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HYDROPHOBIC INTERACTION CHROMATOGRAPHY: A NEW METHOD FOR SUNFLOWER PROTEIN FRACTIONATION

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SUMMARY

Hydrophobic interaction chromatography has been exploited to fractionate saline-soluble sunflower proteins and the fractions obtained have been analysed further by SDS–polyacrylamide gel electrophoresis. Using octyl-Sepharose Cl-4B, the less hydrophobic fractions are bound only when the ionic strength is increased with a strong salting-out cation (NH_4^+) or anion (PO_4^{3-}). The strong hydrophobic fractions are normally bound to the matrix and could be eluted with a chaotropic agent. With 20% saturated $(NH_4)_2SO_4$ solution containing 10% NaCl and 0.02 *M* borate, pH = 7.4, about 98% of the salt-soluble proteins are bound to the matrix and most of the chlorogenic acid (98%) passes straight through the column. The elution of five low hydrophobic protein fractions is achieved by selective decrease of the ionic strength. Then, two strong hydrophobic fractions are eluted using $MgCl_2$ and 2-methoxyethanol as a polarity-reducing agent. Results obtained in SDS–polyacrylamide gel electrophoresis indicate that these seven protein fractions are composed partly of similar subunits and partly of different subunits.

INTRODUCTION

Few investigations have been conducted on the nature and biochemical properties of the globular proteins in sunflower flour[1–3]. High concentrations of chlorogenic, caffeic and quinic acids interact with proteins[4,5] and they must be eliminated in such studies[6–8]. Proteins of seeds are usually separated according to their solubility[9]. Most sunflower flour proteins are salt extractable[10] and protein fractions have been isolated by dialysis against water as salt-soluble proteins (globulins) and water-soluble proteins (albumins)[11]. Salt-soluble proteins from sunflower kernels are usually fractionated by gel filtration after chlorogenic acid has been removed by a preliminary Sephadex G-50[2], G-25[12] gel filtration or not[1]. Sephadex G-50 filtration permits at the same time a partial separation of sunflower proteins and the isolation of three main groups of proteins after dialysis against water: globulins, heavy albumins and light albumins[2].

The Osborne classification[9], although useful, is quite arbitrary and variable

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results are obtained unless the pH, salt concentration and extraction procedure are rigorously constant. On the other hand fractionations based on the differences in molecular weights are not related to the physico-chemical properties of seed proteins. The main fractions obtained by this method undergo dissociation under various experimental conditions and thus appear to be heterogeneous.

Despite the fact that vegetable proteins are known to have significantly different amino acid compositions to animal proteins, most of the usual fractionation methods used for animal proteins are applied to seed proteins. Although the distribution and the reactivity of apolar residues has been little studied[13], some experiments involving hydrophobic interaction chromatography have been performed[14–17]. The low water solubility of sunflower proteins suggests that some apolar interactions play an important rôle in the physical properties of these compounds.

In the present paper a new method for separating sunflower proteins is proposed, based on their hydrophobic binding properties. These investigations could lead to a new approach to seed protein fractionation and to an improved study of some of their properties. Chlorogenic acid is readily eliminated and seven protein fractions are isolated by a stepwise procedure using the octyl-Sepharose Cl-4B hydrophobic matrix.

METHODS

Protein extracts

Sunflower seeds (mirasol variety) were manually dehulled and were ground in a Wiley mill so as to pass a 40-mesh sieve. The flour was defatted by stirring with 10 volumes hexane for 48 h at room temperature, with three changes of solvent. Residual hexane was removed under vacuum and the flour was allowed to stand in order to reach a humidity equilibrium at 25°C. A 10-g amount of sunflower flour was then extracted for 30 min under stirring with 10 volumes 0.02 *M* borate buffer, pH 8.6, 10% NaCl and the supernatant was collected after centrifugation (15 min at 10,000 *g*). A second extraction was performed under the same conditions with the pellet and the supernatants were combined, so constituting the crude saline extract.

