CHEMISTRY AND ULTRASTRUCTURE OF A MAJOR ALEURONE PROTEIN OF RAPESEED MEAL

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ABSTRACT

A major storage protein extracted from commercial rapeseed meal (*Brassica campestris* L. var. Span) was characterized both chemically and ultrastructurally. The 12S glycoprotein was found to contain 12.9% (w/w) carbohydrate consisting of arabinose, galactose, glucose, inositol, glucosamine, and mannose. The rapeseed aleurone grains contain globoid bodies which suggest the presence of phytic acid. This observation may be related to the presence of inositol in the 12S oligomer. It may be that the sugars are added to the protein backbone some time after protein synthesis or perhaps could be complexed with the protein by way of a Maillard condensation reaction in the meal during lipid removal. It is also possible that the differences observed between the carbohydrate contents of the 12S protein recovered from commercial meal and that obtained from seed could have resulted from nonenzymatic browning reactions. The amino acid profile of the 12S globulin was dominated by the acidic amino acids, glutamic and aspartic, which is typical for an oilseed aleurin. There was a scarcity of the sulfur-containing amino acids 1/2 cystine and methionine. Tryptophan was not detected from a *p*-toluenesulfonic acid hydrolyzate of protein. The protein aggregate was found to be morula-like with a maximum particle diameter of 120 Å as determined from electronmicrographs of negatively stained specimens.

Rapeseed is now established as Canada's third most valuable grain crop following wheat and barley (1). Although primarily used as a source of vegetable oil, interest has been shown in its protein as a potential source for human nutrition. Physical and chemical characterization of individual proteins are a prerequisite to effective utilization. Many of the early rapeseed protein studies were concerned with taxonomic differences, utilizing whole seeds as a source for protein recovery. Bhatty *et al.* (2) extracted the proteins of rapeseed with 0.01 *M* sodium pyrophosphate (pH 7.0) and isolated two major fractions, a neutral high molecular weight protein and a basic 1.7S fraction with a molecular weight of 13,800. The larger protein possessed an observed sedimentation coefficient of 12S in 0.1 *M* borate buffer (pH 8.6). The 12S protein was also recovered in a 10% NaCl extract of rapeseed and represented 21% of the nitrogen recovered. Amino acid profiles, sedimentation characteristics and electrophoretic behavior of the 12S proteins of different species and varieties of rapeseed were reported by Finlayson *et al.* (3). Further characterization of the 12S rapeseed glycoprotein from whole seeds was reported by Goding *et al.* (4).

Since rapeseed will likely always be cultivated primarily as a source of oil with secondary protein production utilizing the inexpensive defatted meal by-product, characterization of the 12S protein recovered from this highly denatured source is most relevant. The subcellular localization of glycoprotein (5) as well as the electrophoretic, ultrastructural, and rheological properties of a 12S glycoprotein isolated from commercial rapeseed meal (6) have recently been

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reported. The current study was undertaken to further elucidate the nature of the 12S protein of rapeseed meal.

EXPERIMENTAL

The 12S protein was extracted in 0.1 *M* borate buffer pH 9.2 and subjected to gel filtration in the same buffer on Sephadex G-100. The details of the procedure have been previously described by Gill and Tung (6). The 12S protein was collected, lyophilized, and identified by sedimentation velocity ultracentrifugation in the extraction buffer at 20°C.

Amino acid analyses were carried out as follows. Samples of the freeze-dried isolate were derivatized with 4-vinylpyridine (7). It has been found that the S-β-(4-pyridylethyl)-L-cysteine formed from the selective alkylation of cysteine and reduced cystine is stable under conditions of acid hydrolysis. The reduced alkylated rapeseed protein was hydrolyzed by the method of Liu and Chang (8) utilizing 3*N* *p*-toluenesulfonic acid as a means of protecting tryptophan from hydrolytic destruction. Both samples and standards including pyridylethyl cysteine were analyzed on a Hitachi Model KLA-3B amino acid analyzer utilizing a single column elution system.

