Purification and Characterization of a Glycine-rich Protein from the Aleurone Layer of Soybean Seeds

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Glycine-rich protein (GRP) was extracted with hot water from cell walls of the aleurone layer of soybean seeds and solubilized by pectinase treatment. GRP was purified by Sephadex G-100 gel chromatography, anion exchange HPLC, and reverse-phase HPLC. Two GRP fractions that had almost the same amino acid composition were found by gel chromatography. The high-molecular-mass GRP seems to be an associated form of the low-molecular-mass GRP (30-kDa). Thirty-kDa GRP was separated into a major GRP-I and a minor GRP-II by anion exchange HPLC. The major amino acids of GRP-I purified by reverse-phase HPLC were glycine (68%) and serine (12%). GRP-I contained a small proportion of sugar, approximately 9% (w/w), and mannose, arabinose, glucose, xylose, and galactose were included in the sugar moiety. The N-terminal amino acid sequence of GRP-I was a novel polyglycine structure including at least 20 glycine-repeated sequence. The GRP-I might be a novel type of extracellular matrix protein specific to the aleurone layer.

Extensin, a hydroxyproline-rich protein in plant cell walls, is well known. Glycine-rich protein (GRP) is another type of plant cell wall protein discovered recently. The isolation and characterization of GRPs have scarcely been done except for a few reports on strawberries and pumpkin. On the other hand, genes encoding GRPs have been isolated from several plants including petunia (Petunia hybrida), French bean (Phaseolus vulgaris), and arabinodispis (Arabidopsis thaliana). Moreover, it was found that the expression of plant genes encoding GRPs is induced by water stress, freezing, wounding, light regulation, and pathogen infection. GRPs seem to be stress proteins. However, the relationship between the structure and physiological function of GRP has not been resolved because of delay of investigation of the protein structure. Therefore, more information on the properties of GRP isolated from plant materials is required.

Seed coats of some plant species contain a large amount of glycine. Varner and Cassab reported that GRP exists in the cell walls of seed coats. We also separated GRP from the soybean aleurone layer, the adjacent tissue of seed coats, and reported a part of the properties of the GRP in our previous paper. In a preliminary communication, we reported the amino acid composition and N-terminal amino acid sequence of the GRP. This paper describes in detail the purification and characterization of soybean aleurone GRP.

Materials and Methods

Materials. Soybean seeds (Glycine max L. cv. Enrei) produced in Toyama Prefecture were used. Aspergillus nigerinase was purchased from Sigma Chemical Co. (St. Louis).

Separation of aleurone layer from soybean seeds. The seed coat tissue associated with the aleurone layer was detached from soybean seeds immersed in distilled water at 4°C overnight. Then the aleurone layer was separated. A small amount of spongy parenchyma tissue remaining on the aleurone layer was carefully removed. Ten grams of lyophilized aleurone layers were obtained from 100 g dry weight of seed coats and adjacent tissues collected from 4000 seeds. The separated aleurone layers were lyophilized and observed by a Hitachi scanning electron microscope (type S-530) at 20 kV.

Separation of GRP. GRP was extracted with hot water (80°C) after removal of other proteins with warm water (40°C) from the aleurone layer of soybeans as previously reported. The purification of GRP reported in our previous paper was partially modified as follows. The hot water extract was treated at 25°C for 2.5 h at pH 4.0 with pectinase, and then passed through a DEAE-Sephadex A-50 column (1.6 x 3.0 cm) equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The column was washed with the same buffer and the adsorbed proteins were eluted with the same buffer containing 0.5 M NaCl. The eluate was dialyzed against distilled water and lyophilized.

Sephadex G-100 gel chromatography. The lyophilized powder was dissolved in 0.1 M NaCl. The protein solution was chromatographed on Sephadex G-100 gel column (1.0 x 120 cm) at a flow rate of 0.5 ml/min with 0.1 M NaCl, and the GRP fraction was collected.

Anion exchange and reverse-phase HPLC. Anion exchange HPLC was done with a Toso HPLC apparatus using a DEAE-PSW column. Reverse-phase HPLC was done with a Toso HPLC apparatus using an ODS-120T column.

SDS-polyacrylamide gel electrophoresis (PAGE). GRP was treated in 2% SDS containing 1% mercaptoethanol and 6 M urea and was electrophoresed in 0.1 M Tris-glycine buffer (pH 8.6) containing 0.1% SDS using a 7.5% gel, and was stained with Coomasie brilliant blue G-250 and a sugar detection kit (G.P. Sensor, Hiden). Amino acid and sugar analyses. The fractions separated by chromatographies were hydrolyzed with 6 N HCl at 110°C for 24 h to analyze the amino acid composition of GRP. For the measurement of serine and threonine, the GRP fraction was hydrolyzed for 48 h and 72 h as well as 24 h. The hydrolyzate was analyzed on an amino acid analyzer (Hitachi, Model 835). Half cystine was measured as cystic acid after performic acid oxidation. Tryptophan was not measured. The same fractions used for amino acid analysis were hydrolyzed with 4 M HCl.