Materials

Octyl-Sepharose Cl-4B and phenyl-Sepharose Cl-4B were purchased from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade or the best available commercially. All chromatographic procedures were performed at room temperature, and the column effluents were monitored at 280 nm with an ISCO Type 6 optical unit and recorded with an ISCO Model UA 5 absorbance monitor.

Analytical methods

Protein (nitrogen × 6.25) in the crude extract was determined by the micro-Kjeldahl procedure. After the chromatographic step, protein was determined simultaneously from the absorbance at 280 nm and by a modified biuret method for vegetable proteins[18]. Chlorogenic acid was determined from the absorbance at 345 nm.

SDS–polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)–polyacrylamide-gel electrophoresis was per-

formed at 220 V (ISCO 492 current supply) in an electrophoresis apparatus constructed in our laboratory by using slabs of gel (180 nm × 150 nm × 1.5 mm). Samples were dissolved in a solution of 15% (v/v) glycerol, 2.5% (w/v) SDS, 6 *M* urea, 5 m*M* 2-mercaptoethanol or not and 50 m*M* Tris–borate, pH 8.8, and then heated for 5 min at 100°C. SDS–polyacrylamide gel electrophoresis was carried out on gels containing 12% (w/v) acrylamide (0.5% bisacrylamide). The gels were stained overnight in 0.003% Coomassie Brillant Blue R250 and destained in methanol–acetic acid–water (40:5:55, v/v/v). The molecular weights were estimated by comparing the mobilities of the different proteins with those of markers of known molecular weights.

RESULTS

Fractionation of sunflower proteins on octyl-Sepharose Cl-4B

Preliminary studies. Hydrophobic chromatography on octyl-Sepharose Cl-4B was performed with a column (10 × 2.5 cm) equilibrated with 10% NaCl, 0.02 *M* borate, pH 7.4. Protein extracts (250 mg in 5 ml at pH 7.4) were placed on the column which was washed with the equilibration buffer. Unbound material, which contains most of the proteins and all of the chlorogenic acid, is washed out. Elution with 10^{-3} *M* borate, pH 7.4, gave a small protein peak. The use of a chaotropic solvent [50% aqueous methyl Cellosolve (2-methoxyethanol), MCS] permits the elution of another protein fraction (Fig. 1A) which represents about 15% of the starting material. We name it the "strong hydrophobic fraction". Thus, under the previous starting conditions (0.02 *M* borate, 10% NaCl, pH 7.4) only a minor part of the applied protein is retained by the hydrophobic matrix.

Increasing the salt concentration with salting-out ions results in an increase in hydrophobic interactions between the proteins and the matrix.

Tests were performed by adding to the initial crude extract three concentrations of ammonium sulphate (10%, 15% and 20% in saturation), the octyl-Sepharose column being equilibrated with 10% NaCl, 0.02 *M* borate, pH 7.4, containing 10, 15 or 20% saturated $(NH_4)_2SO_4$. As shown in Fig. 1B, C, D the progressive increase in ammonium sulphate concentration leads to a parallel increase in the amount of bound proteins. These proteins can be desorbed by washing the column with 10^{-3} *M* borate, pH 7.4.

Thus in 20% $(NH_4)_2SO_4$, 95–98% of the proteins are retained on the hydrophobic matrix, and washing with the starting buffer only permits elimination in the void volume of the chlorogenic acid, whose absorbance at 280 nm is always important. Elution with 10^{-3} *M* borate, pH 7.4, gives an important protein fraction (Fig. 1D) which we name the "low hydrophobic fraction" because it is not normally bound on octyl-Sepharose and is only retained when the hydrophobic interactions have been artificially increased with salting-out ions. The low hydrophobic fraction represents about 85% of the protein extract.

