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Characterization of Sunflower Seed Proteins by Electrophoretic Techniques

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ABSTRACT. The seed proteins of sunflower *(Helianthus annuus)* were qualitatively and quantitatively investigated. Qualitative studies were carried out using different electrophoretic techniques (SDS-PAGE, Poro-PAGE, 2-D SDS-PAGE, Isoelectric focusing, Mapping gels). Analysis of the water extracted flour on SDS-PAGE and SDS-Poro-PAGE gave five major polypeptides with MWs of 63.5 KD, 60 KD, 58 KD, 55 KD and 51 KD. The pattern of the buffer extract exhibited 8 major polypeptides with MWs of 65.5 KD, 63.5 KD, 60 KD, 58 KD, 55 KD, 54 KD, 51 KD and 42.5 KD. Second dimension gel showed that the polypeptides with MWs of 65.5 KD, 60 KD, 58 KD, 55 KD, 54 KD, 51 KD are legumin-like proteins. Isoelectric points of the majority of the sunflower seed proteins were between 5 and 7. Mapping gels, however, showed that sunflower seed proteins were highly heterogeneous, especially the major bands. A quantative study indicated that the albumin, globulin, prolamin and glutelin fractions amounted 38.32%, 39.04%, 5.53% and 17.09% respectively of the extracted proteins.

Oilcrops have a world importance. Some of them are directly used as food, while most of them are utilized to obtain fats or oils and cake or flour (Lennerts 1984, Hatje 1989).

Sunflower *(Helianthus annuus)* is an annual oilcrop (belonging to the family Compositae (Lennerts 1984)). The defatted flour of sunflower is used as a source of highly digestible and nutritive protein for poultry (Smith 1968).

Key words: Sunflower seed globulin, *Helianthus annuus;* molecular weights (MWs); two dimension-PAGE (2-D SDS-PAGE); SDS (Sodium Dodecyl Sulphate); 2-ME (2-mercaptoethanol).

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It has been reported that the major proteins (legumin-like proteins) of sunflower have sedimentation constant of about 11S and that their MWs range from 300 KD–350 KD (Joubert 1955, Gheyasuddin *et al.* 1970, Sabir *et al.* 1973, Young and Huang 1981 and Hatje 1989). Heterogeneity and quaternary structure of the major storage proteins have been investigated by Baudet and Mosse' (1977), Rahma *et al.* (1981), Plietz *et al.* (1983), Dalgalarrondo *et al.* (1984) and Abasary (1992).

As far as is known, all the studies carried out on sunflower seed proteins were done with the major seed proteins. The present work, therefore, is made to investigate new major and minor components of the sunflower seed proteins.

Experimental

Materials

Sunflower seeds were obtained from the Agriculture Research Center, Giza, Egypt. The seeds were dehulled, and ground well in a mortar. The flour was defatted by three hexane extractions (10 ml hexane/g flour), each for 2 hours with slow stirring at 4°C. After the n-hexane layer was discarded, the flour was air-dried, brushed through a sieve of 125 um (115 mesh) and then stored at –10°C until used.

Water and Buffer Extracts

A portion (30 mg) of defatted flour was mixed with 0.5 ml distilled water containing 0.02% (w/v) sodium azide in an Eppendorf tube for 30 min at 4°C, and then centrifuged at 23 000 xg (Heraeus Christ Labofuge I-cooling centrifuged) for 20 minutes at 4°C. The residue was re-extracted another two times under the same conditions. Another portion of the defatted flour was extracted with 0.125 M Tris/borate buffer pH 8.9 containing 0.02% (w/v) sodium azide as described for water extraction. Analogous extracts were made with 2-mercaptoethanol (2-ME). For SDS-PAGE, the buffer and water extracts were treated with 2% (w/v) SDS by boiling for 5 min. For complete protomer formation, extracts were boiled for 3 min with 2% SDS and 2% 2-ME.

