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PROTEINS OF COTTONSEED *(GOSSYPIUM BARBADENSE)* : EXTRACTION AND CHARACTERIZATION BY ELECTROPHORESIS

By

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البروتينات البذرية للقطن *(GOSSYPIUM BARBADENSE)* الفصل والتمييز باستخدام تقنيات الإستشراد

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في هذا البحث درست البروتينات البذرية للقطن نوعياً وكمياً . تم إجراء الدراسة النوعية بإستخدام تقنيات الاستشراد . وقد أوضحت الدراسة الكمية للبروتينات المستخلصة بالماء المقطر ، الملح ، الكحول ، والقلوي أن كمية هذه البروتينات على التوالي ١,٠١ ± ١٢٦ ، ٦,٢٥± ٧ . و٦٧ ، ٤,٢ ٣٠,٧٨± ، ٣,١ ٧٩,٤± مليجرام لكل جرام دقيق بذري . وأعطت البروتينات المستخلصة بالماء على رقائق الجل بروتينات ذات أوزان جزيئية تتراوح بين ١٠ - ٢٠ كيلووالتن . إلا أن البروتينات المستخلصة بالمحلول المنظم في وجود أو غياب 2-mercaptoethonol اعطت بروتينات ذات أوزان جزيئية تتراوح بين ١٠ - ٥٢ كيلو والتن . وأوضح الفصل الكهربائي على رقائق الجل في اتجاهين متعامدين احدهما تحت ظروف غير اختزالية والآخر تحت ظروف اختزالية أن بروتينات القطن تحتوي على بروتين له روابط كبريتية ثنائية ذات وزن جزيئي ٤٥ كيلوم والتن . كما أوضحت الدراسة باستخدام Isoelectric Focusing أن البروتينات ذات الوزن الجزيئي ٤٨,٥٢ كيلو والتن نقطة تعادلها الكهربائي تتراوح بين $PH_{g.0}$-$PH_{g.5}$ ، بينما البروتينات الأخرى تتراوح نقطة تعادلها الكهربائي بين PH_5 - PH_7 الخرائط الجلية (Mapping Gels) أوضحت أيضاً أن البروتينات ذات الوزن الجزيئي ، ٤٨,٥٢ كيلووالتن تقع نقطة تعادلها الكهربائي بين $PH_{g.0}$ - $PH_{g.5}$.

Key Words: Globulin, Albumin, 2-Mercaptoethano (2-ME) Molecular weights (MW(s), Kilodalton (KD), Two dimension-PAGE (2-D SDS-PAGE).

ABSTRACT

The seed proteins of cotton *(Gossypium barbadense)* were quantitavely and qualitavely investigated. The qualitative study was carried out using different electrophoretic techniques ((PAGE, SDS-PAGE, Poro-PAGE, SDS-Poro-PAGE, 2-D SDS-PAGE, isoelectric focusing gels, and mapping gels). The quantities of the distilled water (albumin), salt (NaCl) (globulin), alcohol (prolamin) and alkaline solution (glutelin) wer 126.4 +/- 1.51, 6.7.06 +/- 6.25, 30.78 +/- 4.2 and 79.40 +/- 3.1 mg/g dry meal respectively. Water soluble protein gave MW(s) of the reduced and unreduced buffer extracted proteins lies between 10 and 52 KD Second dimension gel showed that cottonseed proteins contain a disulphid bonded polypeptide with MW(s) 45 KD. Isoelectric points of the major polypeptides (the ploypeptides with MW(s) weights 52 KD and 48 KD) were between 9 and 9.5. The other polypeptides had pI-values between 5 and 7. Mapping gels, however indicated that the major bands were highly homogeneous with pI-values between 9-9.5.

INTRODUCTION

Cotton *(Gossypium barbadense L.)* is a perennial shrub belonging to the family Malvaceae. Cotton seeds are predominant source of oils and fats. Its oil is used for human consumption[1].

Cotton is an important oilseed crop which provides plant proteins as by-products after seed oil extraction [2].

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These proteins were found to contain three major types of proteins having sedimentation constants of 2S, 7S, and 9S and existing in equal amounts [3-4]. The 7S and 9S proteins of cotton seeds are typical globulin storage proteins and have similar amino acid compositions [4]. The 2S proteins are albumin proteins [3-4]. Cotton seed major polypeptides have been purified and some of their properties were described[2,5,6].

