Fractionation of Sunflower Seed Proteins

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ABSTRACT

Fractionation of sunflower seed salt-soluble proteins, which amount to nearly 80% of the total seed nitrogen, has been performed by a method we proposed in 1970 and which was confirmed by several others. Three varieties of seeds have been investigated: 'Armavirec,' 'Peredovik,' and a pure strain. The occurrence of three groups of proteic fractions was confirmed. Their proportions, which fluctuate with varieties, are roughly: 20% for "light" (low molecular weight) albumins, 5-10% for "heavy albumins," and 70-80% for globulins. The first group was isolated by Sephadex G-50 chromatography from the other two, which were separated by dialysis. A second chromatography of these three groups on Sephadex G-200 has been realized (with preliminarily calibrated columns for molecular weight evaluations). "Light" albumins appear as a rather homogeneous constituent with a molecular weight of 14,000 and an aminoacid composition showing high amounts of methionine, cystine, arginine and glutamine. "Heavy" albumins, which are still mixed with globulin fractions after dialysis, have a molecular weight of 48,000 and a very different aminoacid composition with a high level of lysine. Globulins are composed of at least four different fractions, two of which (M = 12,000 and M = 25,000) are presumably subunits of the other two and have significantly different aminoacid compositions.

INTRODUCTION

Sunflower culture, yield, production, and utilization are increasing regularly. In the world, during the last 20 years, harvested areas have been extended more than 50%, mean yield has been increased from 700 to 1200 kg per hectare (and it can still be improved to considerably higher levels), and production has increased from 5 to 12 million metric tons, so that it ranks fourth in importance as an oilseed after soybean, cottonseed, and peanut (1) and second as a source of vegetable oil.

Its oil cake is rather rich in protein (45-50%) of which the essential aminoacid ratios are higher than those of cereal proteins. In contrast to the major oilseeds, its kernel does not contain any toxic substance (2) so that it can be eaten (as it is by people of several countries) without any cooking or other processing.

In spite of its interest as a source of protein for food (3), and even though sunflower oil cake can be extruded and kernel protein isolates can be spun (4,5), sunflower kernel proteins have been the subject of such a small number of studies that a quick, almost exhaustive review of them is rather easy.

The pioneer work of Osborne and Campbell (6) was followed by studies concerning sedimentation velocity (7), isoelectric points (8), and partial aminoacid composition (9). It was only in 1970 that Gheyasuddin et al. (10,11) began the study of dissolving sunflower proteins. At the same time, we proposed a laboratory method for extraction of the whole seed proteins (98% of total nitrogen) in perfectly clear solution (12). We demonstrated that it is possible, by a preliminary G-50 Sephadex gel chromatography of the soluble protein extract, to remove chlorogenic acid and prepare practically pure, chlorogenic-free protein samples. Chlorogenic acid is indeed important in sunflower kernels (13-16), and its phenolic or carboxylic functions readily bind with proteins.

This way of eliminating chlorogenic acid was confirmed by Schwenke et al. (17), who used Sephadex G-25 instead of G-50. In the same work (12), we also showed that this Sephadex G-50 gel chromatography of salt-soluble extracts not only removed chlorogenic acid but also gave two protein peaks; the first one containing a mixture of globulins and albumins, named "heavy albumins" (HA), and the second containing low molecular weight albumins, named "light albumins" (LA). Further chromatography with G-100 or G-200 Sephadex columns allowed fractionation of globulins and heavy albumins, among which some had widely differing aminoacid compositions (18).

These results were confirmed by Sabir et al. (19) who did not remove chlorogenic acid as a preliminary, but used a reducing agent. By a similar method (Sephadex G-200 gel chromatography), Schwenke et al. (20) confirmed the existence of a 11-S globulin fraction that they further succeeded in fractionating into 2-S and 3-S components (21).

From a technological point of view, Baudet et al. (22) detected a significant rate of lysinoalanine formation between cysteine and lysine of sunflower proteins, under certain drastic conditions of pH and temperature. Such "isopeptide crosslinks," as they were named by Asquith et al. (23) were confirmed in sunflower protein isolated by Provansal et al. (24). The disadvantages of lysinoalanine in food were underlined by Sternberg et al. (25) and Mossé (26).

