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Characterization of Storage Proteins Extracted from *Avena sativa* Seed Protein Bodies

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Protein bodies have been isolated from *Avena sativa nuda* (cv. Rhea) caryopses by differential centrifugation. Their protein content has been compared to proteins extracted from the meal. Albumins, globulins, prolamins (avenins), and glutelins have been sequentially extracted and characterized both quantitatively and qualitatively by electrophoresis and amino acid analysis. Avenins have also been compared by ion-exchange chromatography. The isolated protein bodies were free from starch, and the proportion of their proteins was different from that of proteins extracted from meal. The protein body proteins are mainly composed of acetic acid soluble glutelins and globulins. Extracted from protein bodies, the albumins, globulins, and acetic acid soluble glutelins form a continuous family contrary to the meal-extracted corresponding groups. The avenin polypeptide chains are all located within protein bodies.

Oat seed storage proteins (Kim et al., 1978), as compared to other cereals, exhibit some peculiarities. The proportion of avenins (the oat prolamins) is quite low compared to prolamins from other species such as wheat gliadins, barley hordeins, or maize zeins (Mossé, 1966). Avenins are not as characteristic as other prolamins, when compared to other storage protein groups on the amino acid composition and on electrophoretic bases. As for other prolamins, avenins belong to the alcohol-soluble protein group, but all polypeptide chains extracted by ethanol mixtures are not typical prolamins. Their definition needs further characterization by electrophoresis and amino acid analysis (Kim et al., 1978). Consequently, we call avenin (or prolamin) the typical fraction of the alcohol-soluble group and not all the proteins extracted by ethanol mixtures.

The interest of prolamins does not only consist in the fact that they represent one of the major storage protein groups which conditions the nutritional value of the seed but also avenins have been shown to constitute a useful tool for phylogenetic studies of the genus *Avena* (Kim et al., 1979b; Kim and Mossé, 1979).

Storage proteins and especially prolamins are located within typical storage organelles, called protein bodies, which occur in the seed endosperm (Pernollet, 1978). Nevertheless, some polypeptide chains belonging to the albumin and globulin groups may be deposited outside these organelles. Within cereal endosperm, one can usually distinguish two different kinds of protein bodies. In the aleurone layer, the protein bodies, also called aleurone grains, exhibit globoid inclusions containing phytin. In the starchy endosperm no such inclusions (nor as much phytin) can be detected within the protein bodies.

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Few studies have dealt with the characterization of oat protein bodies. Sraon (1972) has evidenced protein bodies within oat starchy endosperm, whereas Pomeranz (1972) has failed in locating protein bodies in this tissue. Buttrose (1978) has used electron dispersive X-ray analysis to characterize the protein bodies in the seed embryo of *Avena sativa*. This technique has widely been developed for seed studies. It is a proper tool to differentiate protein bodies within the starchy endosperm from those located in the aleurone layer of *A. sativa* seed (Pernollet and Mossé, 1980). Moreover, Pernollet and Mossé have shown that protein bodies are still present at maturity in the starchy endosperm of oat (Aveneae), contrary to the case in cereals belonging to the *Triticeae* tribe like wheat, barley, or rye.

The isolation of protein bodies from oat seeds has never been performed, whereas this investigation has already been done in other cereals (Pernollet, 1978). This paper describes the isolation of protein bodies from *A. sativa* caryopses, the cytological location of storage protein groups, and the relationships between these groups.

Differential centrifugation has deliberately been used to isolate protein bodies because it is possible to obtain large amounts of materials, which compensates for the low yield of the homogenization steps. We have preferred a gentle homogenization procedure to prevent protein bodies from alteration.

MATERIALS AND METHODS

Plant Material. The seeds of naked oat (*Avena sativa nuda*, cv. Rhea) were obtained from the INRA Plant Breeding Station of Rennes. Mature dry seeds were used throughout this investigation. The nitrogen content of the meal, obtained by the use of a cutting mill, was 2.39% on a dry weight basis.

Optical Microscopy. A M 20 Wild optical microscope equipped with phase contrast and polarized light measurements was used to follow protein body isolation.