As far is known all the published data were concerned with the major storage proteins. The present study therefore, is devoted to investigate the different component of the cotton seed proteins.

MATERIALS AND METHODS

A. Materials

Cottonseeds were obtained from Agriculture Research Center, Giza, Egypt. The seeds were broken in a mixer and the seed coats were removed manually. The seeds free of testa were ground well in a mortar and then defatted by three hexane extractions (10 ml hexane/g meal) for 2 hours with slow stirring in the refrigerator. After filtering off hexane, the meal was air dried, brushed through a sieve of 125 um (115 mesh) and then stored at -10 C until use.

B. EXTRACTION

Water and Buffer Extracts

30 mg (25 ug protein) of the defatted and fried meal was shaken with 500 ul distilled water containing 0.02 sodium azide (w/v) in Eppendorf tube overnight at room temperature. The mixture was centrifuged for 20 minute at 4 C and 10,000 hg (Heraues Christ Labofuge I-cooling centrifuge). Anther portion was extracted with 0.125 M Tris/ borate buffer pH 8.9 containing 0.02% sodium azide (w/v) as described for water extraction. Analogous extracts were made with 2-ME.

SDS-Extract

For SDS-extraction, 30 mg (25 ug protein) of the defatted and dried meal was shaken with 500 ul of an aqueous solution of 5% or 15% SDS (w/v) respectively in Eppendorf centrifuge tube for 20 minutes at 10,000 g and room temperature. The supernatant was used for electrophoresis. Analogous extracts were made with ME.

Urea-Extract

Samples of 30 mg (25 ug protein) defatted meal wer stirred for 30 minutes in an Eppendorf tube with 500 ul of an aqueous solution of 9 M urea, 2% ampholyte (v/v) phH 3-10 and 5% 2-ME (v/v). The mixture was centrifuged (20 min, 22 C, 10,000 g), and the supernatant was used for electrophoresis. Analogous extraction was done without 2-ME or 2-ME/ampholyte [7]. All the extracts are stored at -10 C.

Protein Determination

Distilled water (albumin), salt (NaCl) (globulin), alcohol (prolamin) and alkaline solution (glutelin) were extracted by the same protocol used by Shah and Stegemann [7]. Total proteins were determined by the same method of Lowry et al. [8] using bovine serum albumin as standard proteins.

D. Electrophoresis

Protein separation was carried out in vertical slabs using the LKB-2201 Vertical Electrophoresis Unit. The monomer mixture for 10% PAGE (v/v) contains 22.5 ml 1 M Tris pH 8.8, 19.5 ml of a mixture of 30% acrylamide (w/v) and 0.43% bisacrylamide (w/v), 14.5 ml distlled water, 20 ug ammonium persulfate and 30 ul TEMED.

Electrophoresis was performed in 17% SDS-PAGE (w/v) following the same protocol used by Abasary[6]. For the determination of the protomer MW(s) weights a mixture of the following marker proteins, treated with SDS, are used: transferrin human (76,000), bovine serum albumin (67,000), albumin egg (45,000), B-lactoglobulin (36,000), -chymotrypsinogen-A (25,700), myoglobin (17,200) and cytochrome-C (12,700).

Two SDS-PAGE was carried out according to the protocol used by Sammour [9]. In this protocol the extracted sample was analyzed in the first dimension on 12% SDS/ PAGE (w/v). The gels were stained with 0.05% Commassie Blue- R-250 (w/v) in 50% methanol, 7% acetic acid glacial and 43% distilled water overnight and destained by using the solvent of the stain (50% methanol: 7% acetic acid glacial: 43% distilled water)[10]. After destaining the track was cut with a sharp razor and left in sample buffer containing 5% SDS (w/v) and 2% 2-ME (v/v) for 20 minutes. The gel strip was inserted on 17% SDS-PAGE (w/v) and then subjected to 25mA for about 6 hours.

Poro-PAGE and SDs-PoroPAGE were carried out in a 6-26% gradient polyacrylamide (w/v) in 1.0 Tris/borate buffer. In SDS-PoroPAGE the gradient buffer contains 00.1% SDS (w/v). For the determination of the protomer MW(s) the same markers proteins used in SDS-PAGE were applied to SDS-PoroPAGE.