The unbound material is heterogeneous as shown by spectrophotometric analysis (Fig. 2). The beginning of the non-adsorbed peak possesses an absorption spectrum whose maximum near 260 nm is probably due to nucleic acids. The subsequent material comprises a chromogenic compound which possesses an absorption spectrum close to that of chlorogenic acid. So chlorogenic acid appears to be at least partly delayed on the column, but not bound. This is important because if too much ma-

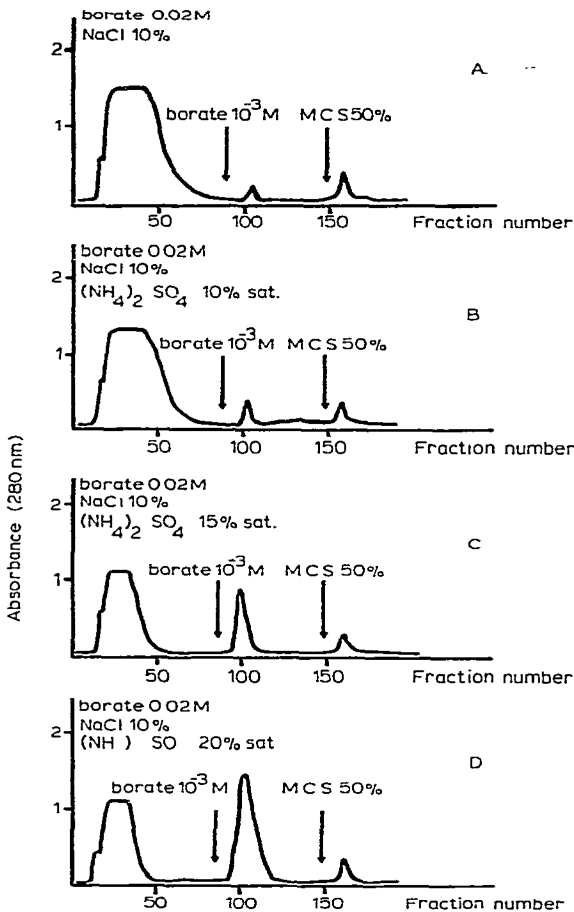

Fig. 1. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B column (10 × 2.5 cm). Buffer: 0.02 *M* borate, 10% NaCl, pH 7.4 (A), containing 10% (B), 15% (C) or 20% (D) saturated $(NH_4)_2SO_4$. Sample: 250 mg of saline-soluble sunflower proteins in 5 ml. Fractions: 1 ml. Flow-rate: 7.5 ml/cm^2·h.

terial is used and if the column is short, a partial competition occurs between proteins and chlorogenic acid and the presence of too much pigment could disturb the protein fixation. To avoid this we recommend that the column be filled step by step and partially washed after each step.

Gradient elution studies. The fractionation of sunflower proteins could be increased if the elution of the low hydrophobic and strong hydrophobic fractions were gradually performed. In one experiment, 5 ml (250 mg of protein) of crude extract in 10% NaCl, 20% saturated $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4, were loaded on the octyl-Sepharose column. After washing with the starting buffer (Fig. 3), elution was carried out with a decreasing linear gradient of NaCl from 10 to 0% in 20% saturated $(NH_4)_2SO_4$, 0.02 *M* borate, pH 7.4. A single protein peak (peak 1) was eluted. Then a gradient decreasing, in ionic strength from 20% $(NH_4)_2SO_4$, 0.02 *M* borate, pH 7.4, to 10% NaCl, 0.02 *M* borate, pH 7.4, gave a second protein fraction (peak 2). Another decreasing gradient, from 10 to 0% NaCl (0.02 *M* borate, pH 7.4) permitted

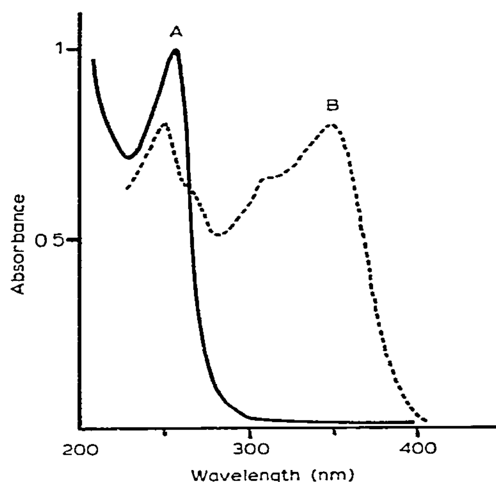

Fig. 2. Absorption spectra of the unbound material: A, beginning of the peak (——); B, following fractions (– – –).

the elution of a third single protein peak. A fourth fraction (peak 4) was eluted when the molarity of borate was reduced from 0.02 *M* to 10^{-3} *M*, pH 7.4, and a final protein fraction (peak 5) was obtained with water. So the low hydrophobic fraction could be fractionated into five separate peaks.

