

CHARACTERIZATION OF PROTEINS RELEASED FROM LEGUME SEEDS IN HOT WATER

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Abstract—When immersed in water at 50–60°, mature soybean seeds release a large amount of protein. The major protein released was basic 7S globulin (Bg), which is present in the cotyledons of soybean seeds. The released Bg consisted of the 27 000 and 16 000 subunits which were linked by disulphide bonding and glycosylated. The released Bg exhibited an identical structure with the mature Bg which was synthesized in the normal developing seeds. Proteins like Bg were also found to be released into hot water from the seeds of legume species such as azuki-bean, cowpea, mung-bean and winged-bean. Besides Bg and Bg-like proteins, a few proteins including the 9 000 hydrophobic protein in soybean, ubiquitin in cowpea and mung-bean, and Kunitz trypsin inhibitor in winged-bean, were released from the seeds in hot water.

INTRODUCTION

When immersed in water at 50–60°, mature soybean seeds swell rapidly. Thereafter, the seeds apparently start to release several proteins into the surrounding water. Asano *et al.* [1] showed by SDS-PAGE that the major released protein is basic 7S globulin (Bg) which accounts for ca 3% of the total protein of mature seeds. They, however, have not confirmed whether or not Bg is released in completely identical form with the mature Bg which is synthesized during normal development of the seeds.

On the other hand, in the previous study, Bg-like proteins which cross-reacted with the anti-Bg antibody were identified in the seeds of legume species such as azuki-bean, cowpea, mung-bean and winged-bean [2]. It has not been known whether or not these proteins are released from the seeds into hot water as in the case of soybean Bg.

In the present study, we confirmed that the released Bg is identical in structure with the mature Bg synthesized during development of the seeds, and found that Bg-like proteins are released from the seeds of several legume species in response to heat treatment. We also structurally characterized all proteins which are released from the seeds in hot water.

RESULTS

When immersed in water at 50–60°, mature soybean seeds released a large amount of protein into the surrounding water. SDS-PAGE revealed that the water contained three major polypeptides designated as S1, S2 and S4 (ca 90%) and two minor polypeptides, S3 and S5 (Fig. 1).

When immersed in water at 60° for 2 hr, one seed absorbed on average 0.5 ml water. Some of the water

(ca 85 µl) accumulated between the seed coat and the cotyledon. Major components which were contained in the accumulated water were the same proteins as those released by the seeds; some cotyledon proteins were found as minor components. Among these proteins only the polypeptides S1, S2, S3, S4 and S5 selectively passed through the seed coat to be released into the surrounding water.

The released proteins were analysed by 2D-PAGE under non-reducing conditions in the first dimension and reducing conditions in the second dimension (Fig. 2). S3 and S5 appeared on the diagonal line in the electrophoretic pattern, confirming that these polypeptides are not linked to any other proteins by disulphide bonding. In contrast, S1, S2 and S4 appeared below the diagonal line, indicating that S1/S2 and S4 are linked by disulphide bonding.

The released proteins were separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane filter. The electroblotted proteins were examined by the lectin-peroxidase method to determine whether those are glycoproteins or not. S1, S2 and S4 reacted strongly with jack-bean concanavalin A and weakly with lentil lectin, castor-bean lectin (ricin), common-bean lectin (E₄) and peanut lectin (anti-T agglutinin).

After separation by SDS-PAGE, these polypeptides were electroblotted and sequenced. The N-terminal amino acid sequences (Table 1) of the major polypeptides S1 and S2 were identical with those of the Bg 27 000 subunit reported previously [3]. The amino acid sequence of S4 was also similar to that of the Bg 16 000 subunit. Furthermore, S1 and S2 reacted strongly with the antiserum raised against the Bg 27 000 subunit, and S4 reacted with the antiserum against the 16 000 subunit. The amino acid compositions of S1 and S4 were determined and found to be consistent with those of the