Interactions of chlorogenic acid with proteins and its elimination from proteic extracts were studied by Cater et al. (27), Sosulski et al. (28,29), and Sosulski (30) as well as by alkaline extraction of proteins from seed or from oilmeal (31-36).

The present communication deals with confirmation of the occurrence of three main groups of proteins in sunflower kernel: globulins, heavy albumins, and light albumins; with the fluctuation of their amounts, heterogeneity, aminoacid composition, and fractionation starting with three different sunflower varieties; finally with chromatographic fractionations of these three protein groups into different, well-separated fractions, some of which appear to be subunits of the others.

MATERIALS AND METHODS

Three varieties of sunflower were used in this study: two common cultivars, 'Peredovik' and 'Armavirec,' and one genetically well defined "line" used in a breeding program ($\delta\, y_2$) we named y_2 line for convenience.

Fully ripened seeds of each variety were manually dehulled; the husks were discarded and the kernels ground then defatted by Soxhlet extraction with petroleum ether for 16 hr.

Globulin and Albumin Preparation

The protein preparations were performed by the previously published method (12), with some modifications. The extraction and fractionation procedure is summarized in Figure 1. Lipid-free meal (10 g) was suspended in 0.02 M (pH 8.6) borate buffered 10% NaCl solution (100 ml). The suspension was stirred for 20 min at room temperature and then centrifuged (about 5,000 g). The sediment resuspended (50 ml) for a second extraction. After centrifugation, the two extracts were combined.

The NaCl extract was applied to a Sephadex G-50 column (8 x 85 cm). Elution with a flow rate of 72 ml/hr was made by the same NaCl buffered solution. Fractions of 18 ml were collected (Fig. 2). Four distinct peaks were obtained: the first two being proteic, the last two containing small molecules among which was chlorogenic acid.

As shown earlier (12), the second peak contained only proteins remaining in solution after dialysis, having a particular aminoacid composition and low molecular weight (suggested by their slow migration on the G 50 column). For this reason, they were designated as "light albumins." After dialysis against deionized water for 6-8 days at 5 C, the pooled fractions corresponding to this peak were freeze-dried.

The eluted fractions corresponding to the first peak were pooled and dialyzed against deionized water at 5 C for 6-8 days. After centrifugation of the contents of the dialysis bags, the sediment ("globulins") and the clear supernatant ("heavy albumins") were freeze-dried.

Fractionation on Sephadex G-200 and Molecular Weight Evaluations

A 3 x 58 cm column was used, with 0.05 M SDS, 0.05 M tris, 10^{-4} M parachloromercuribenzoate and HCl buffered at pH 8. Standards for calibration were: cytochrome C (mol wt: 13,800), α-chymotrypsinogen (25,000), ovalbumin (45,000), conalbumin (76,000) and γ-globulin (150,000). Samples of 100 mg were applied to the column and the fractions containing UV-absorbing material were collected (2 tubes 1 hr, with a constant flow rate of 9 ml/hr), dialysed against deionized water for 5 days at 5 C, and freeze-dried.

Protein and Aminoacid Analysis

Protein contents in the eluted fractions from Sephadex gel chromatography were evaluated by absorbance measurements at 280 nm with a Philips Pye Unicam Spectrophotometer. For meals and freeze dried material, it was measured by nitrogen analysis by the semi-micro Kjeldahl method.

For aminoacid determinations, protein samples were hydrolyzed in sealed tubes under nitrogen at 115 C with 6 N HCl for both 24 and 48 hr. Sulfur aminoacids were estimated after oxidation of the sample by performic acid (37). Chromatographic analyses were performed on a Phoenix model K 8800 analyser. Tryptophan was not determined. In one case, ribonucleic acid was removed from the sample before aminoacid analysis, by the perchloracetic acid extraction method of Ogur and Rosen (38).

Results were expressed as numbers of residues of each aminoacid per 1,000 residues recovered. Yields of all analyses (sum of the nitrogen of every aminoacid and ammonia in comparison with Kjeldahl nitrogen of the sample) fluctuated between 95 and 98%.