The strong hydrophobic fraction could also be fractionated. A linear increasing gradient of $MgCl_2$ from 0 to 0.2 *M* gave a minor protein fraction (peak 6) at 0.1 *M*

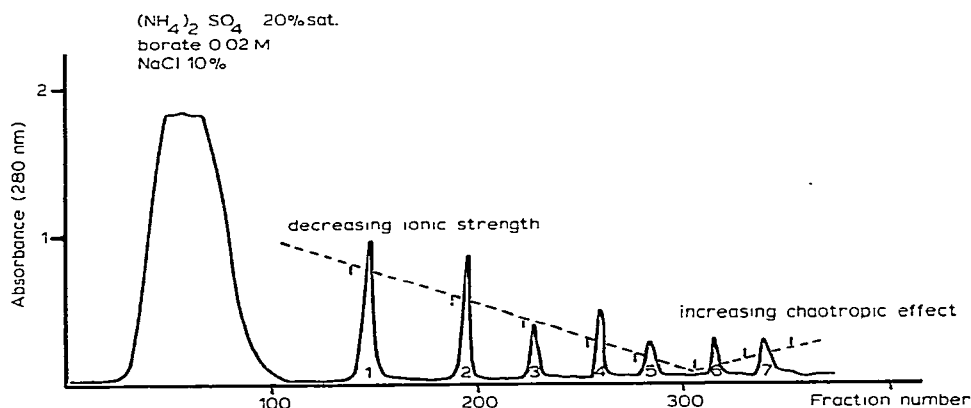

Fig. 3. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B. Column: 10 × 2.5 cm. Buffer: 0.02 *M* borate, 10% NaCl, 20% saturated $(NH_4)_2SO_4$, pH 7.4. Sample: 250 mg of saline-soluble sunflower proteins in 5 ml. The elution was performed as follows: 1, decreasing linear gradient in NaCl, from 0.02 *M* borate, 10% NaCl, 20% saturated $(NH_4)_2SO_4$, pH 7.4, to 0.02 *M* borate, 20% saturated $(NH_4)_2SO_4$, pH 7.4; 2, decreasing linear gradient in ionic strength, from 0.02 *M* borate, 20% saturated $(NH_4)_2SO_4$, pH 7.4, to 0.02 *M* borate, 10% NaCl, pH 7.4; 3, decreasing linear gradient in NaCl, from 0.02 *M* borate, 10% NaCl, pH 7.4, to 0.02 *M* borate, pH 7.4; 4, decreasing linear gradient from 0.02 *M* borate, pH 7.4, to 10^{-3} *M* borate, pH 7.4; 5, decreasing linear gradient from 10^{-3} *M* borate, pH 7.4, to water; 6, increasing linear gradient in $MgCl_2$ (0 to 0.2 *M*); 7, increasing linear gradient from 1% to 50% MCS. Fractions: 1.5 ml. Flow-rate: 7.5 ml/cm^2 · h.

$MgCl_2$ due to the chaotropic effect of the Mg^{2+}, and 50% MCS solution gave a final protein fraction (peak 7).