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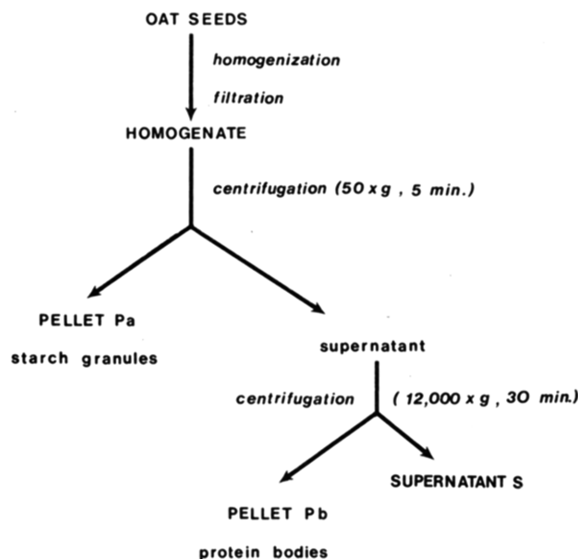

Figure 1. Isolation of protein bodies.

Protein Body Isolation. This procedure is summarized in Figure 1. Oat seeds (50 g) in 500 mL of 0.1 M potassium phosphate buffer (pH 7.2) containing 6 mM $MgCl_2$ (Christianson et al., 1969) were homogenized for 1 min at 4 °C with the aid of a MX 130 National homogenizer at full speed. Cell walls and seed debris were removed by filtering through nylon bolting cloth (Tripette et Renaud; 35-μm average size opening). The homogenate was centrifuged at room temperature for 5 min at 50g in a SS 34 Sorval rotor. The pellet of starch granules (Pa) was stored for further analyses, and the supernatant was centrifuged 30 min at 20 °C and 12000g in the same rotor. The protein bodies were concentrated in a brownish pellet (Pb) which was submitted to microscopical examination prior to analyses; the last supernatant (S) was treated in the same way. In this way 0.8–1 g of protein body dry matter was obtained from 50 g of seeds.

Protein Group Extractions. Prior to protein extraction, the sample (1 g on a dry matter basis) was defatted with water-saturated 1-butanol, and the excess salts were eliminated with distilled water. This last step was omitted when extraction was done from meal. Each extraction has been performed twice, using a volume of solvent equal to 10 times the dry weight of starting material (either protein body pellet or meal). Extractions were performed by 1 h of gentle magnetic stirring at room temperature (except for aqueous extractions which were done at 0–4 °C), followed by 30 min of centrifugation at 3000g in a SS 34 Sorval rotor. Albumins and globulins were first solubilized by 0.5 M NaCl, and globulins were separated from albumins by dialysis against distilled water and centrifugation. Then prolamins were extracted by a binary ethanol–water mixture of 45% (w/w) which corresponds to the highest extractability of avenins (Kim et al., 1978). Lastly, acetic acid soluble glutelins (HAc glutelins = glutelins soluble in 0.1 N acetic acid) were solubilized by 0.1 N acetic acid. At each solvent change the pellet was washed once by distilled water and centrifuged as previously described. All the protein groups were dialyzed against distilled water and freeze-dried.

Analytical Methods. Nitrogen content of fractions was determined by the micro-Kjeldahl method. Starch gel electrophoresis was carried out in 12% starch gel with aluminum lactate buffer (pH 3.2)–3 M urea as described by Landry et al. (1965). Acrylamide gel electrophoresis was performed in 6% acrylamide gels (Kim et al., 1979a) using the same buffer as for starch gel electrophoresis.

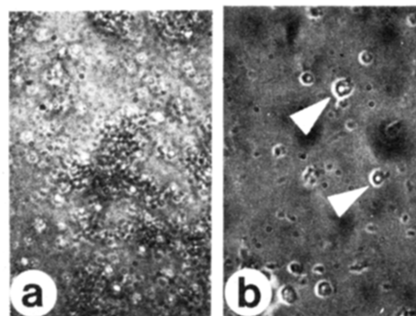

Figure 2. Optical microscopy of the protein body pellet. (a) Magnitude 280×. (b) Magnitude 700×. Arrowheads show protein bodies with inclusion.