RESULTS

Although different by their genetic and agronomic characteristics, the three lipid-free meals used in this study were very close in their nitrogen contents (nearly 9%) and their aminoacid compositions were almost identical.

Globulins, HA, and LA of the Three Varieties

Differences can be seen in the aminoacid compositions of the different fractions–globulins, HA and LA separated by Sephadex G-50 chromatography and dialysis–for each of the three varieties.

In Table I are indicated (expressed as residues per 1,000 residues of the whole) first, the aminoacid composition of the meal (as already mentioned, aminoacid compositions of the three meals were identical, differences being smaller than analytical accuracy); then, the aminoacid compositions of the globulin group for each variety, plus the mean values of the three compositions. It can be seen that globulin compositions are practically the same, whatever the variety.

Table II shows that the aminoacid compositions of HA, 'Armavirec' variety (third column), appear somewhat different from 'Peredovik' (first column) with respect to alanine, valine, leucine, cystine, lysine, aspartic acid or asparagine, and mostly glycine.

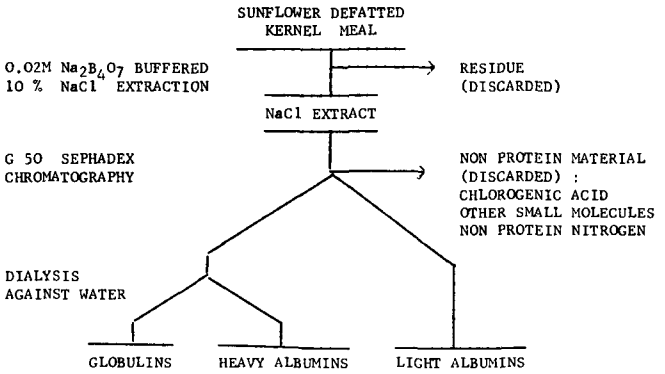

FIG. 1. Flow diagram showing extraction and fractionation of salt soluble sunflower proteins.

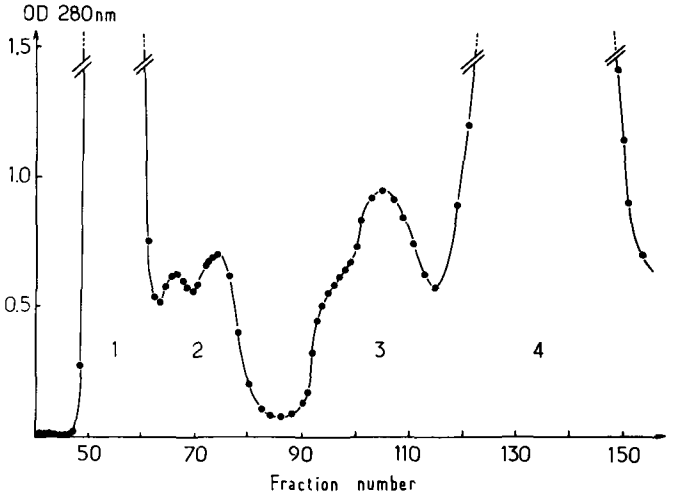

FIG. 2. Elution pattern of G 50 Sephadex chromatography of crude NaCl extract from defatted sunflower kernel flour.

TABLE I

Aminoacid Composition (R °/₀₀) of Defatted Kernels and of Globulin Group of Different Sunflower Varieties[a]

	Defatted kernel	Globulin			
		'Peredovik'	'Armavirec'	'Line y_2'	Mean value
Gly	102	83	87	86	85
Ala	65	69	66	75	70
Val	63	65	68	71	68
Leu	65	71	72	79	74
Ile	45	55	50	52	52
Ser	55	50	53	51	51
Thr	41	41	38	37	39
Tyr	20	24	21	23	23
Phe	37	49	46	53	49
Pro	49	45	49	48	47
Met	20	14	17	14	15
Cys	20	10	14	14	13
Lys	36	29	30	23	27
His	22	23	22	20	22
Arg	68	60	65	57	61
Asx	93	130	104	122	119
Glx	196	181	196	176	184

[a]Expressed as residues per 1000 total residues.