Stepwise elution studies. Since the different protein fractions were eluted near the end of the gradients (except for fraction 6 which is eluted by 0.1 *M* $MgCl_2$) a stepwise procedure was used in the last experiment. On a column (15 × 2.5 cm) of octyl-Sepharose equilibrated with 10% NaCl, 20% saturated $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4, 40 ml of crude extract (under the same starting conditions) were loaded by a step by step procedure (10 ml × 4). When all the chlorogenic acid had been removed by washing the column with the starting buffer, elution was performed with 20% $(NH_4)_2SO_4$ and 0.02 *M* borate, pH 7.4 (peak 1), then with 10% NaCl and 0.02 *M* borate, pH 7.4 (peak 2), with 0.02 *M* borate, pH 7.4 (peak 3), with 10^{-3} *M* borate (peak 4) and finally water (peak 5). The strong hydrophobic fractions were then eluted with 0.1 *M* $MgCl_2$ (peak 6) and 50% MCS solution (peak 7). The resulting chromatographic profile is exactly the same as in Fig. 3. Low hydrophobic proteins represent 86% (peak 1, 52%; 2, 19%; 3, 7%; 4, 5%; 5, 3%) and strong hydrophobic fractions 14% (peak 6, 3%; 7, 11%) of the adsorbed substances.

When hydrophobic chromatography on octyl-Sepharose was performed without ammonium sulphate under the starting conditions (10% NaCl, 0.02 *M* borate, pH 7.4), the unbound material was much more important (Fig. 4). Only three low hydrophobic fractions were eluted (with 0.02 *M* borate, pH 7.4, then 10^{-3} *M* borate, pH 7.4, and finally water) followed by two strong hydrophobic fractions. On the other hand, the same elution profile was obtained when NH_4^+ (high salting-out cation) was replaced by PO_4^{3-} (high salting-out anion). Thus, potassium phosphate is able to replace ammonium sulphate at a concentration of 0.6 *M* in the starting buffer (0.02 *M* borate, pH 7.4, 10% NaCl). Under these conditions, 90% of the proteins are bound to the matrix and the elution was performed with 0.6 *M* K_2HPO_4, 0.02 *M* borate, pH 7.4, and successively with the six other solutions in the same manner as before (results not shown).

Phenyl-Sepharose Cl-4B is also suitable, and the sunflower proteins are adsorbed at a lower ammonium sulphate concentration. In this case, however, the proteins are bound so firmly that elution at decreasing ionic strength and with a

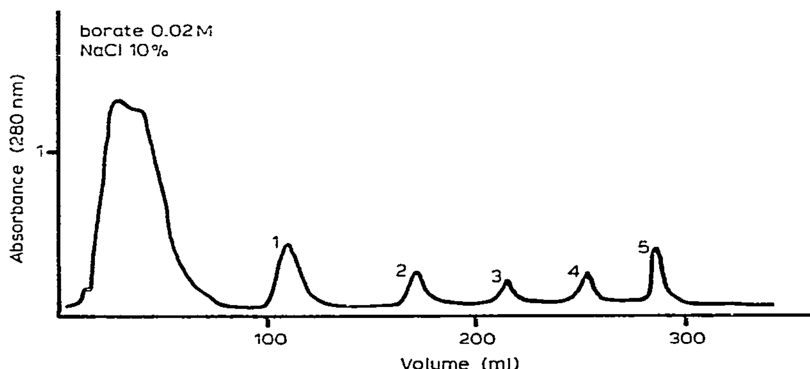

Fig. 4. Hydrophobic interaction chromatography on octyl-Sepharose Cl-4B. Column: 10 × 2.5 cm. Sample; 250 mg of saline-soluble sunflower proteins in 5 ml. Buffer: 0.02 *M* borate, 10% NaCl, pH 7.4. The elution was performed as follows: 1, 0.02 *M* borate, pH 7.4; 2, 10^{-3} *M* borate, pH 7.4; 3, water; 4, 0.1 *M* $MgCl_2$; 5, 50% MCS. Fractions: 2.5 ml. Flow-rate 7.5 ml/cm^2·h.

polarity-reducing agent give a lower resolution than those obtained with the octyl-Sepharose matrix (results not shown).

SDS-polyacrylamide gel electrophoretic analysis

The seven separated fractions were analysed by SDS-polyacrylamide gel electrophoresis in order to establish the compositional differences among these fractions.