PAGIF was run in tubes (gel cylinders). Isoelectric focusing was carried out, as described by Stegmann *et al.* [11] on 6% ppolyacrylamide tube gels (w/v) containing 8 M urea.

Mapping: Isoelectric focusing in the first and SDS in the second dimension is run as described by Stegmann *et al.* [12] and Laemmli[10].

The gels wer stained with 0.05% Commassie Blue- R-250 (w/v) in 50% methanol, 7% acetic acid glacial and 43% distilled water overnight and destained by using the solvent of the stain (50% methanol: 7% acetic acid glacia: 43% distilled water) [10].

RESULTS AND DISCUSSION

The total seed proteins successively extracted with dis-

Table 1.
The protein contents of the albumin, globulin, prolamin and glutelin of the cotton flour.

Protein species	Quantity mg/g seed flour	Percent to the total protein content
Albumin	126.40 =/- 1.51*	41.60
Globulin	067.06 +/- 6.25*	22.09
Prolamin	030.78 =/- 4.20*	10.14
Glutelin	079.40 +/- 3.10*	26.15

Total protein content 303.64 mg/g seed flour
*This figure represents the mean and standard deviation of six readings.

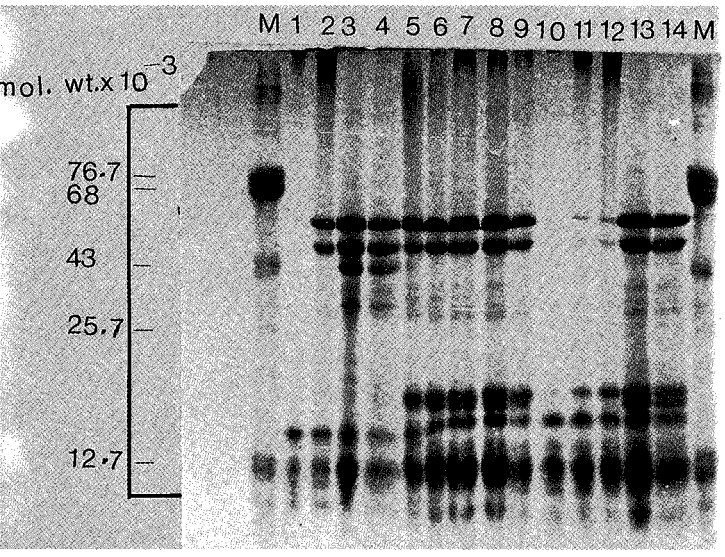
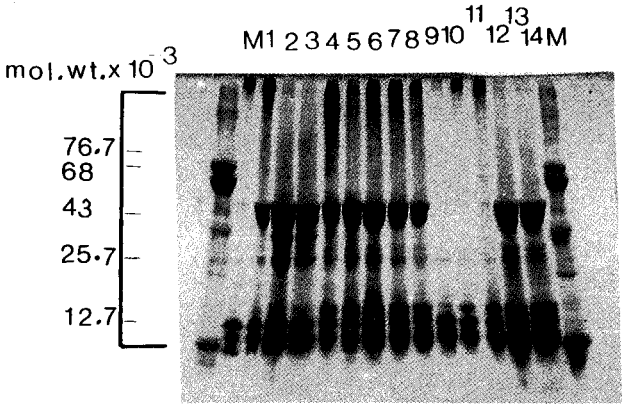


Fig. 1 SDS-Polyacrylamide gel electrophoresis (17% SDS-PAGE) of different extracts of cottonseed proteins. 1. water extract, 2. Tris/borate buffer (pH 8.9) extract, 3. 5% SDS extract, 4. 15% SDS extract, 5. Urea extract, 6. Urea/ampholyte extract, 7. Urea/2% ampholyte/2% 2-ME extract, 8. Urea/2% 2-ME 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 (Transferrin human, Bovine Serum Albumin, Ovaalbumin, Chymotrypsinogen-A, and Cytochrome-C).



