accordingly, after heat treatment, the total amount of the basic 7*S* globulin is increased 2·5-fold. Compared with the major seed proteins, glycinin and β-conglycinin, the basic 7*S* globulin is rich in sulfur-containing amino acids; it consists of approximately 5·8% cysteine and approximately 2·3% methionine. Consequently, heating the seeds increases the amount of sulfur-containing amino acid significantly. We deduce that, after heat treatment, the sulfur-containing amino-acid content may be about 30% more than the content in the dry mature seeds. It is important to note that the induction of the basic 7*S* globulin by heat shock during food processing could drastically improve the nutritional quality of soybean seeds and seeds of other legume plants. There is also

the possibility of synthesizing a new protein (and effecting, for example, its functionality in foods, texture and medicinal drugs) in the seed by using a basic 7*S*-globulin promoter.

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