Hydrolysis of the protein and analysis of the liberated neutral and amino sugars were carried out using a modification of the procedure described by Porter (9). The method enabled the simultaneous gas chromatographic determination of the sugars as their alditol acetates. The monosaccharides were liberated from the protein in the presence of a cation exchange resin and the amino sugars released from the resin through a nitrous acid deamination reaction. Gas-liquid chromatography was carried out on a Tracor MT220 gas chromatograph equipped with 6.4 mm × 183 cm dual silanized glass columns packed with 3% ECNSS-M on 100/120-mesh Gas Chrom Q (Applied Science Laboratories) (10). The injection port temperature was 230°C and the flame ionization detector was 280°C. Oven temperature was programmed from 150 to 190°C at 1 C° min^{-1}. The nitrogen carrier gas was adjusted to 40 ml min^{-1} and all samples were applied via on-column injection. Alditol acetate derivatives were identified by retention times as compared to standards prepared from reagent grade sugars. An examination of a wide range of all common neutral and amino sugars indicated that xylose and glucosamine possessed identical retention times although this was the only coincidence observed.

An independent procedure for the quantitation of glucosamine (11) was used to detect this sugar. Hydrolysis of the glycoprotein was carried out as described for gas chromatography and separation of the amino sugars from the neutral sugars was carried out according to the ion exchange method of Boas (12). Sialic acid determinations were carried out on hydrolyzed samples by the method of Warren (13). The rapid micro Kjeldahl method of Concon and Soltess (14) (%*N* × 6.25) was used for protein determinations.

In order to investigate the molecular structure of the 12S glycoprotein more fully, ultrastructural examination of the aggregate was carried out on negatively stained specimens. Copper grids (400-mesh) were washed thoroughly with acetone, dried, and dropped onto a floating layer of collodion. Filter paper was used to pick up the collodion film which supported the grids. After air drying, the collodion-coated grids were placed in a Balzers Micro BA3 high vacuum coating

unit and coated with carbon. Immediately before use, the collodion was removed from the grids with a 1.5-min dip in acetone. A drop of protein solution (approximately 0.001% w/v in 0.1*M* borate buffer pH 9.2) was placed on each grid and the excess removed after 4.5 min by touching the edge of the grid with a torn piece of filter paper. A drop of 0.5% aqueous uranyl acetate (freshly dissolved) was placed on each grid for 2.5 min and the excess liquid removed as before. After air drying, the negatively-stained grids were examined on a Zeiss 10 transmission electron microscope with an accelerating voltage of 60 kV and images recorded at magnifications of 40,000–60,000 diameters.

RESULTS AND DISCUSSION

The amino acid profile for the 12S protein isolated from commercial meal *(Brassica campestris* L. var. Span) is tabulated in Table I. The maximum nitrogen recovery was 94% for 5 samples which were hydrolyzed in *p*-toluenesulfonic acid for periods of 24–36 hr. The highest recovery was observed at 27-hr hydrolysis. Results obtained for the commercial meal compared favorably with results published for the 12S proteins isolated from *B. campestris* L. var. Echo (3) and the related species *B. juncea* L. var. Coss (15) which is regarded as being derived from the hybridization of *B. nigra* and *B. campestris*. The 12S protein would appear to be a typical oilseed aleurin (16) in that relatively high levels of glutamic acid, aspartic acid, and arginine are present. The amino acid composition of the aleurins is often biased toward the more readily utilizable

TABLE I
Amino Acid Composition of the 12S Glycoprotein from Rapeseed Meal (*B. campestris* L. var. Span)

Amino Acid	g Residue per 16g *N* recovered	mM per 16g *N* recovered
Aspartic acid	9.56	83.0
Threonine	3.82	37.7
Serine	4.91	56.4
Glutamic acid	20.9	162
Proline	4.20	43.3
Glycine	4.65	81.4
Alanine	3.87	54.4
Valine	3.86	38.9
Methionine	1.88	14.3
Isoleucine	3.60	31.8
Leucine	7.42	65.6
Tyrosine	3.13	19.2
Phenylalanine	4.93	33.5
Ammonia	2.68	158
Lysine	2.96	23.1
Histidine	2.87	20.9
Tryptophan	0	0
Arginine	6.03	38.6
Pyridylethyl-L-cystine	Trace	Trace
Total	91.3	
Total recovery (%*N*)	94	

amino acids since they are considered to have a storage function supplying the seedling with organic nitrogen rather than structural or catalytic functions. Other notable features of the 12S amino acid profile are the absence of tryptophan and the scarcity of 1/2 cystine and methionine.

