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Studies on Persicae Semen. IV.[1] Separation and Characterization of Globulin Polypeptides from Persicae Semen

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Some properties of globulin polypeptides from Persicae semen were investigated. PR-A was separated into acidic polypeptides A_1 and A_2, and basic polypeptides, a mixture of A_3, A_4 and A_5 (A_3—A_5), by ion-exchange chromatography in 6 M urea. N-Terminal amino acids were determined as alanine for A_1, alanine and glutamine for A_2, and glycine for A_3—A_5 by the use of a gas-phase sequencer. As regards amino acid composition, A_1 and A_2 showed higher glutamine (and glutamic acid) content, and lower contets of basic amino acids (lysine, histidine and arginine) as compared to A_3—A_5. It seemed that PR-A exists as disulfide-linked A_1A_3-, A_2A_4- and A_2A_5- species with molecular weights of 65000, 59000 and 59000, respectively.

Keywords——Persicae semen; *Prunus persica*; globulin; polypeptide; gel-filtration chromatography; ion-exchange chromatography; electrophoresis

Persicae semen (Tohnin in Japanese) is an important crude drug commonly used for the treatment of Oketsu syndrome as an anti-coagulant, antiphlogistic and anodyne in the traditional Chinese system of medicine. We reported that a globulin PR-A (molecular weight (M.W.) 300000) isolated from water extract of Tohnin showed strong anti-inflammatory and analgesic activities, and afforded five major bands, A_1, A_2, A_3, A_4 and A_5 of polypeptides, showing molecular weights of 45000, 37500, 22000, 20000 and 19000, respectively, on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.[1a] However, the investigation was not extended to the physico-chemical properties or the structure of PR-A and these polypeptides. The purpose of this study was to separate and characterize the polypeptides, and to clarify the structural relationship between PR-A and its polypeptides.

Experimental

Preparation of PR-A——PR-A was prepared by the reported procedure.[1a]

Reduction and S-Carboxyamidemethylation[2a]——PR-A (3 g) was dissolved in 0.15 M Tris-HCl buffer (pH 8.7, 375 ml) containing 5 M guanidine–HCl and disodium ethylenediaminetetraacetate (139.5 mg) under a nitrogen stream. To this solution, 2-mercaptoethanol (3 ml) was added, and the mixture was stirred for 2 h at room temperature. Then, iodoacetamide (8.04 g) was added, and the reaction mixture was stirred in the dark for 15 min, dialyzed against deionized water in the dark, and then lyophilized to give S-carboxyamidomethylated PR-A (SM, 3.04 g).

SDS–Polyacrylamide Gel Electrophoresis——PR-A, SM and their components obtained by chromatographic separation as described below were subjected to electrophoresis according to the method of Laemmli[3] for 3 h at 170 V in 12.5% slab gel. The gel was fixed and stained with 0.025% Coomassie brilliant blue R-250 in 10% acetic acid and 50% methanol, and then destained with 5% acetic acid and 7.5% methanol. For the determination of molecular weights, the following proteins were used as standards (M.W.): cytochrome c (12400), lysozyme (14400), soybean trypsin inhibitor (21500), α-chymotrypsinogen (25700), carbonic anhydrase (31000), ovalbumin (45000), catalase (60000), bovine serum albumin (66200), phosphorylase (92500) and β-galactosidase (116250).

Gel-Filtration Chromatography on Sephadex G-100——SM was applied to a Sephadex G-100 column. The column was eluted with 0.02 M Na_2HPO_4–NaH_2PO_4 buffer (pH 7.5) containing 6 M urea. The eluates were monitored

with an ultraviolet (UV) detector at 280 nm.

Ion-Exchange Chromatography on Diethylaminoethyl (DEAE)-Sepharose CL-6B——SM and PR-A were separately applied to a DEAE-Sepharose CL-6B column with 0.02 M Tris–HCl buffer (pH 8.0) containing 6 M urea and stepwisely increasing amounts of NaCl.

Ion-Exchange Chromatography Carboxymethyl (CM)-Sepharose CL-6B——Each fraction (V, VI and VII) was separately applied to a CM-Sepharose CL-6B column with 0.02 M Na_2HPO_4–NaH_2PO_4 buffer (pH 6.0) containing 6 M urea and stepwisely increasing amounts of NaCl.