SDS-Extract

For SDS-extraction, 30 mg of the defatted and dried flour were shaken with 0.5 ml of an aqueous solution of 5% or 15% (w/v) SDS respectively in an Eppendorf centrifuge tube and centrifuged for at 23 000 xg for 20 minutes at room temperature. The supernatant was used for electrophoresis. Analogous extracts were made with 2-ME.

Urea-Extract

Samples of 30 mg defatted flour were stirred for 30 minutes in an Eppendorf tube with 0.5 ml of an aqueous solution of 9 M urea, 2% (v/v) ampholyte pH 3-10 (Pharmacia (Ampholine) and 2% 2-ME. The mixture was centrifuged (20 min, 22°C, 23 000 xg), and the supernatant was used for electrophoresis. Analogous extraction was done without 2-ME or 2-ME/ampholyte (Shah and Stegemann 1983). All the extracts were stored at –10°C.

Protein determination

Albumin, globulin, prolamin and glutelin were extracted using the protocol used by Shah and Stegemann (1983). Total protein was determined by the method of Bradford (1976) using bovine serum albumin as standard protein.

Electrophoresis

Protein separation was carried out in vertical slabs using the LKB-2201 Vertical Electrophoresis Unit. The polymerization mixture for PAGE contained 22.5 ml of 1 M Tris pH 8.8, 19.5 ml of a mixture of 30% acrylamide and 0.43% bisacrylamide, 14.5 ml distilled water, 20 ug ammonium persulfate and 30 ul TEMED.

Two-dimensional SDS-PAGE was carried out according to Sammour (1985). In this protocol the extracted sample was analyzed in the first dimension on 12% SDS-PAGE. The gels were stained overnight with 0.05% (w/v) Coomassie Blue– R–250 in methanol, acetic acid and distilled water (50:7:43, by v/v) and destained in methanol, acetic acid water (Laemmli 1970). After destaining, the track was cut with a sharp razor-blade and left in sample buffer containing 5% SDS and 2% 2-ME for 20 minutes. The gel strip was then inserted onto a 17% SDS-PAGE and developed with a constant current of 25 mA.

Electrophoresis was performed in 17% SDS-PAGE following the same protocol as that used by Abasery (1992). For the determination of the protomer MWs a mixture of the following marker proteins, treated with SDS, were used: human transferrin (76.7 KD), bovine serum albumin (68 KD), albumin egg (43 KD), ∝-chymotrypsinogen-A (25.7 KD), and cytochrome-C (12.7 KD).

Poro-PAGE was carried out in a 6-26% gradient polyacrylamide in 0.125 M Tris/borate buffer. For determination of protomer MWs the same protein markers used in SDS-PAGE were applied to SDS-Poro- PAGE.

Isolectric focusing was carried out as described by Stegmann *et al.* (1988) using 6% polyacrylamide tube gels containing 6M urea.

Mapping: Isoelectric focusing in the first dimension and SDS in the second dimension was run as described by Stegmann *et al.* (1981) and Laemmli (1970). The gels were stained with Coomassie Blue- R-250 as described above.

Results and Discussion

Sunflower seed flour was successively extracted with distilled water (albumin), salt (NaCl) (globulin), alcohol (prolamin) and alkaline solution (glutelin) using the method of Bradford (1976). Protein contents of 134.43 ± 13.62 mg/g seed flour and 137.39 ± 6.34 mg/g seed flour were found respectively in the water and salt extract (Table 1). The protein extracted with the alkaline solution represented nearly half of the protein content found after salt extraction. Protein analysis cannot be carried out in extracts containing ampholyte, since the ampholytes complex with copper ions (Shah and Stegemann 1983). A protein content in sunflower of 16.6 to 20.3% has been reported by Hatje (1989) which contradicts our findings (35%). Youle and Huang (1981) reported that 33% of sunflower seed protein was albumin. However the variation between their data and the data reported in the present work could be due to methodological or varietal differences.