Table I. Kjeldahl Nitrogen Distribution within the Subcellular Fractions

fraction	nitrogen, %	protein content[a]
homogenate	100	20.6
starch granule pellet (Pa)	32	7.2
protein body pellet (Pb)	32.5	70.6
supernatant (S)	29.5	57.6

[a] Protein content as 6.25 × N g for 100 g of dry weight.

Proteins were stained in Coomassie blue at 1% in water diluted into 40 volumes of 6% trichloroacetic acid in a mixture of acetic acid, methanol, and water, 7:20:80 (v/v/v). Destaining was done with a mixture of acetic acid, methanol, and water 1:6:14 (v/v/v).

Amino acid analyses, performed on a Phoenix Model K 8000 B amino acid analyzer, and ion-exchange chromatography of avenins on SP Sephadex C-50 are described elsewhere (Kim et al., 1978).

RESULTS AND DISCUSSION

Protein Body Isolation. In our conditions the seed homogenization is not exhaustive but is gentle enough to prevent organelles from disruption. The nitrogen yield is low (22% of meal nitrogen) but starch granules are unbroken, which is essential for good further differential sedimentations.

The protein body pellet is mainly composed of small spherical organelles which do not polarize light and of a few sparse starch granules. When observed by phase contrast, two kinds of protein bodies are revealed: the major part of them is homogeneous while some show spherical inclusions (Figure 2). Scanning electron microscopy and electron dispersive X-ray analysis of this pellet, exhibiting sulfur, phosphorus, potassium, and magnesium, provide an indirect proof that the protein bodies in Pb originate as well from the aleurone layer as from the starchy endosperm (results not shown). Protein bodies isolated by differential centrifugation are a mixture of organelles from starchy endosperm and from aleurone cells. Those with spherical inclusions may come from the aleurone layer, as it is for the other cereals (Pernollet, 1978), and the presence of phosphorus, detected by electron dispersive X-ray analysis, is a good specific index for the presence of aleurone protein bodies in the pellet Pb. The supernatant is free from organelles.

Roughly, nitrogen is equally distributed among Pa, Pb, and S (Table I). The protein body pellet is characterized by a higher protein content (>70% of dry weight) compared to the starch pellet (7.2%). The comparison between the amino acid analysis of the protein body pellet and the

Table II. Amino Acid Compositions of Oat Meal, Protein Body Pellet, and Starch Pellet

	no. of residues/1000 total residues		
amino acids	defatted meal	protein body pellet (Pb)	starch pellet (Pa)
Lys	36	34	31
His	18	19	19
Arg	50	57	54
Asx	80	76	78
Thr	40	41	36
Ser	63	58	58
Glx	195	211	228
Pro	62	59	59
Gly	89	73	70
Ala	71	68	65
Cys	36	25	29
Val	65	65	65
Met	15	17	14
Ile	41	45	46
Leu	73	78	78
Tyr	25	28	24
Phe	41	46	46
N yield, %	96.9	92.7	100.6

oat meal is reported in Table II. The difference between these compositions is significative, indicating that the protein content of the protein bodies is not identical with that of the meal: for instance, protein bodies are richer in arginine, glutamic acid, and phenylalanine but poorer in serine, glycine, and cysteine.

On the contrary, the amino acid composition of the starch granule pellet is far less different from the protein body pellet composition. This result indicates that the starch pellet is largely contaminated by protein bodies which adhere to starch granules (Adams and Novellie, 1975; Pernollet and Mossé, 1980). Assuming that all nitrogen found in the starch pellet is coming from protein body contamination, the results from Table I allow us to ascertain that about half of the protein bodies are lost in the first centrifugation step. Reciprocally, during protein hydrochloric acid hydrolysis, the presence of starch gives rise to levulinic acid which reacts with ninhydrin and is therefore detected by the amino acid analyzer (Schram et al., 1953; Hamilton, 1963). A comparison of the levulinic acid peak areas found in Pa and Pb hydrolysates allows us to estimate the contamination of protein body pellet by starch granules to be lower than 3% which is consistent with the optical microscopy observations. Taking into account losses due to homogenization and filtration, one can assume that the pellet Pb represents less than 10% of the protein bodies of the whole seed. This estimation is made either on a nitrogen distribution basis or on the comparison of the avenin levels between meal and protein bodies where all the avenins are located. The yield we obtained is quite comparable with those published in the literature: this technique of protein body isolation has already been mentioned as not exhaustive (Thomson et al., 1978; Rao and Pernollet, 1981); nevertheless the purity of the protein body pellet is fair.