Sequential Amino Acid Analysis of the Subunits from Their N-Terminal to the Third Amino Acid——Automated Edman degradation was carried out on a Model 470A protein sequencer (Applied Biosystems, U.S.A.). Phenylthiohydantoin (PTH) amino acids were analyzed on an SP 8700 high performance liquid chromatography (HPLC) system with an Aquasil SEQ-4 (K) column (300 × 4.6 mm i.d.) at 45 °C using the following developing solvents: for the first 12 min, a CH_3CN gradient from 36 to 55% in 40 mM NaOAc buffer; for the next 1 min, 65% CH_3CN in 40 mM NaOAc buffer; for the last 6 min, 36% CH_3CN in 40 mM NaOAc buffer.

Amino Acid Analysis——After hydrolysis of the sample by heating in 6N HCl solution in a sealed tube at 105 °C for 18 h, amino acid analysis was carried out on the hydrolyzate with a Hitachi KLA-5 automatic amino acid analyzer.

Results and Discussion

In order to separate polypeptides corresponding to the five major bands A_1, A_2, A_3, A_4 and A_5, PR-A was reduced and carboxyamidomethylated, and the resulting polypeptide mixture (SM) was gel-filtered. Two apparent polypeptide classes, namely a mixture of the high-molecular-weight polypeptides (A_1 and A_2), and a mixture of the low-molecular-weight polypeptides (A_3, A_4 and A_5), were separated on Sephadex G-100 using 0.02 M phosphate buffer (pH 7.5) containing 6 M urea (Fig. 1).

On a DEAE-Sepharose CL-6B column equilibrated with Tris–HCl buffer (pH 8.0) containing 6 M urea, A_1 and A_2 were retained, and then eluted with the buffer containing 0.05 M and 0.1 M NaCl, respectively (Fig. 2). Furthermore, on CM-Sepharose CL-6B chromatography using 0.02 M phosphate buffer (pH 6.0) containing 6 M urea, A_2 was eluted in the unadsorbed fraction, while A_1 was gradually eluted, as shown in Fig. 3. These results on ion-exchange chromatography indicate that A_1 and A_2 are acidic polypeptides, and A_2 is

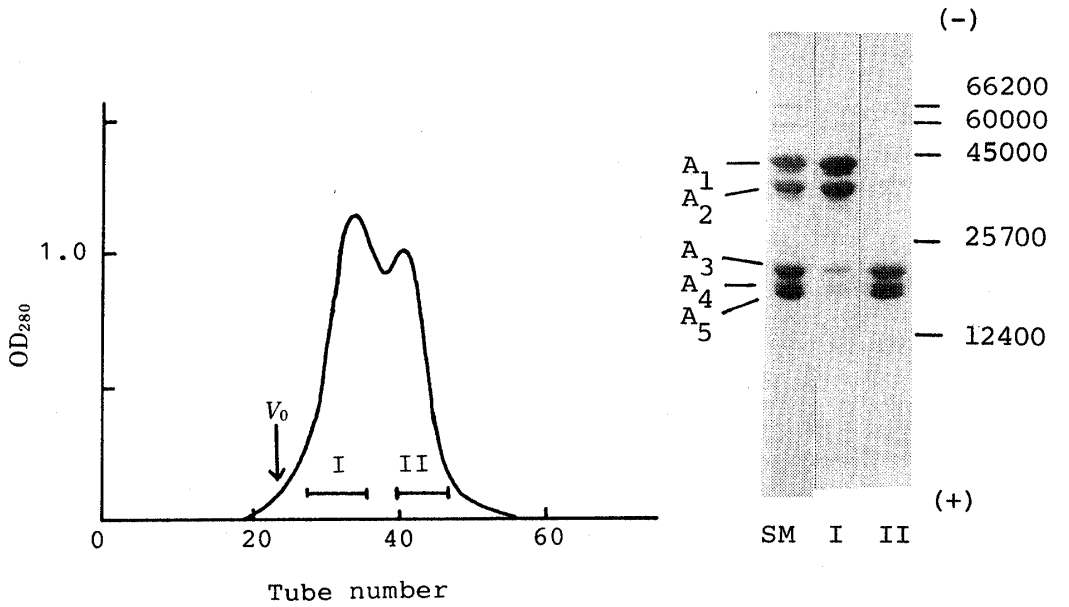

Fig. 1. Column Chromatogram of SM on Sephadex G-100

Column size, 2.5 × 50 cm; volume of tube, 3 ml; flow rate, 10 ml per h; buffer, 0.02 M phosphate buffer (pH 7.5) containing 6.0 M urea; sample, 100 mg of SM dissolved in 2 ml of the buffer; I (tube No. 28—35, 40 mg; A_1 and A_2), II (tube No. 40—46, 34 mg; A_3, A_4 and A_5). Insets are electrophoretic patterns of SM and the fractions (I and II).