TABLE II

Amino Acid Composition (R °/₀₀) of Heavy Albumin Group of Different Sunflower Varieties[a]

	'Peredovik'	'Peredovik' (RNA free)	'Armavirec'	'Line y_2'	Mean[b] value
Gly	189	100	97	141	113
Ala	54	59	76	63	66
Val	51	59	66	58	61
Leu	49	56	58	56	57
Ile	42	48	45	47	47
Ser	59	56	58	56	57
Thr	35	42	49	37	43
Tyr	20	19	17	18	18
Phe	34	36	33	39	36
Pro	43	45	44	44	44
Met	18	16	19	16	17
Cys	26	18	15	31	21
Lys	34	37	45	33	38
His	20	21	21	20	21
Arg	57	69	55	55	60
Asx	90	95	110	114	106
Glx	182	226	191	173	203

[a]Expressed as residues per 1000 total residues.

[b]Calculated as 1/3 of the sum (Peredovik' RNA free + 'Armavirec' + 'Line y_2').

TABLE III

Amino Acid Composition (R °/₀₀) of Light Albumin Group of Different Sunflower Varieties[a]

	'Peredovik'	'Armavirec'	'Line y_2'	Mean value
Gly	56	64	60	60
Ala	44	39	46	43
Val	43	47	48	46
Leu	67	59	61	62
Ile	42	42	44	43
Ser	40	39	40	40
Thr	33	29	35	32
Tyr	15	15	15	15
Phe	15	20	22	19
Pro	58	60	60	59
Met	44	35	28	36
Cys	70	51	59	60
Lys	34	35	36	35
His	11	13	10	11
Arg	78	86	81	82
Asx	83	72	83	79
Glx	267	292	274	278

[a]Expressed as residues per 1000 total residues.

On the other hand, the extinction coefficient of HA solutions at UV wavelengths has suggested that presence of a significant amount of nucleic acid. One could consider that the very high level of glycine in this fraction might arise from the degradation of nucleic acid during HCl hydrolysis of the samples. Sabir et al. (19) have already pointed out the presence of nucleic acid in a fraction they obtained from a sunflower meal extract. Using the perchloric acid extraction method (38), we extracted the nucleic acid components (RNA) from a sample of 'Peredovik' HA, showing the highest content in glycine: in the RNA-free protein fraction (Table II), the glycine content falls from 189 to 100 residues per 1,000 total residues.

Table III lists LA aminoacid compositions that are rather similar for the three varieties, despite a few discrepancies for alanine or threonine, comparing 'Armavirec' with the 'y_2 line.'

With regard to the meal composition, globulins appear highest in phenylalanine and aspartic acid (or asparagine) and lowest in cystine. HA in Table II are the protein group of which the composition is closest to that of the meal. (We shall see later that this group is quite contaminated by globulin components). On the other hand, LA exhibit amounts very different from those of the two other groups, particularly high levels of sulfur aminoacids and glutamic acid (or glutamine). Carrying out the protein fractionation for each of the three groups–globulins, HA and LA–one notices that the groups are not only heterogeneous, but also incompletely separated from each other. The HA and LA, of which the amount is much less important than the predominant globulin group, are contaminated by a part of the globulin group, but in proportions differing according to the varieties.

Fractionation of Globulins, HA and LA

This fractionation was conducted with proteins from the 'Armavirec' variety. Under the conditions described above, globulins are fractionated in four well-separated proteic peaks; HA in two and LA in two, of which the second is split into two fractions during further dialysis (fraction 2a corresponds to soluble and fraction 2b to the precipitated proteins).

Table IV lists for each group: (a) the protein ratio of each fraction per 100 g protein of the group, (b) the molecular weight measured by the peak elution volume, (c) the aminoacid composition of the fraction.

For the globulin group, the four separated fractions are slightly different in composition. It can be noted that fractions 1 and 3 (mol wt 95,000 and 25,000 respectively) have quite similar compositions, likewise fractions 2 and 4 (mol wt 43,000 and 13,000 respectively).