Distribution of Protein Groups. The nitrogen repartition among extracted nitrogen groups is compared in Table III between the meal, the protein bodies (Pb), and the supernatant (S). The protein body pellet is comparatively rich in water-saturated 1-butanol-soluble nitrogen, which is compatible with the presence of liposoluble proteins coming from the protein body membrane. Nonprotein nitrogen (NPN) is high in the supernatant and low in the protein body pellet. The typical storage proteins of protein bodies appear to be HAc glutelins, residual glutelins, and prolamins. As we shall see further, alcohol-soluble proteins extracted from the supernatant are very poor in typical prolamins. Prolamins and HAc glutelins found in this fraction are likely to be due to contamination (non centrifuged protein bodies or disrupted organelles). At first view, the avenin proportions seem to be identical whatever their origin (meal or protein bodies) may be. This can be explained by the increase of the HAc glutelin proportion which is counterbalanced by the attendant decrease of the nonprotein nitrogen proportion. We must not forget that we have extracted proteins from a heterogeneous mixture of organelles which differ in their polypeptide chain content (Pernollet, 1978): the lack of degradation of aleurone cell protein bodies by the ethanol–water mixture (Pernollet and Mossé, 1980) is another indication that prolamins are only located within starchy endosperm protein bodies and, therefore, it is likely that starchy endosperm protein bodies have a higher avenin content than the amount determined in the pellet Pb.

Table III. Distribution of Nitrogen among Protein Groups Obtained by the Micro-Kjeldahl Method

		N, %		
extraction steps	protein groups	meal	protein bodies (Pb)	supernatant (S)
water-saturated 1-butanol, defatting		1.4	4.7	2.5
trichloroacetic acid supernatant	NPN	11.7	0.6	15.5
salt elimination	albumins and globulins		3.3	
water and saline extraction	albumins and globulins	9.7	4.6	35.9
ethanol extraction	alcohol-soluble proteins	10.8	10.1	1.2
0.1 N acetic acid extraction	HAc glutelins	20.1	46.9	4.1
residue	residual glutelins	46.0	30.0	40.7

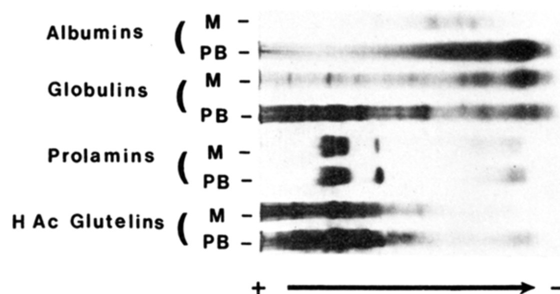

Figure 3. Polyacrylamide gel electrophoresis of protein groups from isolated protein bodies (PB) and from meal (M). Medium for electrophoresis: aluminum lactate–3 M urea pH 3.2 buffer.

Qualitative Data on Albumins, Globulins, and Acetic Acid Soluble Glutelins. Electrophoretic patterns of protein groups obtained at acidic pH in 3 M urea are presented in Figure 3. Albumins extracted from protein bodies are qualitatively different from the meal-extracted ones, but they seem to have common constituents with meal globulins when comparing the fast-moving bands. Protein body globulins are composed of slower moving bands than meal globulins, and these bands are partly similar to those of protein body HAc glutelins. Meal and protein body HAc glutelins show almost identical patterns except in the fast-moving bands. The slowest and the fastest electrophoretic bands of the protein body globulins are respectively common to the HAc glutelins and the albumins extracted from these organelles. This continuity is supported by the amino acid analysis (Table IV). The