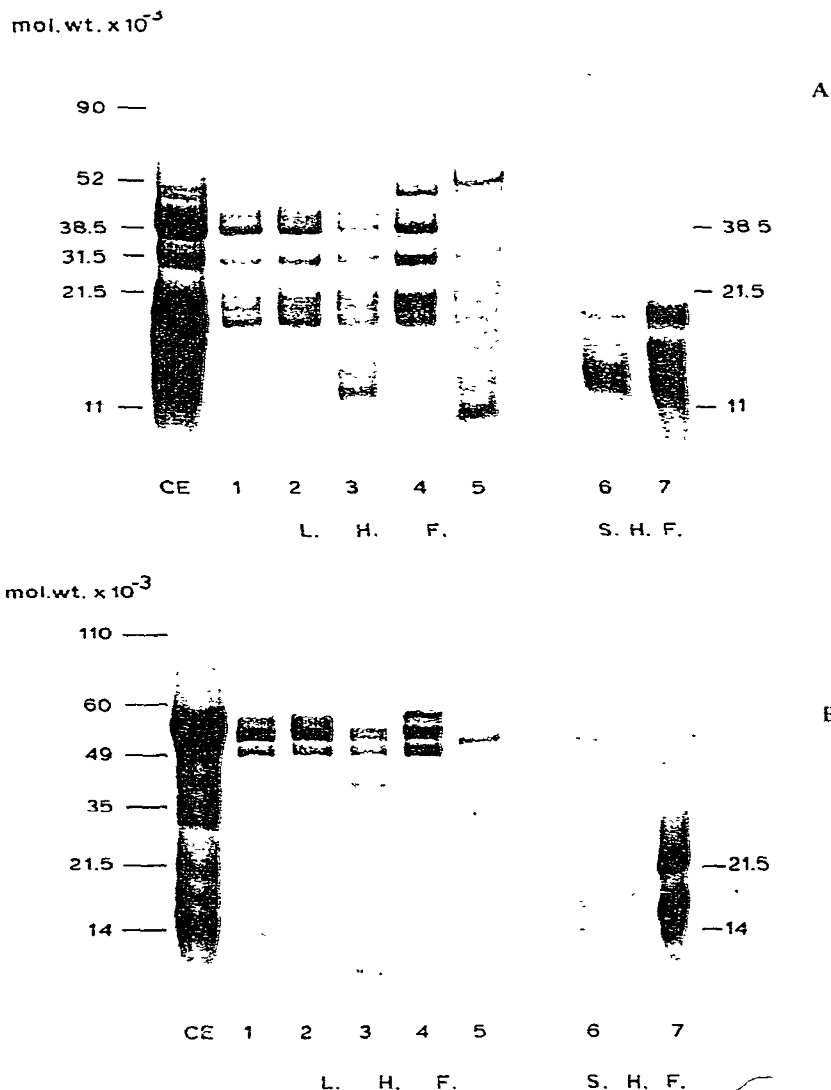

Fig. 5. SDS-polyacrylamide gel electrophoresis patterns of the different protein fractions obtained by hydrophobic chromatography on octyl-Sepharose Cl-4B according to the procedure described in Fig. 3. CE = Crude extract. The seven eluted fractions were analysed and are numbered 1–7 from the lowest to the strongest hydrophobicity. A, Non-reduced samples; B, reduced samples. One hundred and twenty micrograms (fraction CE), 50 μg (fractions 1, 2, 3, 4, 6, 7) and 30 μg (fraction 5) of protein were loaded. L.H.F. = Low hydrophobic fractions; S.H.F. = strong hydrophobic fractions.

Without prior reduction with 2-mercaptoethanol, the SDS electrophoresis pattern (Fig. 5A) shows that the proteins of the crude extract are separated into several subunits with molecular weights ranging from 60,000 to 49,000 and only a few with lower molecular weights. With previous reduction with 2-mercaptoethanol, the highest-molecular-weight protein bands disappear and simultaneously some smaller-molecular-weight polypeptides (52,000 to 10,000) appear (Fig. 6B).