Table 1. Protein contents of the albumin, globulin, prolamin and glutelin fractions of sunflower seed flour

Protein species	Quantity mg/g Seed flour	Percent to the total protein content
Albumin	134.43 ± 13.2*	38.33
Globulin	137.39 ± 10.7*	39.04
Prolamin	019.39 ± 05.4*	05.54
Glutelin	059.95 ± 07.3*	17.09
Total protein content 351.16		

* This figure represents the mean and SD of six readings

In SDS-PAGE the seed proteins extracted with distilled water showed five major polypeptides with MWs of 63.5 KD (kiloDalton), 60 KD, 58 KD, 55 KD and 51 KD (Fig. 1, lane 1). The pattern of the buffer exract (lane. 2) exhibied 8 major polypeptides with molecular weights of 65.5 KD, 63.5 KD, 60 KD, 58 KD, 55 KD, 54 KD, 51 KD and 42.5 KD. The extraction with SDS (Fig. 1, lane 3) extracts gave electrophoretic patterns similar to that of the buffer extract, but with higher intensity. There was also an additional strong band at 15 KD. The greater amount of seed proteins extracted with SDS extracts could be due to the ability of SDS to dissociated cell membrane-binding proteins (Gennis and Jonas 1977). The pattern of the urea extract showed an increase in the numbers and intensity of the bands (Fig. 1, lane 5). However, the electrophoretic pattern of the urea extract in the presence of ampholyte shwoed an electrophoretic pattern similar to those of the extracts analyzed under reducing conditions (Fig. 1, lanes 6-14). This shows the reducing nature of the ampholyte. The extracts with urea, water, buffer or SDS in the presence of 2-ME showed similar electrophoretic patterns (Fig. 1, lanes 7-14). However there was a variation in the quantity of the protein extracted. Urea extracts in the presence of 2-ME showed high protein quantity and a new protein band denoted with arrow in Fig. 1, lanes 7-9.

Two- dimensional SDS-PAGE gel was done for further resolution, first by SDS-PAGE, and in the second dimension by SDS-PAGE under reducing conditions (Fig. 2). The polypeptides which dissociated under reducing conditions (65.5 KD, 60 KD, 58 KD, 55 KD, 54 KD, 51 KD) are legumin-like proteins. These polypeptides are separated into acidic subunits (designated 1a, 2a, 3a, 4a, 5a, 6a) and basic subunits (designated 1b, 2b, 3b, 4b, 5b, 6b) (see Table 2). These polypeptides can be defined as falling into three groups corresponding to bands G1, G2 and G3 in Fig. 1.

Table 2. Subunits molecular weights of sunflower legumin-like proteins

No.	Polpeptides MWs	Subunits			
		Acidic Subunits		Basic Subunits	
		Code	MWs	Code	MWs
1	65.5	a1	50.5	b1	15.0
2	60.0	a2	43.0	b2	17.0
3	58.0	a3	40.0	b3	18.0
4	55.0	a4	40.0	b4	15.0
5	54.0	a5	37.0	b5	17.0
6	51.0	a6	36.0	b6	15.0

The first group includes subunit 1a, 2a, 3a, 4a; the second a5 and a6; and the third 1b, 2b, 3b, 4b, 5b, 6b. These analysis are not in a good agreement with the work of Dalgalarrondo *et al.* (1984) and Allen *et al.* (1985) who reported that legumin-like proteins of sunflower seeds consists of four polypeptides wit MWs of 60 KD, 54 KD, 48 KD, 40 KD. The appearance of the band with molecular weight 130 KD (denoted with an arrow in Fig. 1) was found to consist of disulphide linked pairs of