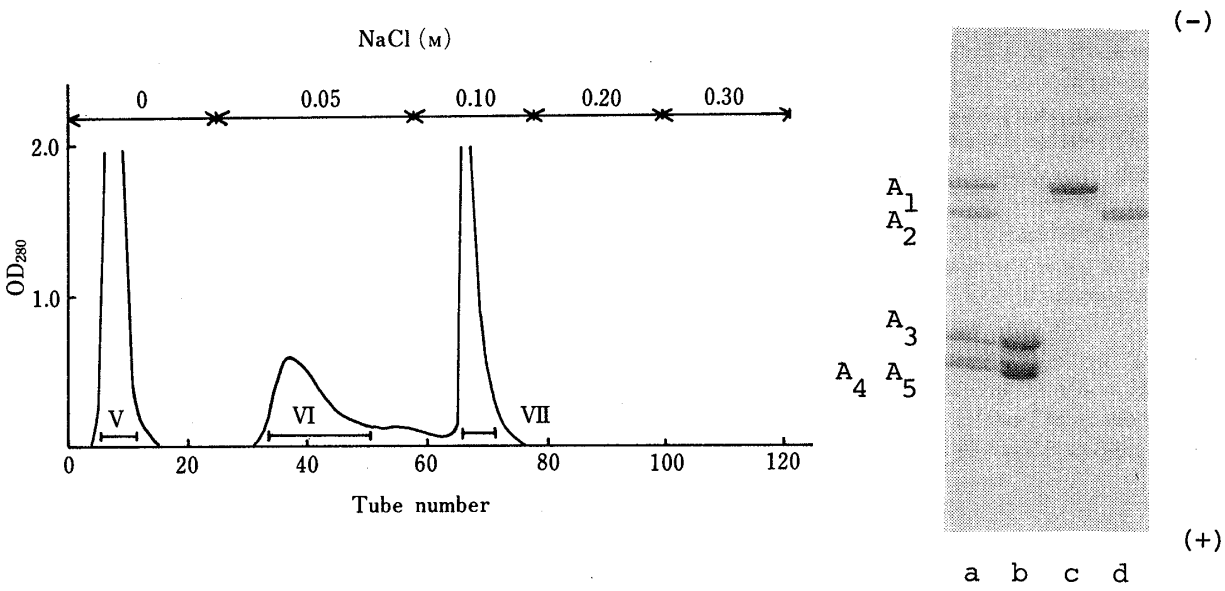

Fig. 2. Stepwise Column Chromatogram of SM on DEAE-Sepharose CL-6B

Column size, 2.6 × 12.5 cm; volume of tube, 10 ml; flow rate, 110 ml per h; buffer, 0.02 M Tris–HCl buffer (pH 8.0) containing 6 M urea; sample, 500 mg of SM dissolved in 10 ml of the buffer; V (tube No. 5—11, 130 mg; a mixture of A_3, A_4 and A_5), VI (tube No. 33—47, 132 mg; A_1), VII (tube No. 66—71, 109 mg; A_2). The NaCl concentration in the buffer was changed in a stepwise manner from 0 to 0.3 M. Insets are electrophoretic patterns of SM and the fractions (V, VI and VII). SM (a), V (b), VI (c), VII (d).