Finlayson *et al.* (3) reported differences in the amino acid compositions of the 12S proteins isolated from different species and varieties of rapeseed. There were considerable differences in cystine and methionine contents noted between species, and cultivars within the same species. It was concluded that because obvious differences did occur in structurally important amino acids, the 12S proteins from the different species probably had different structures although similar sedimentation coefficients were observed. Although the method of recovery was similar to that reported by Bhatty *et al.* (2), the 12S isolate in the second study contained almost 2% more nitrogen than in the first study. No apparent reason for this phenomenon was given. The protein in the present study contained substantially less nitrogen, the lyophilized isolate containing 12.5% nitrogen while a value of 13.4% was obtained for an oven dried sample.

The gas chromatographic analysis of both neutral and amino sugars indicated the presence of 6 components. A typical gas chromatogram of the monosaccharide derivatives (alditol acetates) is illustrated in Fig. 1. The peaks identified by retention times as compared to those of authentic sugars were (a) arabinose, (c) mannose, (d) galactose, (e) glucose, and (f) inositol. A peak (b), emerging shortly after arabinose, was found to have a retention time equal to that of both xylose and glucosamine. The determination of glucosamine was therefore carried out colorimetrically by the Elson-Morgan reaction (11). Results of the sugar analyses are tabulated for the freeze dried isolate in Table II

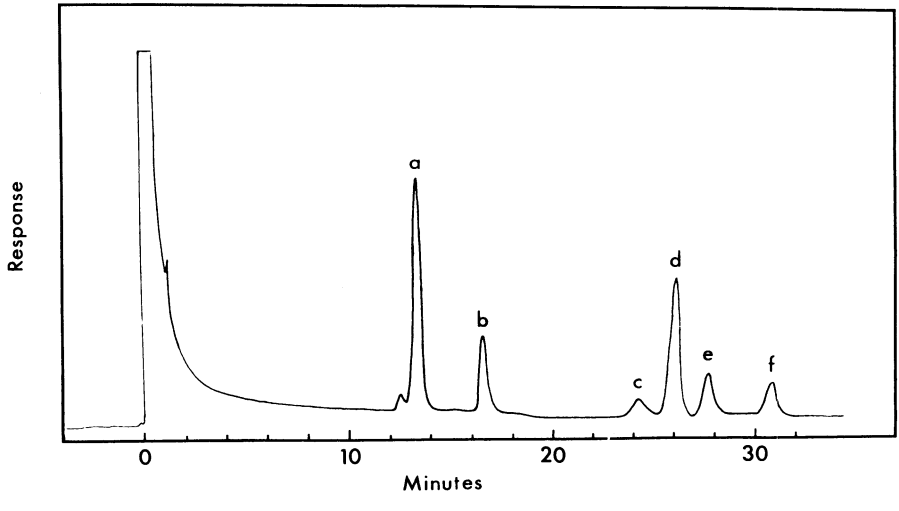

Fig. 1. A typical gas chromatogram of the alditol acetate derivatives of the neutral and amino sugars in the 12S glycoprotein. (a) arabinose, (b) unidentified, (c) mannose, (d) galactose, (e) glucose, (f) inositol.

along with amino acid content (% w/w), assuming 100% recovery. A calculation of theoretical partial specific volume (Vp) (17) utilizing monosaccharide partial specific volumes (18) is included in Table II. The total sugar content of the 12S glycoprotein was found to be 12.9% while the remaining 87.1% was presumed to be amino acids. This figure would appear reasonable since the lyophilized isolate contained only 12.5% nitrogen.

Comparison of results from this study with those of previous rapeseed protein reports suggests large varietal differences, or differences between seed and meal. Although amino acid profiles are similar, the carbohydrate and nitrogen contents differ considerably from those reported by Goding *et al.* (4) for the 12S protein isolated from the seeds of *B. campestris* L. var. Echo and *B. napus* L. var. Target. Instead, the 12S protein isolated from commercial meal more closely resembles "fraction A" isolated from *B. nigra, B. juncea,* and *B. hirta* by

TABLE II
Monosaccharide[a] and Amino Acid[b] Composition of the 12S Glycoprotein from Rapeseed Meal (*B. campestris* L. var. Span)