The unbound protein material is essentially composed of low-molecular-weight peptides. The patterns obtained in non-reduced form for the first and second fraction were essentially the same (Fig. 5A). Upon SDS gel electrophoresis, these fractions appear to consist of the main subunits found in the crude extract. However, there are obvious differences in the intensity of the stained bands in the region below molecular weight 35,000. On the other hand, reduced fractions 1 and 2 (Fig. 5B) also showed differences in their electrophoretic patterns. As in the case of the crude extract, some smaller polypeptides appear after reduction. Fractions 1 and 2 seem to be identical in the number of subunits, however their proportions are different, particularly in the lower-molecular-weight range (below 21,000).

Compared with fractions 1 and 2, the electrophoretic patterns of fractions 3, 4 and 5 (Fig. 5A, B) show more important differences in composition as well as in the proportions of the subunits, and each protein group has a characteristic pattern.

The electrophoretic patterns of fractions 6 and 7 (the strong hydrophobic fractions) are also very different. Fraction 7 is the only eluted fraction which doesn't possess any polypeptidic band of high molecular weight (60,000 to 49,000) and the pattern of reduced fraction 7 shows a large number of low-molecular-weight polypeptides.

The fractions obtained when the chromatographic procedure was conducted without NH_4^+ (see Fig. 4) were also submitted to electrophoretic analysis. As shown in Fig. 6 the exclusion peak contains most of the subunits found in the crude extract.

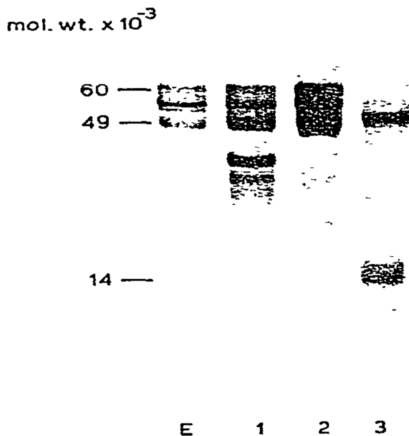

Fig. 6. SDS–polyacrylamide gel electrophoresis patterns of eluted protein fractions from octyl-Sepharose Cl-4B without prior addition of $(NH_4)_2SO_4$ as described in Fig. 4. E = Exclusion fraction. Fractions 1, 2 and 3 were respectively eluted with 0.02 *M* borate, pH 7.4, 10^{-3} *M* borate, pH 7.4, and water. A 50-μg amount of protein in non-reduced form was loaded.

Fractions 1, 2 and 3 respectively eluted with 0.02 *M* borate, pH 7.4, then 10^{-3} *M* borate, pH 7.4, and finally water have similar electrophoretic patterns to those of fractions 3, 4 and 5 previously described.

SDS–polyacrylamide gel electrophoresis shows that all the eluted fractions exhibit similar patterns despite the fact the chromatographic procedure has been conducted in the presence of NH_4^+ or PO_4^{3-} (results not shown).

The described procedure allowed us to fractionate proteins from other vegetable origins. For example, electrophoretic patterns of soybean and broadbean protein fractions obtained by this procedure are shown in Fig. 7A and B.