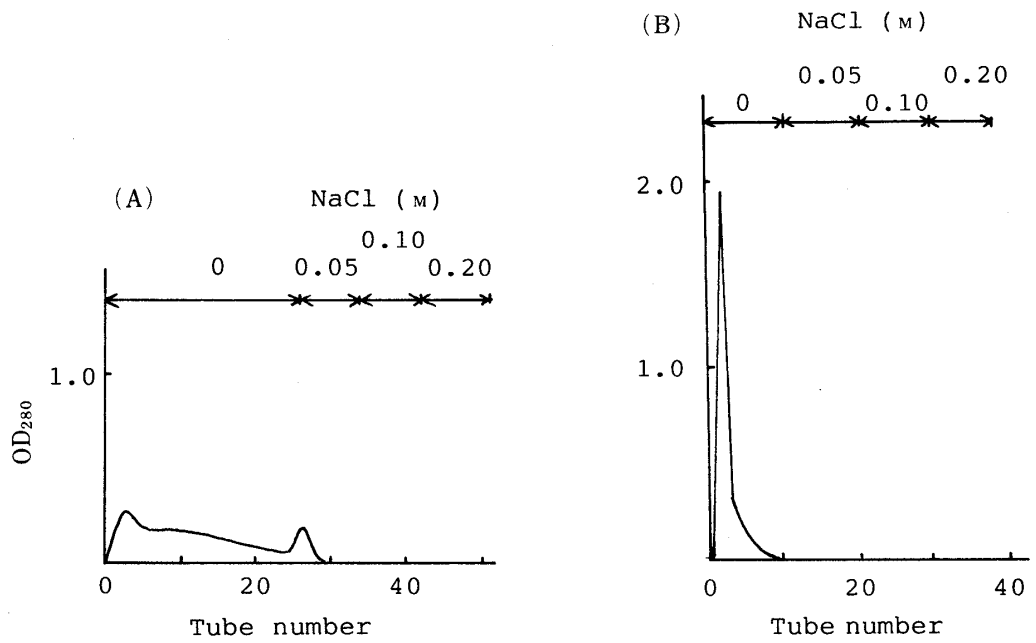

Fig. 3. Stepwise Column Chromatogram of Fractions VI (A) and VII (B) in Fig. 2 on CM-Sepharose CL-6B

Column size, 1.7 × 4 cm; volume of tube, 3 ml; flow rate, 30 ml per h; buffer, 0.02 M phosphate buffer (pH 6.0) containing 6 M urea; sample, 20 mg of fraction VI or VII dissolved in 2 ml of the buffer. The NaCl concentration in the buffer was changed in a stepwise manner from 0 to 0.2 M.

more acidic than A_1. On the other hand, A_3, A_4 and A_5 polypeptides (A_3—A_5) were eluted in the unadsorbed fraction on the same chromatography, as shown in Fig. 2. A_3—A_5 were weakly retained on a CM-Sepharose CL-6B column equilibrated with 0.02 M phosphate buffer

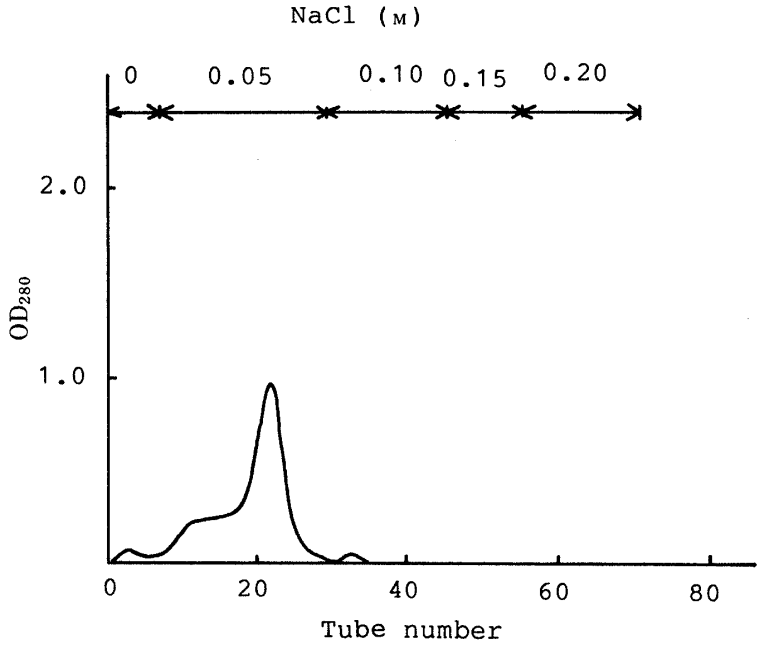

Fig. 4. Stepwise Column Chromatogram of Fraction V in Fig. 2 on CM-Sepharose CL-6B

Column size, 1.7×4 cm; volume of tube, 3 ml; flow rate, 30 ml per h; buffer, 0.02 M phosphate buffer (pH 6.0) containing 6 M urea; sample, 50 mg of fraction V dissolved in 2 ml of the buffer. The NaCl concentration in the buffer was changed in a stepwise manner from 0 to 0.2 M.