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Abbreviations: GRP, glycine-rich protein; SDS PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin.
Fig. 1. The Aleurone Layer of Soybean Seeds.
A: × 60; scale bar indicates 500 μm. B: × 500; scale bar indicates 100 μm.

Fig. 2. Chromatogram of Glycine-rich Fraction on Sephadex G-100 Column.
The glycine-rich fraction eluted from the DEAE-Sephadex column after pectinase
treatment was chromatographed on a Sephadex G-100 column (1.0 × 120 cm).
trifluoroacetic acid at 100°C for 3 h. The sugars of the hydrolyzate were
acetylated and pyridylaminated to measure as fluorescent derivatives. The
derivatives were analyzed by HPLC by the method of Suzuki et al.18)

Amino acid sequencing. Automatic Edman degradation was done with
a gas-phase sequencer (Applied Biosystems, Model 470A). Phenylthiohy-
dantoin (PTH) derivatives of amino acids were identified by HPLC.

Results
Aleurone layer
The aleurone layer separated from soybean seeds is shown in Fig. 1. The top surface (Fig. 1A) was on the
side of the seed coat. When the purity of the aleurone layer was checked with a scanning electron microscope,
spongy parenchyma tissue was not observed. Cell walls of the aleurone layer were obviously thicker than ones of seed
coat tissues.

Isolation of GRP
Although GRP of the aleurone layer was not extracted
with acidic, alkaline, or neutral salt solutions, GRP was
effectively extracted with hot water at more than 80°C. The
GRP extracted with hot water from the aleurone layer was
approximately 22 mg/g of aleurone total protein. The vis-
cosity of the hot water extract was decreased by pectinase
treatment. The elution profile of GRP obtained from 2 g of

Fig. 3. Elution Profile of GRP-I and GRP-II from the DEAE-SPW
Column.
A DEAE-SPW column (7.5 × 75 mm) was equilibrated with 0.02 M Tris HCl buffer
(pH 8.0). F-2 was chromatographed by anion exchange HPLC with the column using
a linear gradient of 0 to 0.5 M NaCl in the same buffer. The flow rate of the buffer
was 1.0 ml/min.

Fig. 4. Isolation of GRP-I by a Reverse-phase HPLC.
GRP-I was isolated by a reverse-phase HPLC with a TSK-gel ODS-120T column
(4.6 × 150 mm). The column was eluted with a linear gradient of acetonitrile from 0
to 80% in 0.1% trifluoroacetic acid. The flow rate of the buffer was 1.0 ml/min.
Characterization of Glycine-rich Protein from Soybean

Fig. 5. Molecular Mass Estimation of GRPs.
A: Sephadex G-100 gel filtration of F-2. B: SDS-PAGE pattern of F-2 (the low-molecular-mass GRP fraction separated by Sephadex G-100 gel chromatography). Lane 1: Marker protein; Lane 2: F-2. BSA, 66.0-kDa; egg albumin, 45.0-kDa; β-lactoglobulin, 36.8-kDa; cytochrome c, 12.4-kDa; aprotinin, 6.5-kDa.

Table Amino Acid and Sugar Compositions of GRP-I

<table>
<thead>
<tr>
<th>Amino acid (mol%)</th>
<th>Sugar (mol%)</th>
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<tbody>
<tr>
<td>Asx 1.7</td>
<td>Man 31.4</td>
</tr>
<tr>
<td>Thr 0.5</td>
<td>Ara 23.5</td>
</tr>
<tr>
<td>Ser 11.8</td>
<td>GIm 20.0</td>
</tr>
<tr>
<td>GIIx 5.8</td>
<td>Xyl 15.5</td>
</tr>
<tr>
<td>Pro 0.8</td>
<td>Gal 9.7</td>
</tr>
<tr>
<td>Gly 68.2</td>
<td></td>
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<tr>
<td>Ala 4.3</td>
<td></td>
</tr>
<tr>
<td>1/2Cys* 0.6</td>
<td></td>
</tr>
<tr>
<td>Val 0.4</td>
<td></td>
</tr>
<tr>
<td>Met 0.0</td>
<td></td>
</tr>
<tr>
<td>Ile 0.1</td>
<td></td>
</tr>
<tr>
<td>Leu 0.2</td>
<td></td>
</tr>
<tr>
<td>Tyr 1.9</td>
<td></td>
</tr>
<tr>
<td>Phe 0.0</td>
<td></td>
</tr>
<tr>
<td>Lys 2.3</td>
<td></td>
</tr>
<tr>
<td>His 0.9</td>
<td></td>
</tr>
<tr>
<td>Arg 0.5</td>
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Total 100.0       Total 100.1

* Measured as cysteic acid.

aleurone layer on a Sephadex G-100 column is shown in Fig. 2. The separation of GRP on the column chromatography was done better than that without pectinase treatment. GRP was found in two fractions (F-1 and F-2) by the column chromatography. Both fractions showed almost the same amino acid composition. Therefore, F-1 and F-2 seem to be oligomers and monomers of GRP, respectively. F-2 gave two peaks by anion exchange HPLC (Fig. 3), which were GRPs. GRPs in a major and a minor peak were temporarily named GRP-I and GRP-II, respectively. The elution profile of GRP-I on reverse-phase ODS-column was shown in Fig. 4. GRP-I showed a single peak. Homogeneity of the purified GRP-I was confirmed by the reverse-phase HPLC.