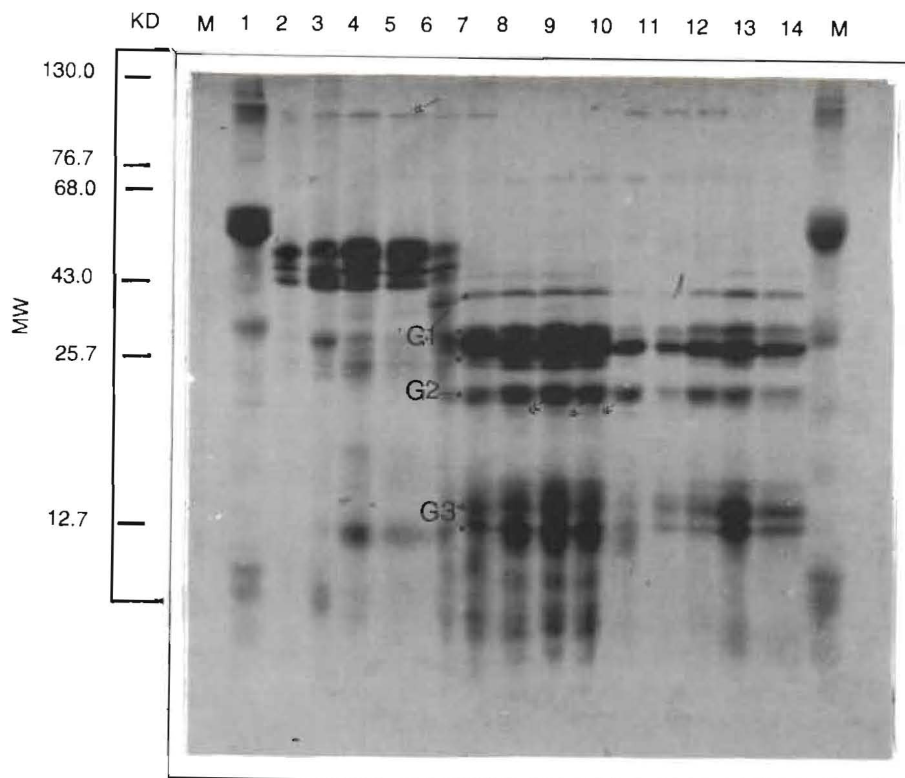

Fig. 1. SDS-Polyacrylamide gel electrophoresis (17% SDS-PAGE) of different extracts of sunflower seed proteins. 1) water extract, 2) Tris/borate buffer (pH 8.9) extract, 3) 5% SDS extract, 4) 15% SDS extract,'5) Urea extract, 6) Urea/ampholyte extract, 7) Urea/2% ampholyte/2% 2-ME extract, 8) Urea/ 2% 2-ME extract, 9) Urea/ 2% 2-ME extract (after boiling for 3 min. 2% 2-ME is added), 10) water/ 2% 2-ME extract (after 30 min. 2% 2-ME is added), 11) water/ 2% 2-ME extract, 12) Tris/borate buffer (pH 8.9) /2% 2-ME extract, 13) 5% SDS/ 2% 2-ME extract, 14) 15% SDS/ 2% 2-ME extract, M. Protein standards (Human transferrin. Bovine Serum Albumin, Ovaalbumin ∝-Chymotrypsinogen-A, and Cyochrome-C).

large acidic and small basic subunits. However these linked pairs of acidic and basic subunits were dissociated into their components in Fig. 2.