Table IV. Amino Acid Compositions of Protein Groups Extracted from Meal and Protein Bodies

	no. of residues/1000 total residues				
amino acids	meal globulins	protein body globulins	meal HAc glutelins	protein body prolamins	meal prolamins
Lys	43	34	29	10	10
His	20	21	22	8	9
Arg	68	85	62	27	28
Asx	78	71	95	21	21
Thr	45	35	39	18	17
Ser	58	64	66	28	27
Glx	174	209	202	373	363
Pro	56	56	53	101	104
Gly	84	82	76	18	19
Ala	69	68	65	57	55
Cys	38	27	17	36	38
Val	59	61	58	78	77
Met	28	14	11	15	20
Ile	43	40	48	31	34
Leu	74	71	74	106	108
Tyr	29	26	32	17	17
Phe	35	37	53	56	55
N yield, %	95	104	101	101	95

composition of protein body extracted globulins is roughly intermediary betwen meal globulins and meal HAc glutelins ones, although some amino acids are significantly found out of the range: Arg is 20% higher, Asx 9% lower, and Ile 7% lower than in meal globulins and Thr 10% lower than in meal HAc glutelins. Nevertheless, these amino acids represent less than 23% of the number of residues and are not very far from the meal composition.

The greatest difference between meal and protein body protein groups lies in the continuity of albumins, globulins, and HAc glutelins. When extracted from protein bodies, these proteins share in common numerous electrophoretic bands, whereas the meal-extracted protein groups are more clearly individualized. The extractability of globulins and HAc glutelins has been discussed in the literature, some authors finding that globulins are the major protein group of oat meal (Wu et al., 1972; Peterson and Smith, 1976) while others claimed glutelins are the main storage group (Osborne, 1924; Csonka, 1941; Ewart, 1978; Draper, 1973). Kim (1978) has shown that the extractability of globulins and HAc glutelins depends drastically on salt concentration. It is likely that globulins and HAc glutelins extracted from protein bodies are closely related to each others and that the increase of salt concentration makes higher the solubilization of protein body HAc glutelins as globulins.

Comparison of Avenins Extracted from Meal and Protein Bodies. Avenins extracted from protein bodies are devoid of pigments, whereas meal avenins exhibit a yellow brownish color. Table IV gives the amino acid composition of the two batches of avenins and ascertains the identity of the proteins obtained from the two extracts. Figure 4 exhibits ion-exchange chromatographic elution profiles of the two batches of avenins. Electrophoretic patterns of the fractions (figure not presented) show that peaks III–V correspond to the avenin fractions obtained by Kim et al. (1978). The difference between the two batches in the peaks I and II corresponds to glutelin contaminants. Protein body crude avenins, devoid of pigments and of glutelins, are therefore purer than meal prolamins.

Electrophoretic patterns of avenins from meal and protein bodies are rigorously identical whatever kind of gel (starch or acrylamide) is used. Only acrylamide electrophoretic patterns are shown in Figure 5. With a supernatant protein deposit 3 times greater than that for other samples, only some faint typical avenin bands can be seen as compared to important rapid trailings, which are not typical avenins. Avenins are therefore only located within protein bodies.

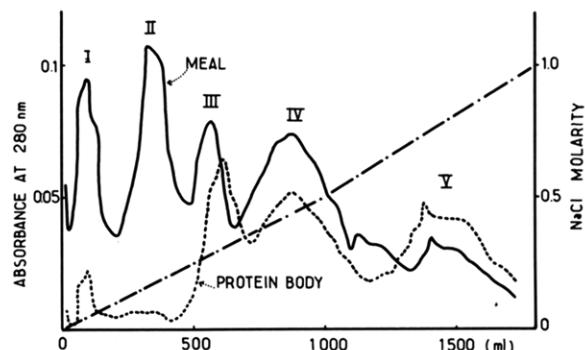

Figure 4. Ion-exchange chromatography of avenins on SP Sephadex C-50: elution patterns of alcohol-soluble proteins extracted from isolated protein bodies (---) and from meal (—). (-·-) NaCl gradient.

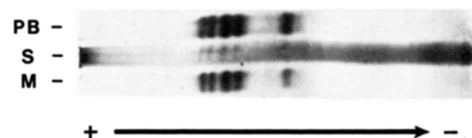

Figure 5. Polyacrylamide gel electrophoresis of ethanol-soluble proteins extracted from protein bodies (PB), meal (M), and the supernatant (S). For the supernatant the protein sample was 3 times more concentrated than for meal or protein body prolamins. Medium for electrophoresis: aluminum lactate–3 M urea pH 3.2 buffer.