TABLE I. Amino Acid Composition of Polypeptides

Amino acid	(Residues/100 residues)			Amino acid	(Residues/100 residues)		
	A_1 (Acidic)	A_2 (Acidic)	A_3—A_5 (Basic)		A_1 (Acidic)	A_2 (Acidic)	A_3—A_5 (Basic)
Lys	0.7	0.7	1.5	Ala	3.4	4.0	5.8
His	1.8	1.5	2.3	Cys	—a)	—a)	—a)
Arg	12.5	10.8	13.8	Val	3.2	4.4	4.2
Asp	10.8	12.0	14.2	Met	—a)	—a)	—a)
Thr	1.6	1.7	4.0	Ile	2.3	2.3	5.0
Ser	3.7	4.1	5.7	Leu	5.8	6.5	9.8
Glu	39.7	33.4	17.1	Tyr	—a)	1.9	2.4
Pro	4.1	5.5	3.9	Phe	4.6	5.3	6.2
Gly	5.8	5.8	4.1				

a) Not detected.

(pH 6.0) containing 6 M urea (Fig. 4), but could not be separated from each other. Accordingly A_3, A_4, A_5 were considered to be basic polypeptides having very similar electrical properties.

On the other hand, A_1 and A_2 polypeptides were similar in amino acid composition with a high content of glutamine (and glutamic acid), and lower contents of basic amino acids (lysine, histidine and arginine) as compared to those of A_3—A_5 (Table I). On sequential amino acid analysis of A_1, alanine, arginine and glutamine were detected from the N-terminal to the third amino acid, respectively, while those of A_2 were pairs of alanine·glutamine, arginine·leucine and glutamic acid·glutamine, respectively. Although A_2 could not be separated by several chromatographies (data not shown), and A_2 showed a single band on