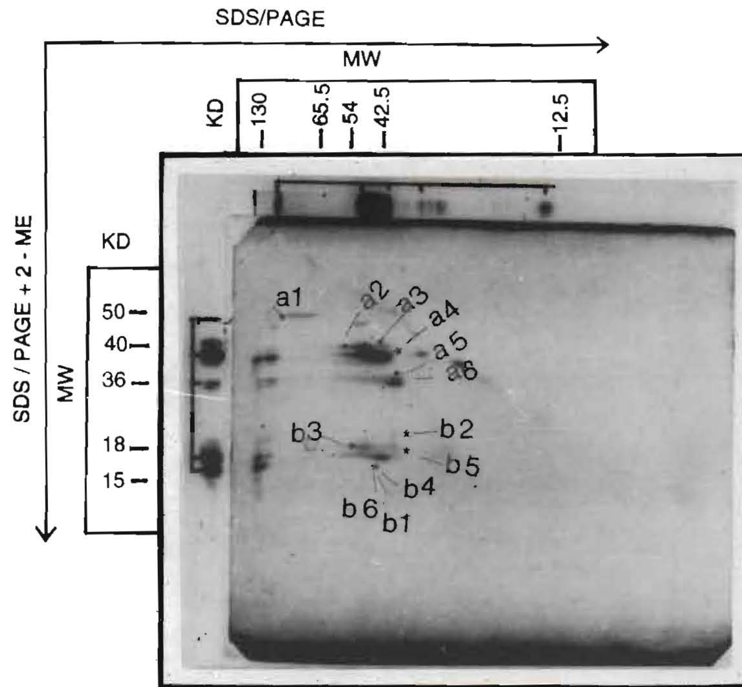

Fig. 2. Two dimensional SDS-PAGE of the total sunflower seed protein extract. 1st-D: SDS-PAGE under non-reducing conditions, and 2nd-D: SDS-PAGE under reducing conditions.

Poro-PAGE revealed a variation in the electrophoretic patterns of the various unreduced sunflower extracts (Fig. 3, lanes 1-4). After reduction with 2-ME, however, the pattern was strikingly similar; fewer bands in the upper part of the gel and more bands in the lower part were observed (lanes 5-7). As expected, meals extracted with 2-ME showed more bands than did extracts with 2-ME. However urea extraction out in the presence of ampholyte increased the dissociation of the proteins (Fig. 3, lane 4). The close similarity of the protomers of the extracts in lanes 5-7 and 10 does not mean that these proteins are the same classes of the proteins, since the separation in the Poro-gel is dependent partly on the shape and charge and partly on the molecular, weight of the polypeptides (Derbyshire *et al.* 1976 and Sammour 1985).

Isoelectric focusing was carried out between pH 3-10 in cylindrical tubes containing 8 M urea (Fig. 4). As with *Vicia faba* and potato tuber, the patterns for the urea extracts did not allow for good differentiation or clear separation of the

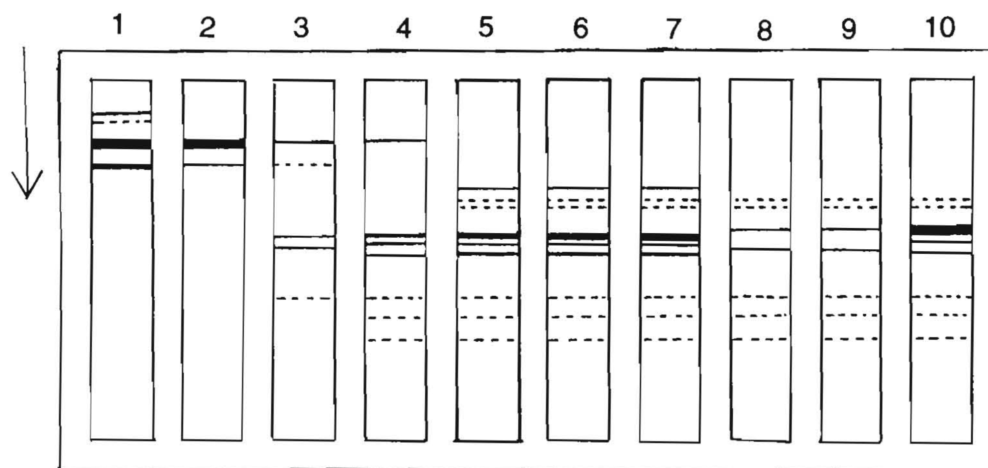

Fig. 3. Porosity gradient polyacrylamide gel electrophoresis (6-26% Poro-PAGE) of different extracts of sunflower seed proteins. 1) water extract, 2) Tris/borate buffer (pH 8.9) extract, 3) urea extract, 4) urea/ 2% ampholyte extract, 5) urea/ 2% ampholyte/ 2% 2-ME extract, 6) urea/ 2% 2-ME extract, 7) urea/2% 2-ME extract (after boiling for 3 min. 2% 2-ME is added), 8) water/ 2% 2-ME extract (2% 2-ME is added after 30 min), 9) water/ 2% 2-ME extract 10) Tris/borate buffer (pH 8.9)/ 2% 2-ME extract.