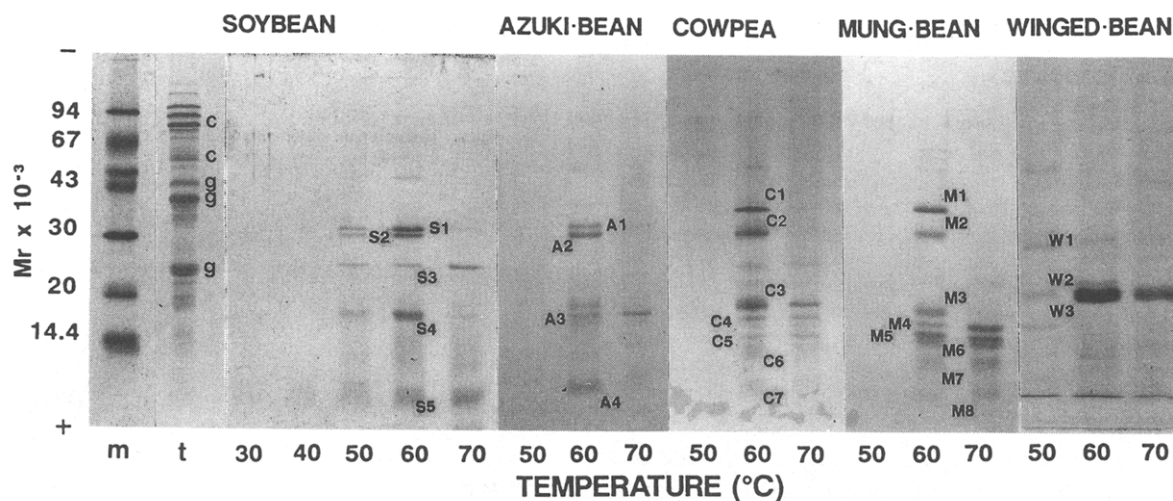


Fig. 1. SDS-PAGE of the proteins released from the seeds of soybean, azuki-bean, cowpea, mung-bean and winged-bean in hot water. Seeds were incubated in water (seed/water 1:10, w/v) for 1.5 hr. After incubation, 20 μ l of the protein soln was lyophilized. The proteins were dissolved in 5 μ l of SDS sample buffer (see Experimental), heated at 90° for 5 min, and applied to the gel. The polypeptides, S1, S2, S3, S4 and S5 had apparent M_r s of 31 000, 31 000, 24 000, 16 000 and 9 000, respectively. The M_r s of the S1 and S2 corresponding to the Bg HI and HII subunits, respectively, was found to be higher than the value of 26 000 reported previously [16]. m, Molecular mass marker proteins; t, total protein of soybean seeds; c, β -conglycinin; g, glycinin.

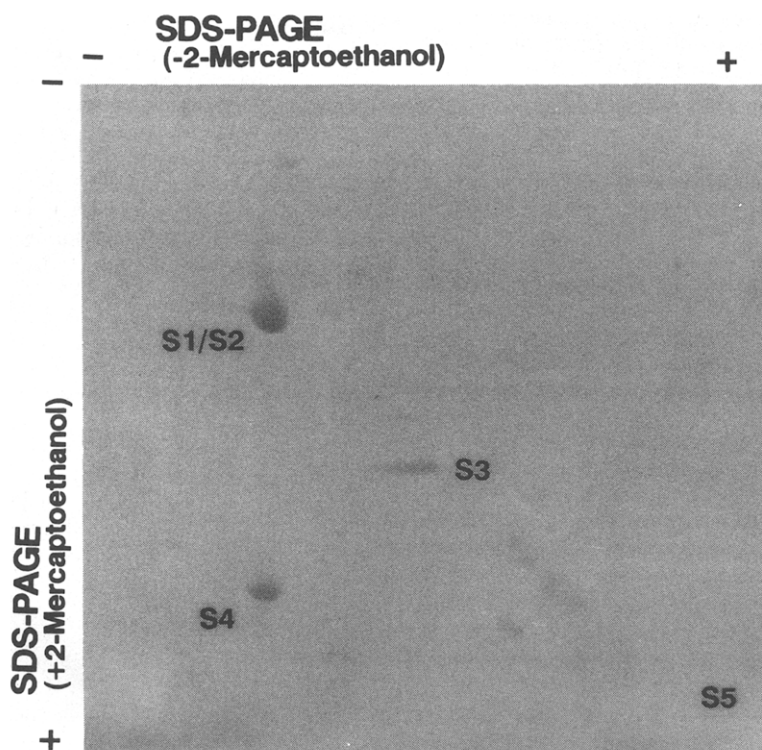


Fig. 2. 2D-PAGE of the proteins released from the seeds of soybean. Seeds were incubated in water (seed/water 1:10, w/v) at 60° for 1.5 hr. After incubation, 20 μ l of the protein soln was lyophilized. The proteins were dissolved in 5 μ l of SDS sample buffer without 2-mercaptoethanol, and separated by PAGE in the first dimension. After the PAGE, the gel was equilibrated with the buffer containing 5% 2-mercaptoethanol and subjected to 2D PAGE.