Molecular mass estimation
The average molecular size of F-2 obtained by gel chromatography was estimated as approximately 30-kDa (Fig. 5A). F-1 seems to be an associated form because it was eluted at near the void volume. Three protein bands, approximately 90-kDa, 60-kDa, and 30-kDa, appeared on SDS-PAGE of F-2 (Fig. 5B), because the association and aggregation of F-2 arose on the concentration treatment for SDS-PAGE. Ninety and 60-kDa GRPs seem to be trimers and dimers of 30-kDa GRP, respectively. Sugar was also observed in the three GRP bands on SDS-PAGE using sugar detection kit (data was not shown). The broadness of the GRP band on SDS-PAGE seems to depend on the microheterogeneity of the sugar content.

Amino acid and sugar compositions
GRP-I isolated by reverse-phase HPLC showed glycine (68%) and serine (12%) as shown in the Table. GRP included 9% (w/w) sugar. The major sugar moiety of GRP-I was composed of mannose (31%), arabinose (24%), glucose (20%), xylitol (16%), and galactose (10%) as shown in the Table.

N-Terminal amino acid sequence
On the analysis of N-terminal amino acid sequence of GRP-I, the Edman degradation was repeated till the 20th cycle. Therefore, GRP-I showed polyglycine structure, at least a 20 glycine-repeated sequence, in the N-terminus.

Discussion
Extensin is widely distributed in plant tissues, but GRP is distributed in limited tissues, stem, root, and seed coat. While the structure and function of extensin in cell walls were well known and have been established, those of GRP have not been established. Recently, the structures of some plant genes encoding GRPs have been reported. The amino acid compositions estimated from DNA sequences of these GRPs were very different in the serine percentage from that of GRP which we purified from the aleurone layer of soybean seeds. Ye and Varner reported the isolation of two GRPs containing 50% glycine from soybean seed coats. Although the detailed data on these GRPs have not been shown, they were distinct from the aleurone GRP because of the smaller glycine percentage and the distribution in vascular tissue.

Keller et al. reported that the gene encoding GRP in
French bean was expressed in vascular tissue, and GRP might have a specific role in the functional specialization of the vascular tissue.\(^4,23\) Ye and Varner also reported the occurrence of GRP in the vascular tissue of the soybean root and seed coat.\(^20\) However, the vascular system was not found in the aleurone layers separated from soybean seeds (Fig. 1). Consequently, it is assumed that the soybean aleurone GRP was a different GRP form from that in the vascular system of seed coat. The occurrence of GRP in the aleurone layer has never reported before our study.

GRP of the aleurone layer could not be extracted with 0.2 M CaCl\(_2\), which was usually used for extraction of soluble hydroxyproline-rich proteins from plant cell walls.\(^29\) The possible extraction of GRP with hot water suggests that GRP is associated with cell wall polysaccharides by non-ionic bonds.

The value of molecular mass of 30-kDa obtained by gel filtration was close to that of French bean GRP 1.0 reported by Keller et al.\(^11\) Although only the higher molecular mass (90-kDa) appeared without 6 M urea treatment, both the 30-kDa and 90-kDa GRPs arose on 6 M urea treatment.\(^15\) Sixty-kDa GRP also appeared on SDS–PAGE in our results (Fig. 5). It is assumed that the association of 30-kDa GRPs might be formed by hydrogen bonds.

Since the isolation and characterization of GRP have scarcely been reported, the occurrence of a sugar moiety of GRP has not been known. We found that GRP is a glycoprotein. The length of continuous glycline sequence found in the aleurone GRP has never been found in amino acid sequences of other GRPs.\(^1,4,5,23,27\) Polyglycine and GRP containing serine are capable of forming a β-sheet structure.\(^11\) It was expected that the aleurone GRP might form a β-sheet structure because of its polyglycine structure. However, we could not show the β-sheet structure of the GRP by CD measurement. It was assumed that the aleurone GRP could not form a β-structure because of the interference of sugar chains.

Plant GRPs have specific roles on response to the environmental stress.\(^6,11\) Therefore, GRP of the soybean aleurone layers is expected to participate in the function of the extracellular matrix of the aleurone tissue like the extracellular protein in carrot seeds reported by Satoh et al.\(^30\).

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**References**