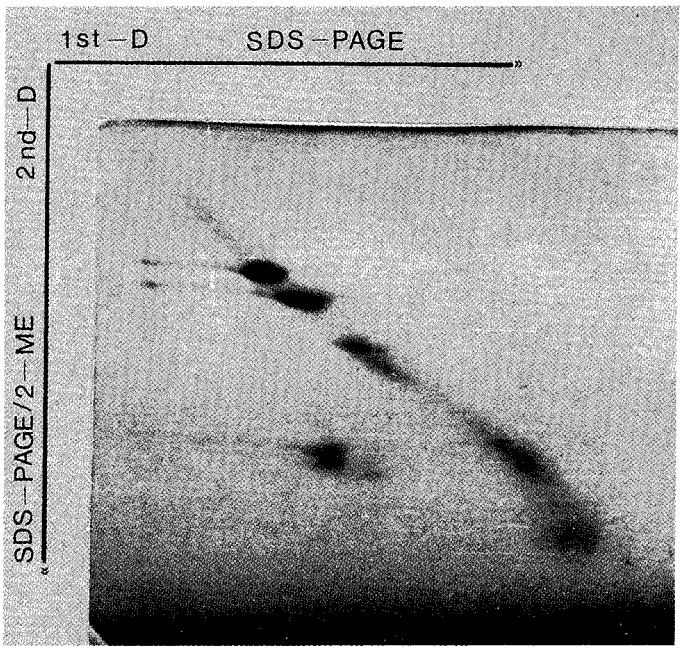


Fig. 2. Two dimensional SDS-PAGE of the total cottonseed protein extract. 1st-D: SDS-PAGE under non-reducing conditions, and 2nd-D: SDS-PAGE under reducing conditions.

Fig. 3. SDS-Porosity gradient polyacrylamide gel electrophoresis (6-26% SDS-PoroPAGE) of different extracts of cottonseed 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 30 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% s-ME extract, 14. 15% SDS/2% 2-ME extract, M. Protein standards (Transferrin human, Bovine Serum Albumin, Ovaalbumin, -Chymotrypsinogen-A, and Cytochrome-C).

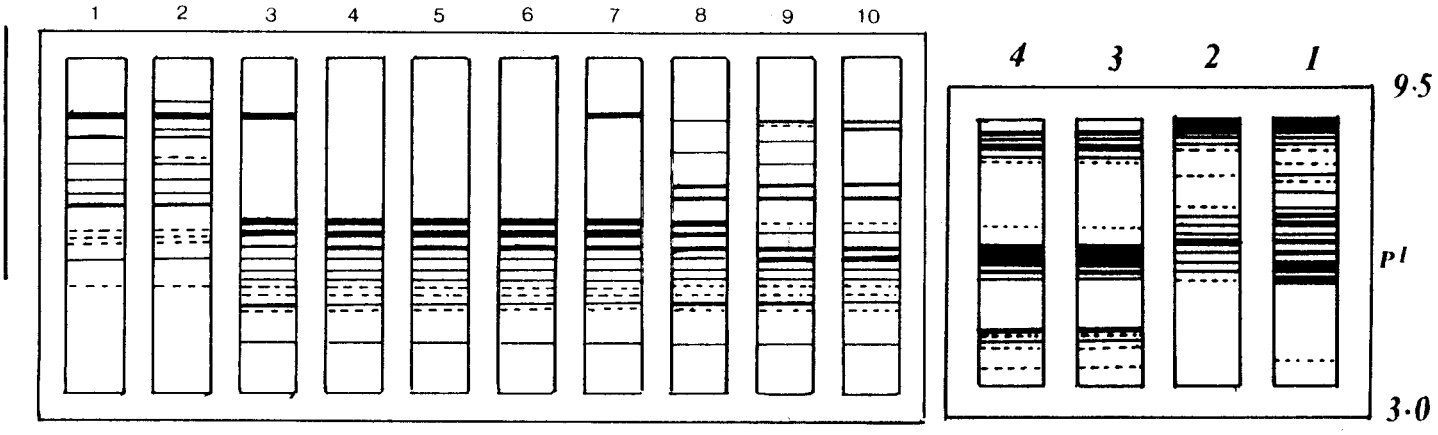


Fig. 4. Porosity gradient polyacrylamide gel electrophoresis (6-26% Poro-PAGE) of different extracts of cottonseed proteins. 1. water extract 2. Tris/borate buffer (pH 8.9) extract, 3. Urea extract, 4. urea/2% ampholyte extract, 5. urea/2% ampholyte/2% 2-ME extract, 6. urea/2% 2-ME extract, 7. urea/2% 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. 5. PAGIF in 6% polyacrylamide, 2% ampholyte pH 3-10. Samples of cottonseed meal extracted with urea (1), urea/2% ampholyte (2), urea/2% ampholyte/2% 2-ME (3) andurea/2% 2-ME.