CONCLUSION

One may consider that protein bodies are mainly composed of two protein groups: the avenins and the globulin–HAc–glutelin group which represent more than the two-thirds of the storage proteins. Insoluble residual glutelins are the major constituent of the last third. Protein groups extracted from protein bodies are purer than those extracted from meal: freeze-dried samples are devoid of pigments, their solubilization is easier, and, as it has been shown by chromatography, avenins are far less contaminated by other proteins.

Whereas the oat protein bodies' and protein groups' degradations have already been studied during the germinative processes (Kim et al., 1979a), the question of the biosynthesis of these proteins during the seed development is still open: do avenins and globulins–glutelins undergo the same mechanism and what is the part of posttraductional modifications (first suggested by Burr et al. (1978) for maize zein) in the heterogeneity of these storage proteins, not only for avenins but also for the other groups? The answer to these questions and the knowledge of the precise distribution of proteins within aleurone or starchy endosperm protein bodies require further purification of these organelles, but the lack of biological characterization of the seed storage proteins implies large-scale isolation of pure organelles which is uneasy to obtain by techniques other than differential centrifugation.

ACKNOWLEDGMENT

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Isolation and Characterization of Two Cryoproteins from Florunner Peanut (*Arachis hypogaea* L.) Seed

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Two cryoproteins (CP I and CP II) from the peanut (*Arachis hypogaea* L.) seed were isolated following the fractionation of a 2 M NaCl, 0.01 M Tris-HCl (pH 7.5), and 0.002% sodium azide protein extract on a Sephacryl S-300 column. The cryoproteins I and II had molecular weights of 500 000 and 380 000 ± 20 000, respectively. The protein composition of the cryoproteins was found to be different after ion-exchange chromatography and gel electrophoresis. Addition of 2-mercaptoethanol did not alter the molecular weights and electrophoretic patterns of the cryoproteins. Amino acid compositions of the cryoproteins I and II were similar. The critical temperature for cryoprecipitation was between 2 and 15 °C. Cryoprecipitation increased with increasing protein concentration and reached a maximum at 40 mg/mL. Cryoprotein I showed higher cryoprecipitation with lower protein concentration compared to the cryoprotein II. Maxium cryoprecipitation was obtained around pH 7.5 and at an ionic concentration of 0.4 M (CP I) and 0.2 M (CP II). Limited protease treatment, although it destroyed the cryoprecipitation property, did not cause a major change in the molecular weights of the cryoproteins.

Cooling of certain protein solutions brings about the precipitation of specific proteins. Such precipitation phenomenon is known as "cryoprecipitation" and is thermally reversible. The occurrence of such phenomenon in seed extracts of certain species is known (Ghetie and Buzila, 1962). Daussant et al. (1969) have suggested that in peanut seed the cryoprecipitation property occurs almost exclusively for α-arachin. Neucere (1969) has employed this property for purification of α-arachin. Although peanut proteins are known to possess the cryoprecipitation property, very little information is available on the process of cryoprecipitation, the number of proteins involved, and the factors affecting the cryoprecipitation of peanut proteins. Hence, a study was initiated to isolate the peanut cryoproteins and to determine their characteristics and the factors influencing the cryoprecipitation property. This paper describes isolation of two cryoproteins from peanut seed and examines various factors affecting the cryoprecipitation property.

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MATERIALS AND METHODS

Seed Material. Seeds of cv. Florunner are a gift from Dr. A. J. Norden of the University of Florida. After removal of the seed coats and embryonic axes, the cotyledons were ground into a meal and defatted with diethyl ether. Defatted meal was stored at −20 °C.

Protein Extraction. Protein from defatted peanut meal (3 g) was extracted with 10 mL of 2 M NaCl, 10 mM Tris-HCl buffer (7.5), 0.002% (w/v) sodium azide (NaN_3), and 2 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 20000*g* for 20 min at 20 °C. The clear supernatant was used for further analysis.

Separation of Proteins. An aliquot (8 mL) of the seed protein extract was placed on a Sephacryl S-300 column (125 × 2.5 cm) equilibrated at room temperature with 0.5

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