Table 1. N-Terminal amino acid sequences of the proteins released from five legume species

Soybean S1	VTPTKPINLV VLPVQNDGST GLHWANLQKR TPL
Soybean S2	VTPTKPINLV VLPVQNDGST GLHWANLQ
Soybean S3	ADPTFGTPLG
Soybean S4	STIVGSTSGG TMISTSTPHM VLQQSVYQAX TQ
Soybean S5	ALITRPSXPD LSIXLNILGG SLG
Azuki-bean A1	NYVMNPAYLL VL
Azuki-bean A2	NYVMNPAYLL VL
Azuki-bean A3	STTVGDSGGT MISTV
Azuki-bean A4	RTXENLANSY REPF
Cowpea C1	Blocked*
Cowpea C2	NNMVNPAYML VLPTQXDAXG
Cowpea C3	STTVGNSGGT MISTXP
Cowpea C4	Blocked
Cowpea C5	Blocked
Cowpea C6	MTVGQVQGNL AQPSGFLQN
Cowpea C7	MQIFVLTLTG KTITLEVESS
Mung-bean M1	NYVMNPAYLL VLPTQXDAXG
Mung-bean M2	NYVMNPAYLL VLPTQXD
Mung-bean M3	STTVGXSGG
Mung-bean M4	STTVGXSGGT MISTV
Mung-bean M5	Blocked
Mung-bean M6	Blocked
Mung-bean M7	MTVGQVQGNL AQPIGFLQKG VVP
Mung-bean M8	MQIFVLTLTG KTITLE
Winged-bean W1	VSPTVPPVLL V
Winged-bean W2	ADDPVYDAEG N
Winged-bean W3	STVPGGG

*None of the PTII amino-acid derivatives released could be identified after 10 cycles of Edman degradation of the peptides, C1, C4, C5, M5 and M6, suggesting the presence of a blocked N-terminal.

Bg 27 000 and 16 000 subunits deduced from the nucleotide sequence of Bg cDNA [4] (Table 2). These results confirm that the released polypeptides, S1 and S2 and S4 are identical with the 27 000 and 16 000 subunits of Bg, respectively.

The amino acid sequence of S5 was found to be consistent with the sequence of the previously reported hydrophobic protein of soybean [5]. Although we compared the N-terminal sequence of S3 with those compiled in the amino acid sequence data base, no protein showed a clear homology with S3.

After the seeds were immersed in hot water, the proteins from the cotyledon, hypocotyl and seed coat were separated by SDS-PAGE. As the Bg 27 000 and 16 000 subunits were identified only in the cotyledon, it is suggested that the cotyledon is the source of the Bg protein.

Similar heat treatment experiments were performed with several other legume species. When immersed in hot water, the seeds of azuki-bean, cowpea, mung-bean and winged-bean also released large amounts of protein (Fig. 2). The N-terminal amino acid sequences of the proteins released from the seeds which were immersed in hot water are shown in Table 1. Among them, the major proteins have highly homologous amino acid sequences with the Bg 27 000 and 16 000 subunits (Fig. 3). All the Bg-like proteins detected in the four legume species strongly reacted with the antiserum raised against Bg.

Table 2. Amino acid compositions of S1 and S4

	S1	(a)	S4	(b)
Asx	23.4	(23)	13.2	(13)
Thr	21.5	(22)	9.3	(9)
Ser	17.8	(18)	14.8	(15)
Glx	28.3	(28)	16.1	(16)
Pro	19.2	(19)	7.4	(8)
Gly	18.2	(18)	13.3	(13)
Ala	14.4	(14)	12.0	(12)
Val	16.0	(16)	16.0	(16)
Cys	8.6	(9)	4.7	(5)
Met	2.7	(3)	5.8	(6)
Ile	10.9	(11)	4.8	(5)
Leu	26.8	(27)	12.2	(12)
Tyr	3.7	(4)	2.0	(2)
Phe	10.9	(11)	6.4	(6)
His	10.3	(10)	2.8	(3)
Lys	7.4	(7)	5.0	(5)
Arg	8.8	(9)	5.0	(5)
Trp	—	(2)	—	(1)
	248.9	(251)	150.5	(152)

The values in parentheses are the numbers obtained from the amino acid sequence deduced by the nucleotide sequence of Bg cDNA [4]. (a) Bg 27 000 subunit; (b) Bg 16 000 subunit.