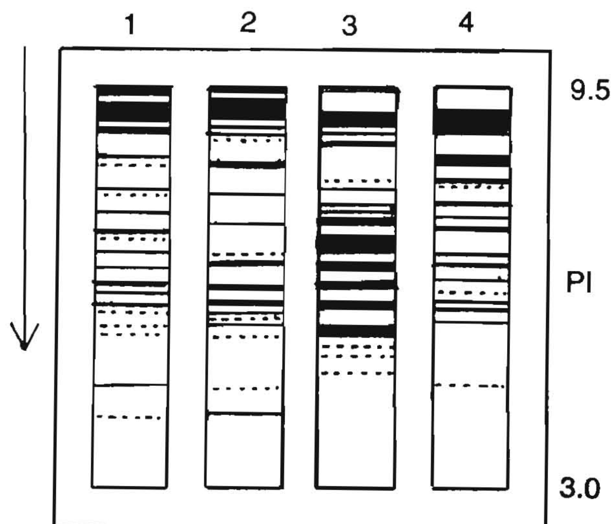

Fig. 4. PAGIF in 6% polyacrylamide, 2% ampholyte pH 3-10. Samples of sunflower flour extraced with water (1), Tris/borate buffer (2), water/2-ME (3) and Tris/borate buffer/2-ME.

bands. This poor differentiation may be attributed to the presence of phytic acid in the extracts (Stegemann *et al.* 1980). The patterns for the water or buffer extracts showed a number of band with pI-values between 9 and 5. The patterns for the water and buffer extracts analyzed in the presence of 2-ME showed little variation.

Mapping was done for further resolution, first by isoelectric focusing and in the second dimension by SDS-PAGE (Fig. 5). The polypeptide distribution of the buffer extracts consists of three groups matching the three groups of polypeptides reported in Fig. 1. It is very interesting to notice that the acidic subunits have pIs between 5-7, whole the basic subunits have pIs between 9.5-7. The gel also show that the basic and acidic subunits are highly heterogeneous. However, the acidic subunits are distributed in a narrow pH range, while the basic subunits are distributed over a wider pH range. The heterogenity can be explained as follows: 1) the subunits could be composed of several nearly identical polypeptides; 2) the proteolytic modifications could produce charge variants and in this event the differences between molecules in each preparations would be evident only at the ends of the molecules; 3) glutamine and asparagine could be diamidated in some peptides.