In the HA group, the aminoacid compositions of the two separated fractions are very different. Fraction 2 which contributes 4/5 of the HA group in 'Armavirec' variety, is so close in composition to the globulin group (especially globulin fraction 1) that it appears as a globulin-like fraction. In contrast, HA peak 1 is possibly one protein having the original aminoacid composition, lower than any other fraction in glutamic acid or glutamine: (121 residues °/₀₀), higher in lysine (65 °/₀₀), serine, and threonine (78 and 51 °/₀₀ respectively). The mol wt is about 48,000.

Finally in Table IV, characteristics of the three LA fractions obtained by chromatography on Sephadex G-200 and subsequent dialysis are also given. All three fractions are similar in composition, which is really unusual when compared with the globulin and HA fractions, especially because of the high level of sulfur aminoacids (between 70 and 100 residues °/₀₀ for the sum methionine + cystine) and in glutamic acid or glutamine (aobut 300 residues °/₀₀).

Amounts of the Three Protein Groups Studied

The nitrogen forms (protein and nonprotein nitrogen) dissolved by buffered NaCl account for nearly 80% of total kernel nitrogen with every variety, the same percentage as that reported previously (12). After freeze-drying, the total proteins (globulins + HA + LA) represent from 50 to 55% (according to varieties) of total defatted meal nitrogen and approximately 75% of salt-soluble proteins. Thus, only 25% of the salt-soluble proteins are lost during the different preparation steps (chromatography, dialysis, freeze drying). Although such a yield is rather good, it is not sufficient to get an accurate idea of the fluctuations of the three different protein groups according to variety. It is noteworthy that, for the three varieties studied, LA were the most constant, representing about 20% of total proteins prepared (20% of salt soluble proteins). Globulin and especially HA amounts were more variable, for reasons we will now discuss.

DISCUSSION

Three main points will be discussed: heterogeneity of the three groups studied, their respective levels within the sunflower kernel, and the possible existence of subunits among different fractions of these groups.

TABLE IV

Amino Acid Composition (R °/₀₀) of 'Armavirec' Sunflower Proteic Fractions[a]

	Globulins				Heavy Albumins		Light Albumins		
Fraction N.	1	2	3	4	1	2	1	2a	2b
%	10	50	25	15	18	82	7	30	63
Mol wt	95000	43000	25000	13000	48000	13000	50000	13000	13000
Gly	94	88	124	83	139	87	65	66	54
Ala	70	65	65	64	75	71	44	42	30
Val	69	69	65	69	66	72	45	46	48
Leu	74	68	66	57	69	54	67	64	57
Ile	53	52	46	41	39	50	42	40	45
Ser	57	51	58	51	78	54	48	45	36
Thr	43	34	37	37	51	41	30	29	18
Tyr	21	24	20	22	23	18	12	16	14
Phe	45	50	47	48	36	32	17	16	21
Pro	50	48	29	48	49	51	58	53	59
Met	10	12	12	5	12	16	24	45	39
Cys	15	7	7	9	14	18	47	56	54
Lys	40	26	30	26	65	42	38	43	24
His	21	23	21	20	19	20	12	15	10
Arg	58	67	58	80	40	58	83	76	99
Asx	110	115	112	130	104	112	77	74	67
Glx	171	202	185	219	121	206	292	274	324

[a]Expressed as residues per 1000 total residues.

Heterogeneity of the Three Groups

As shown in the first four columns of Table IV, at first sight the globulin group seems to be slightly heterogeneous, with at least four fractions, two of which might be subunits of the other two but which appear rather similar by their aminoacid compositions.

The major fraction of 'Armavirec' HA (HA fraction 2), called a globulin-like fraction, comes plausibly from the group of globulins, the separation of which, with HA, is the result of a simple dialysis. Therefore, the globulin group can be considered as a mixture of five fractions, while true HA appear to be one fraction (HA 1), which is the highest in lysine among all of the fractions obtained.

On the other hand, the LA group shows some heterogeneity that was already suggested by the elution pattern from a Sephadex G-50 column (Fig. 2). The second peak containing LA is beginning to split into two peaks. Furthermore, aminoacid compositions and molecular weights are close enough to conclude that at least fractions 2a and 2b are very similar. It is therefore possible, when talking of such proteins, to use the term "isoproteins," as one of us (39) had already proposed, with the aim of broadening the "isoenzyme" concept.