Soybean Bg 27 kD subunit	V T P T K P I N L V V L P V Q N D G S T G L H
Azuki-bean A1 and A2	N Y V M N P A Y L L V L
Cowpea C2	N N M V N P A Y M L V L P T Q X D A
Mung-bean M1 and M2	N Y V M N P A Y L L V L P
Winged-bean W1	V S P T V P P V L L V
Soybean Bg 16 kD subunit	S T I V - G S T - S G G T M I S T S T P
Azuki-bean A3	S T T V - G D - - S G G T M I S T V
Cowpea C3	S T T V - G N - - S G G T M I S T S X P
Mung-bean M3	S T T V - G X - - S G G T M I S T V
Winged-bean W3	S T - V P G - - - G G
Lupin conglutinin γ^{17}	S T S Y H G S G E I G G A L I T T - T
Cowpea C7	M Q I F V K T L T G K T I T L E V E S S
Mung-bean M8	M Q I F V K T L T G K T I T L E V E
Oat ubiquitin ⁷⁾	M Q I F V K T L T G K T I T L E V E S S
Barley ubiquitin ⁸⁾	M Q I F V K T L T G K T I T L E V E S S
Cowpea C6	M T V G Q V Q G N L A Q P S G F L Q N
Mung-bean M7	M T V G Q V Q G N L A Q P I G F L Q K G G V V P

Fig. 3. Structural homology of the proteins released from the seeds of five legume species. The identical amino acids are boxed. The hyphens indicate the gaps that are necessary to maximize the homology among the proteins.

In azuki-bean, cowpea and mung-bean seeds, the N-terminal sequences of the 8 000 proteins were found to be highly homologous with the ubiquitin from animals [6] and plants [7, 8] (Fig. 3).

The soybean seeds also released a protein (S5) which had a *M*, similar to that of these ubiquitins. However, the N-terminal sequence of S5 was not homologous with those of the 8 000 proteins of the three legume species studied. No ubiquitin-like protein was found in soybean seeds in the present experiment.

In winged-bean seeds, besides the Bg-like protein, the Kunitz trypsin inhibitor with a *M*, of 22 000 [9] was also released.

DISCUSSION

Bg released from the seeds into hot water consisted of the 27 000 and 16 000 subunits which were linked by disulphide bonding and glycosylated. The released Bg exhibited an identical structure with the mature Bg which was synthesized during normal development of the seeds [4].

It remains to be determined whether *de novo* synthesized or stored Bg is released. We have not enough evidence to answer this question. However, we detected a polypeptide which has the same molecular mass as the Bg precursor polypeptide [4] and cross-reacts with the anti-Bg antibody, and a slightly increased amount of mature Bg when we immersed the seeds in hot water. Also, we detected mRNA in the seeds immersed in water at 60° [10]. These results suggest that Bg might be produced in response to heat treatment.

In the previous study, we identified Bg-like proteins which cross-reacted with the anti-Bg antibody in the seeds of legume species such as azuki-bean, cowpea, mung-bean and winged-bean [2]. Interestingly, these proteins are also released from the seeds into hot water as in the case of soybean Bg. We have observed that Bg-like

proteins are also released from the seeds of common-bean (*Phaseolus vulgaris* L.) and jack-bean (*Canavalia ensiformis* DC.) into hot water (data not shown).

In common-bean, cowpea and mung-bean seeds, 8 000 proteins highly homologous in the N-terminal sequence with the ubiquitin from plants and animals were detected. Ubiquitin is known to be induced by heat shock [6]. It has been suggested that ubiquitins interact with heat shock proteins for the regulation of their degradation in eukaryotes. The release of the ubiquitin from the legume seeds into hot water might be related to that of the Bg-like proteins.

In winged-bean seeds, the Kunitz trypsin inhibitor was released in a much larger amount than the Bg-like protein, suggesting that the released Bg-like proteins might be also protease inhibitors. However, Bg shows no inhibitory activity against trypsin, α -chymotrypsin, α -amylase and papain. In addition, Bg has no haemagglutinate activity, based on an assay using rabbit peripheral erythrocytes (data not shown).