Component	% (w/w)	Partial Specific Volume (Vi)	% (w/w) × Vi
Arabinose	7.24 ± 0.16	0.613	4.44
Glucosamine	0.29 ± 0.042	0.666	0.193
Mannose	0.340 ± 0.030	0.613	0.208
Galactose	3.32 ± 0.12	0.613	2.04
Glucose	0.870 ± 0.019	0.613	0.533
Inositol	0.840 ± 0.077	0.613	0.515
Sialic acid	0		
Aspartic acid	9.40	0.59	5.55
Threonine	3.75	0.70	2.63
Serine	4.83	0.63	3.04
Glutamic acid	20.6	0.66	13.6
Proline	4.13	0.76	3.14
Glycine	4.57	0.64	2.92
Alanine	3.80	0.74	2.81
Valine	3.79	0.86	3.26
Methionine	1.85	0.75	1.39
Isoleucine	3.54	0.90	3.19
Leucine	7.29	0.90	6.56
Tyrosine	3.08	0.71	2.19
Phenylalanine	4.85	0.77	3.73
Lysine	2.91	0.82	2.39
Histidine	2.82	0.67	1.89
Tryptophan	0		
Arginine	5.93	0.70	4.15
Pyridylethyl-L-cystine	Trace		

Theoretical partial specific volume (17), $Vp = \frac{\Sigma\ \%\ (w/w) \times Vi}{\Sigma\ \%\ (w/w)} = 0.704$

[a]All monosaccharide data except glucosamine reported as a mean of 4 determinations ± standard error of the mean. Glucosamine reported as a mean of 6 determinations ± standard error of the mean.

[b]Amino acid values calculated from those of Table I assuming 100% recovery.

MacKenzie and Blakely (15) and from *B. juncea* by MacKenzie (19). The nitrogen content of this fraction was reported to be 12.7, 13.7, and 14.9% for *B. nigra, B. juncea,* and *B. hirta,* respectively. The differences in carbohydrate content of the 12S aggregates could also be explained by the effects of commercial fat extraction. It has been reported that the content of reducing sugars and available lysine in commercial meal drops appreciably due to the high temperatures employed (20) during fat extraction. This phenomenon was attributed to nonenzymatic browning. However, if the 12S aggregate in the present study were being extensively modified through nonenzymatic browning of the meal, such effects would be expected to be reflected in the sedimentation characteristics and in the homogeneity of the preparation. The criteria for homogeneity will be examined in a later report.

The two most notable features of the carbohydrate composition are the relatively large amounts of arabinose and the presence of inositol. Although there is a lack of detailed information on carbohydrate components of rapeseed, Appelqvist (21) has cited the presence of sugars in aqueous extracts of white mustard. The sugars of white mustard were predominantly arabinose, galactose, and glucuronic acid and were discovered in both hot and cold water-soluble fractions. The presence of inositol may be related to the presence of a large number of globoid bodies found in the rapeseed aleurones (5). A noteworthy binding phenomenon has been described (22) between phytic acid (inositol hexaorthophosphate) and glycinin, the major soybean globulin. The authors proposed that the association was mainly due to electrostatic attraction between

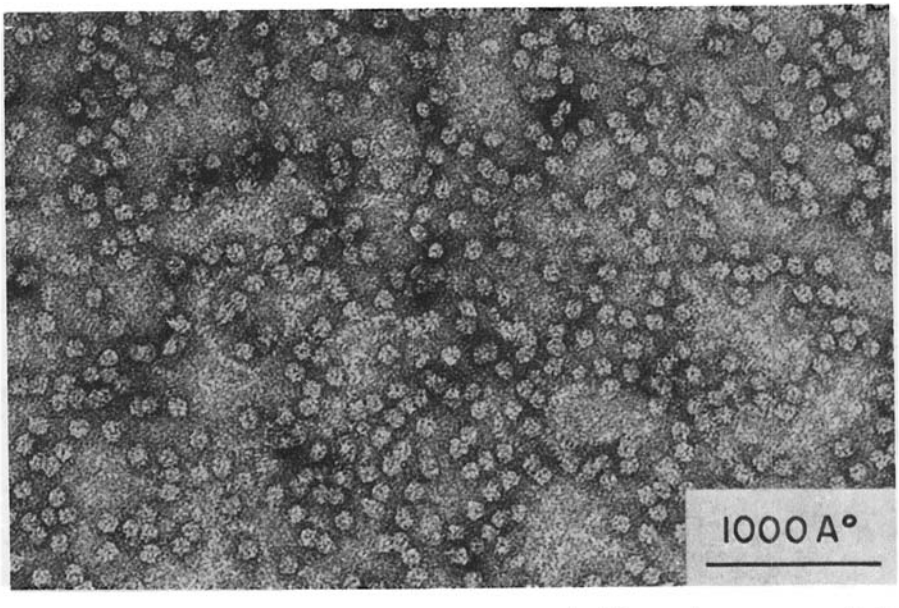

Fig. 2. Electron micrograph of 12S rapeseed glycoprotein. The specimen was negatively stained and supported on a carbon film.