Levels of the Three Groups among the Kernel Proteins

The fact that HA fraction 2 appears as a globulin fraction which already precipitates during dialysis explains that the apparent amount of HA is changing according to both extracting conditions and to variety. Real HA appear as a minor fraction representing only 5 to 10% of the total salt-soluble proteins. Real globulins consequently reach a level corresponding to ca. 3/4 of the total salt-soluble proteins, while the LA level is ca. 20% of the salt-extracted proteins.

As we have shown previously (12), salt-insoluble proteins had an aminoacid composition quite identical to that of whole kernel proteins. It is very plausible to conclude that proportions of the three groups studied among kernel proteins are of the same magnitude as these evaluated in the restricted pool of salt-soluble proteins.

Possible Subunits of Globulins and LA

Among the nine different fractions separated by G-200 Sephadex in the presence of Sodium Dodecyl Suffate (SDS), several show sufficiently low molecular weight to try to calculate the lowest possible value of this molecular weight based upon aminoacid composition.

TABLE V

Number of Aminoacid Residue per mole of Subunits

	Globulins Fraction	Heavy Albumins Fraction	Light Albumins Fraction	Light Albumins Fraction
	4	2	2a	2b
Gly	9	10	8	7
Ala	7	7	5	4
Val	7	7	6	6
Leu	6	6	8	7
Ile	4	5	5	6
Ser	6	6	6	4
Thr	4	4	4	2
Tyr	2	2	2	2
Phe	5	3	2	3
Pro	5	51	7	7
Met	(1)	2	6	5
Cys	1	3	7	7
Lys	3	4	5	3
His	2	2	2	1
Arg	9	8	9	12
Asx	14	11	9	8
Glx	24	26	34	40
Total	109	111	125	124
Calculated mol wt	12,400	12,450	14,600	14,900

Table V gives the results so obtained. The first two columns are relative to globulin fraction 4 and to "globulin like" fraction 2 of the HA group. Instead of 13,000 as found on calibrated Sephadex columns, aminoacid compositions give respectively 12,400 and 12,450 for their mol wt, with a total number of residues per mole of 109 and 111 respectively. The similarity of the aminoacid compositions of these two fractions is an additional confirmation of the globulin nature of HA fraction 2.

Concerning the LA rather close compositions are shown for the two fractions, 2a and 2b, with practically the same calculated mol wt (14,600 and 14,900 respectively, instead

of 13,000 found with Sephadex calibrated columns). This suggests either the existence of only one polypeptide chain with a few proteic impurities or, more probably, the occurrence of two LA fractions which are isoproteins and perhaps even alloproteins, with possibly large identical sequence patterns for their primary structure. In spite of a few differences, their aminoacid compositions are in good agreement with those found by Schwenke et al. (17), and by Sabir et al. (19). This should be compared with the interesting results of Lönnerdal et al. (40) on rapeseed albumins. These authors have indeed shown that rapeseed albumins, which also have rather low molecular weights, constitute heterogeneous group made of a few very similar protein fractions. This raises the important question of whether these rapeseed albumins have any homology with sunflower LA as a proposed by Schwenke et al. (20). This is another problem requiring further study.

In conclusion, globulins, which are the major storage proteins of sunflower kernel, appear to be a mixture of only a few polypeptide chains, of which the essential aminoacid patterns are not as good as those of other oilseed or legumeseed proteins. Nevertheless, the presence of true HA, very rich in lysine, and LA fractions, very rich in methionine and cysteine, suggests the possibility for genetic improvement of sunflower proteins from a nutritional point of view by a systematic search for lines or mutants derepressed for biosynthesis of HA and LA.

On the other hand, high levels of either lysine or sulfur aminoacids in these two kinds of albumins indeed favour the production of lysinoalanine which, if not toxic, can slightly depreciate sunflower proteins during industrial processing. Technology of sunflower seed proteins must take into account both the possible occurrence of lysinoalanine under drastic conditions and the rather low molecular weight of albumin fractions which can lower final yield of industrial preparations. However, because of a few precautions suggested by these particular characteristics, sunflower meal proteins remain a very interesting and important source of vegetable proteins for new foods.

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