Recently, there have been two interesting findings; one is that Bg has an insulin-binding activity [11], suggesting that Bg might be a receptor of insulin-like hormone. Another is that the amino acid sequence of the auxin-regulated extracellular glycoprotein GP57 from carrots was highly homologous with that of Bg [12]. These results suggest that Bg is an important functional protein widely distributed in many plant species.

EXPERIMENTAL

Plant material. Dry mature seeds of soybean [*Glycine max* (L.) Merrill cv Miyagishirome], azuki-bean (*Vigna angularis* L. cv Dainagon), cowpea (*Vigna sinensis* Endl. strain CH-19), mung-bean (*Vigna radiata* L. strain EG23456) (kindly provided by Dr Y. Egawa) and winged-bean (*Psophocarpus tetragonolobus* L.

strain PT 4912), harvested in the previous year, were used for this experiment.

SDS-PAGE. Proteins were dissolved in SDS-sample buffer (0.0625 M Tris-HCl buffer at pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol and 10% glycerol), and heated at 90° for 5 min. The protein soln was subjected to SDS-PAGE as described in ref. [13].

2D-PAGE. Proteins were separated by 2D-PAGE [9]. First, proteins were dissolved in the SDS-sample buffer without 2-mercaptoethanol and separated by PAGE in the first dimension under non-reducing conditions. After electrophoresis in the first dimension, the gel was equilibrated with buffer containing 5% 2-mercaptoethanol and subjected to PAGE in the second dimension under reducing conditions in order to identify the disulphide-linked proteins.

N-Terminal sequence analysis. After separation by SDS-PAGE, proteins were electroblotted from the gel onto a PVDF membrane (Immobilon) (Millipore, Milford, USA) as described in ref. [9] with a slight modification: in the cathode soln for blotting, Tris 2-amino-*n*-caproic acid buffer was substituted for Tris 6-amino-*n*-caproic acid buffer [14] to avoid solubility problems. The protein electroblotted onto the PVDF membrane was detected by Coomassie blue staining [9]. The portions of the PVDF membrane carrying the electroblotted protein were cut out and mounted onto the upper glass block of the reaction chamber in the protein gas-phase sequencer model 470A or 477A (Applied Biosystems, Foster City). Sequential Edman degradation was performed according to the standard program supplied by Applied Biosystems. The released phenylthiohydantoin (PTH) amino acid derivatives were identified by the on-line system of HPLC, model 120A (Applied Biosystems).

Amino acid analysis. Proteins were electroeluted from the SDS-PAGE gels, desalted by HPLC using a reverse-phase C3 column (Beckman, San Ramon) and hydrolysed with 6 M HCl at 105° for 24 hr and the amino acid compositions determined by amino acid analyser model 5500 (Irika, Kyoto, Japan).

Homology search of amino acid sequence. The amino acid sequences obtained were compared with sequences of over 16 000 proteins contained in the amino acid sequence data base SEQDB (Protein Research Foundation, Osaka, Japan).

Detection of glycoproteins. Proteins were separated by SDS-PAGE, electroblotted onto the PVDF membrane, and reacted with peroxidase-coupled concanavalin A, lentil lectin, castor-bean lectin, common-bean lectin or peanut lectin (Honen Oil, Tokyo) according to the procedure described in ref. [15]. Glycoproteins having sugar moieties were detected as coloured bands on the PVDF membrane.

Immunoblotting. Proteins were separated by SDS-PAGE, electroblotted onto the PVDF membrane, and reacted with the antibody raised against Bg 27 000 or 16 000 subunit [2]. Proteins that cross-reacted with the antibody were detected by peroxidase

enzyme immunoassay (BioRad Immunoblot Assay Kit) (BioRad, Richmond).

Heat treatment of the seeds. Seeds were put into a beaker, covered with deionized H₂O (seed/H₂O 1:10, w/v), and the beaker heated in a water bath. Proteins released from the seeds into water lyophilized and subjected to SDS-PAGE and 2D-PAGE. After heat treatment, soybean cotyledon, hypocotyl or seed coat tissues were homogenized in the SDS-sample buffer and heated at 90° for 5 min. After centrifugation at 15 000 *g* for 2 min, proteins in the supernatant were separated by SDS-PAGE.

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