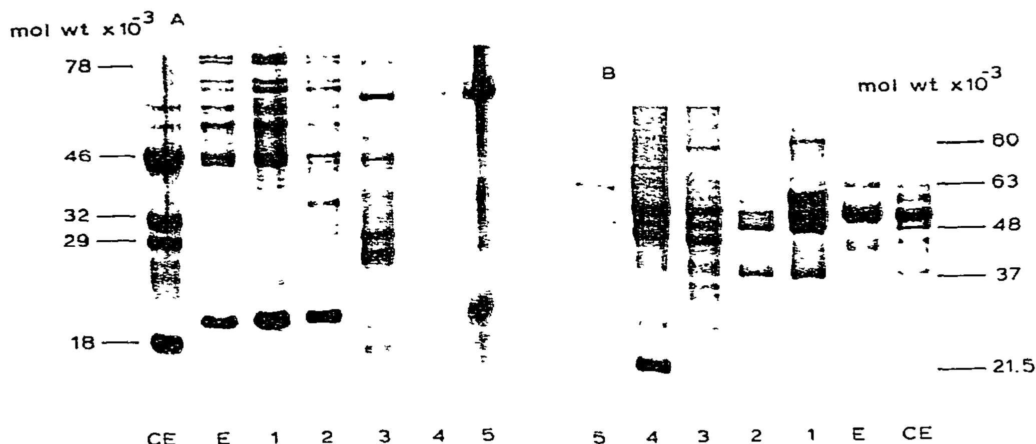

Fig. 7. SDS–polyacrylamide gel electrophoresis patterns of the different protein fractions obtained from soybean (A) and broadbean (B) using hydrophobic chromatography, by the procedure described for sunflower proteins. CE = Crude extract; E = exclusion fraction. Only the five low hydrophobic fractions (1–5) were analysed in non-reduced form.

DISCUSSION

The hydrophobic properties of sunflower proteins seem to be provide an interesting approach to seed protein fractionation. Using octyl-Sepharose Cl-4B as matrix, hydrophobic chromatography permits the separation of two main protein species: the low hydrophobic proteins which are not normally bound to the matrix, and the strong hydrophobic proteins which are retained and only eluted with the help of a chaotropic agent which decreases the hydrophobic interactions between the protein and the hydrophobic groups of the gel. The strong hydrophobic protein fraction represents about 15% of the starting material.

The hydrophobic interactions are known to increase upon increasing the ionic strength of the medium[16]. In this way, our results show that in 20% saturated $(NH_4)_2SO_4$, only about 1% of the crude proteins are not bound to the hydrophobic matrix. Under these conditions chlorogenic acid is readily eliminated in a non-adsorbed fraction. Thus, by this procedure, it is possible to remove chlorogenic acid and prepare almost pure chlorogenic-free protein samples. The elimination of this phenolic acid is of great interest because its presence causes considerable discoloration of sunflower meals, restricting their application in the food industry.

This chromatographic procedure not only removes chlorogenic acid but also permits the fractionation of the salt-soluble protein extract adsorbed on the hydrophobic matrix. The separation is based on the fact that a progressive decrease in the ionic strength of the elution buffer may promote selective desorption of proteins by decreasing their hydrophobic interactions with the gel. Our studies have shown that a stepwise procedure using five different solutions of decreasing ionic strength yields five low hydrophobic protein fractions.

The strong hydrophobic proteins may also be separated in two different fractions by the use of chaotropic agents such as Mg^{2+} and MCS.

Like other seed proteins[19,20], sunflower proteins are heterogeneous and composed of different subunits[1–3]. Thus, seed proteins may be considered as an associating protein system[21,22]. Results obtained on SDS–polyacrylamide gel electrophoresis indicate that the seven protein fractions are partly composed of similar subunits (but in different proportions) and partly of different subunits. The existence of polypeptide chains in these subunits and connected at least partly by disulphide bridges could be proved by SDS gel electrophoresis of reduced proteins. The hydrophobic amino acid content of these different subunits and/or their spacial arrangement in the quaternary structure may explain their hydrophobic chromatographic behaviour.

It has been shown in this study that fixation of the low hydrophobic proteins may be achieved by raising the ionic strength in the starting buffer. The use of salting-out anions or cations (NH_4^+ or PO_4^{3-}) leads to the same fixation effectiveness. On the other hand, the presence of NH_4^+ seems to have an effect upon the fixation of only some specific proteins since the electrophoretic patterns of fractions 3, 4 and 5 are similar to those of fractions 1, 2 and 3 obtained when the chromatographic procedure is carried out without prior addition of ammonium sulphate.

Preliminary results in our laboratory have shown that seed proteins of other species such as broadbean and soybean exhibit, under the described conditions, similar behaviour when they are submitted to octyl-Sepharose chromatography. For these two proteins about 20% of the starting protein material is not retained on the column. The importance of this "hydrophilic" fraction can be related to the high water solubility of legume seed proteins.

The analytical procedure described permits a new approach to the fractionation of sunflower storage proteins which is based on their hydrophobic properties. Octyl-Sepharose seems to be a very suitable matrix for most of the seed proteins studied and further investigations are now being performed to substantiate this fact.

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