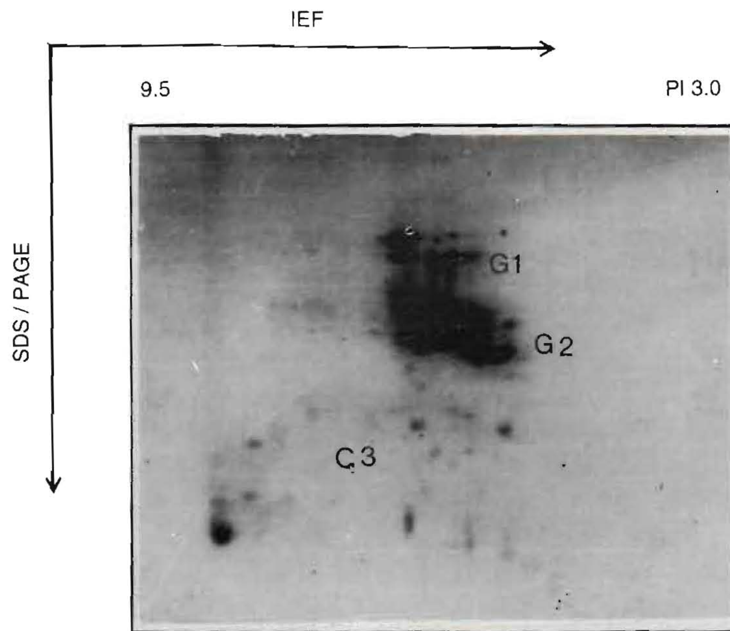

Fig. 5. Mapping of sunflower proteins extracted with Tris/borate buffer

In conclusion, sunflower seed proteins contain approximatly equal amounts of albumin and globulin proteins. These proteins represent about 77% of the total seed proteins. The total seed proteins in turn represent about 35% of the seed meal. This percentage makes sunflower seed proteins a good resource for feeding animals. The richness of the legumin-like proteins in sulphur amino acids makes them highly nutritional. On reduction with 2-ME, the legumin-like proteins were cleavaged into acidic and basic subunits. The pI-values of sunflower seed proteins range between 9.5 and 5.0. Urea and ampholyte (which are denaturating agent) gave electrophoretic patterns similar to those of the proteins extracted with Tris/borate buffer and analyzed under reducing conditions. The extraction with SDS appears to release the proteins binding to membrane.

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تميز بروتينات بذور عباد الشمس باستخدام تقنيات الاستشراد

رضا حلمي أحمد سمور و محمد نبيه الشوربجي و عاطف محمد أبو شادي
و عبد الظاهر أباصيري

قسم النبات - كلية العلوم - جامعة طنطا - طنطا - مصر

اجريت في هذا البحث دراسة كمية ونوعية للبروتينات البذرية لبذور عباد الشمس ، وقد تم اجراء الدراسة النوعية بأستخدام تقنيات الاستشراد PAGE, SDS/PAGE, Poro/PAGE, 2-D/SDS/PAGE,) (Electrophoresis) (Isoelectric Focusing, Mapping Gels . تحليل بروتينات عباد الشمس المستخلصة بالماء باستخدام SDS/PAGE اظهر خمسة عديدة البيتيدات ذات اوزان جزيئية ٥ و٦٣ ، ٦٠ ، ٥٨ ، ٥٥ ، ٥١ كيلو دالتون . كما اظهر مستخلص دقيق البذور بالمحلول المنظم ثمانية بيتيدات ذات اوزان الجزيئية ٥, ٦٥ ، ٥, ٦٣ ، ٥٨ ، ٥٥ ، ٥٤ ، ٥١ ، ٥, ٤٢ كيلو دالتون . الفصل الكهربائي على رقائق الجل في اتجاهين متعامدين احدهما تحت ظروف غير اختزالية والاخر تحت ظروف اختزالية اوضح ان عديدة البيتيدات ذات الأوزان الجزيئية ٥, ٦٥ ، ٦٠ ، ٥٨ ، ٥٥ ، ٥٤ ، ٥١ كيلو دالتون ذات روابط كبريتية ثنائية و تعرف باشباه اللجيمين . كما أوضحت الدراسة باستخدام تقنية Isoelectric Focusing أن غالبية البروتينات البذرية لنبات عباد الشمس ذات نقطة تعادل كهربائي واقعة بين pH5-pH7 ، كما اوضحت الخرائط الجلية من ناحية اخرى ان البروتينات البذرية لبذور عباد الشمس غير

متجانسة بدرجة كبيرة . الدراسة الكمـية أوضحـت ان كميـة الالبومين والجلوبيلـين
و البرولين والجلـوتين بالنسبـة لكمية البروتين الكلـي المستخلـص مـن البذور
٣٨,٣٢٪ ، ٣٩,٤٪ ، ٥,٣٥ ، ١٧,٠٩ على التوالي .