the protonated basic residues of glycinin below its isoelectric point and the anionic phosphate groups of phytate. This, however, would not be likely to occur to any appreciable extent in the case of the 12S rapeseed protein since extraction and purification were carried out at pH 9.2; however, this does not rule out other forms of molecular interaction or the possibility of *in vivo* incorporation of inositol into the molecule following amino acid synthesis.

No sialic acid could be detected in the isolate by the method of Warren (13).

Since very little detail of molecular structure could be obtained from the electron microscopy of positively-stained sections of the 12S rapeseed protein (6), the ultrastructural examination of the unfixed, negatively stained molecular aggregate was undertaken. Figure 2 illustrates a field of view in which several hundred of the 12S particles are distributed on a carbon film while Fig. 3 demonstrates a much higher magnification of the 12S material. The aggregate would appear to be a much more complicated structure than the 11S protein of soybean which has been examined with a similar technique by Badley *et al.* (23). The soybean globulin is an oligomer composed of 12 subunits packed into two identical hexagons placed one upon the other with a maximum particle diameter of 110Å. The rapeseed glycoprotein would appear to be a morula-like structure composed of more than 12 subunits and much more irregularly shaped although more spherical than the soybean protein. The 12S protein has a maximum diameter of 120Å and is more or less spherical. It is of interest to note that although the 12S subunits have not been chemically characterized, the recent work of MacKenzie (19) indicated the presence of at least 11 distinct fractions

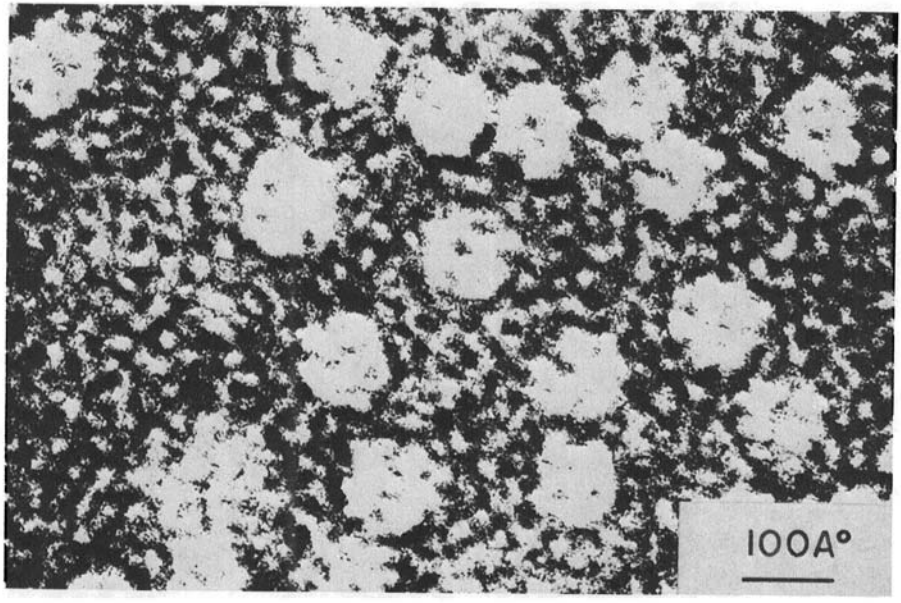

Fig. 3. A higher magnification of the 12S glycoprotein aggregate. Maximum particle diameter ≃ 120Å.

derived from isoelectric focusing experiments on the 12S globulin prepared from *B. juncea*. Catsimpoolas *et al.* (24) have shown that glycinin is composed of only 6 chemically distinct subunits by a similar procedure. Thus, it would not be surprising to find the 12S glycoprotein more chemically heterogenous than the 11S soybean globulin. However, no attempt has been made to study subunit structure in the present investigation.

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