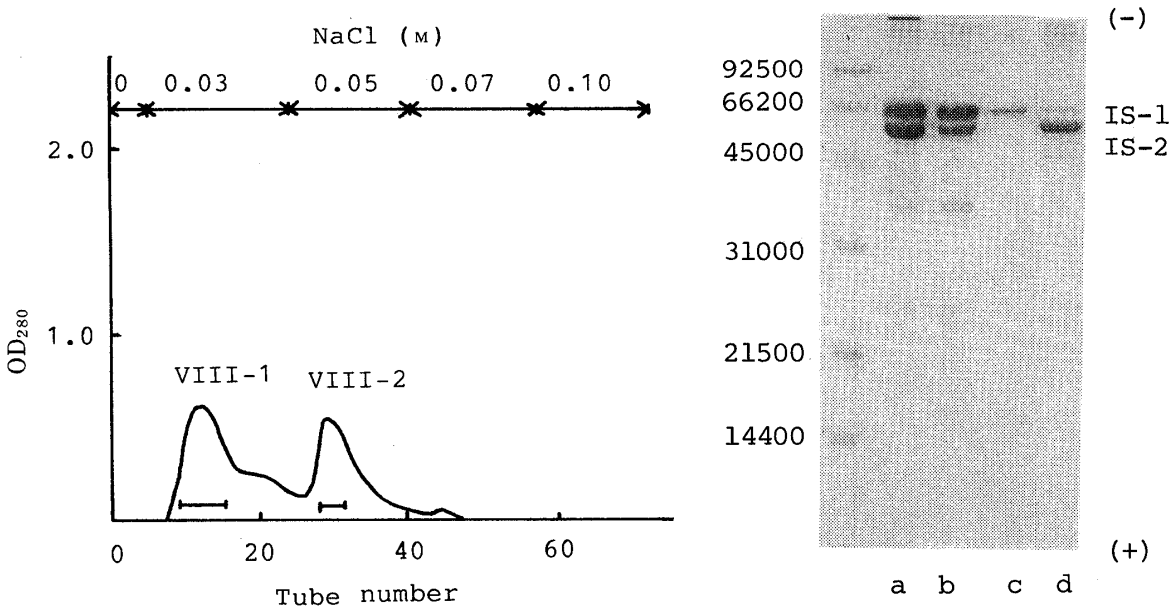

Fig. 5. Stepwise Column Chromatogram of Fraction VIII on DEAE-Sepharose CL-6B

Column size, 1.6 × 6.5 cm; volume of tube, 5 ml; flow rate, 50 ml per h; buffer, 0.02 M Tris–HCl buffer (pH 8.0) containing 6 M urea; sample, 80 mg of VIII dissolved in 5 ml of the buffer. The NaCl concentration in the buffer was changed in a stepwise manner from 0 to 0.1 M. Insets are electrophoretic patterns of PR-A (a), fractions VIII (b), VIII-1 (c) and VIII-2 (d) under nonreducing conditions.[4] IS-1 and IS-2: intermediary subunits. Fraction VIII was obtained by DEAE-Sepharose CL-66 column (2.6 × 10 cm) chromatography of PR-A (500 mg); buffer, 0.02 M Tris–HCl (pH 8.0) containing 6 M urea; volume of tube, 5 ml; flow rate, 50 ml per h; VIII (tube No. 15—21, 164 mg). The NaCl concentration in the buffer was changed in a stepwise manner from 0 to 0.1 M.

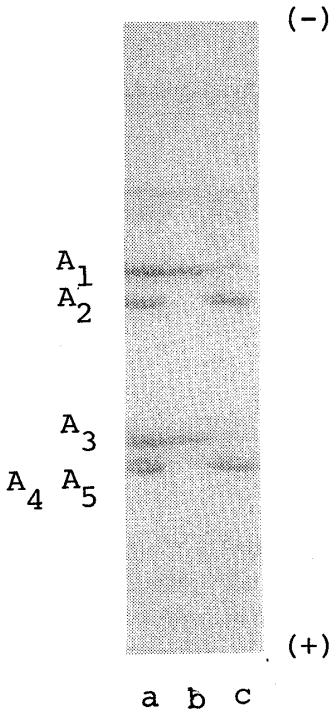

Fig. 6. Electrophoretic Patterns of PR-A and Fractions VIII-1 and VIII-2 under Reducing Conditions

PR-A (a), VIII-1 (b) and VIII-2 (c).

SDS–polyacrylamide gel electrophoresis, the results of the sequence analysis suggested that A_2 is heterogeneous. Only glycine was detected as the N-terminal amino acid for A_3—A_5, and the second and the third amino acids of A_3—A_5 were valine·leucine and glutamic acid,

respectively.

On SDS–polyacrylamide gel electrophoresis without 2-mercaptoethanol, PR-A showed only two major bands, IS-1 and IS-2, which were placed in the higher-molecular-weight region than A_1, A_2, A_3, A_4 and A_5. Consequently these seem to be intermediary subunits. IS-1 (M.W. 65000) and IS-2 (M.W. 59000) were obtained by rechromatography of PR-A on DEAE-Sepharose CL-6B using 0.02 M Tris–HCl buffer (pH 8.0) containing 6 M urea (Fig. 5). On SDS–polyacrylamide gel electrophoresis with 2-mercaptoethanol, IS-1 was separated into A_1 and A_3, and IS-2 into A_2, A_4 and A_5 (Fig. 6). It appears that IS-1 consists of A_1 (acidic, M.W. 45000) and A_3 (basic, M.W. 22000) linked through disulfide bonds, and IS-2 consists of A_2 (acidic, M.W. 37500) and A_4 (basic, M.W. 20000), or A_2 (acidic, M.W. 37500) and A_5 (basic, M.W. 19000). It is assumed that PR-A consists of five or six intermediary subunits on the basis of the molecular weights of the respective intermediary subunits. Legumin-like storage globulins such as soybean 11S globulin (M.W. 362000)[2] and oat globulin (M.W. 326000—369000)[5] were each composed of six of an intermediary subunit, a pair of the high-molecular-weight acidic polypeptide (subunit) and low-molecular-weight basic polypeptide (subunit). Reduced and unreduced PR-A exhibited patterns similar to those of soybean 11S globulin and oat globulin in terms of the molecular weights of polypeptides on SDS–polyacrylamide gel electrophoresis. It can be considered that PR-A is also composed of intermediary subunits with the structures similar to those of legumin-like globulins.

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References and Notes

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