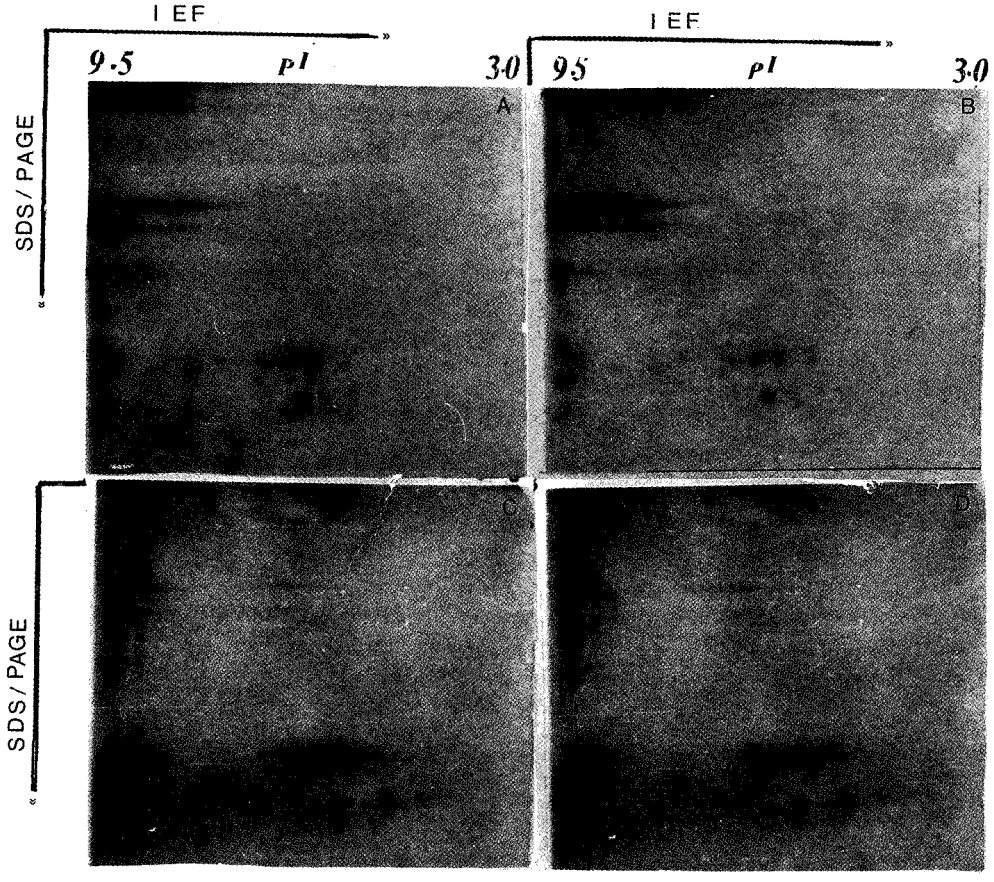


Fig. 6. Mapping of cottonseed proteins extracted with the following solvents: A) urea, B) urea/2% ampholyte, C) urea/2% ampholyte/2% 2-Me, D) urea/2% 2-ME.

tilled water (albumins), salt (NaCl) (globulins), alcohol (prolamins) and alkaline solution (glutelins) were determined by Lowry *et al.* method [8]. A protein content of 126.4 +/- 1.51 mg/g and 67.06 +/- 6.25 mg/g seed meal was found in the water and salt extract respectively. The protein extracted with the alkaline solution showed a slight increase compared to the protein extracted with the salt solution (Table 1). No protein analysis can be carried out in extracts containing ampholyte, since the ampholytes complex with copper ions [7]. A protein content in cottonseed from 28% to 32% (w/w) has been reported from different authors [3-4]. This quantity agrees well with our findings. However the claim that albumins in cottonseed represented 33% (w/w) of the total seed proteins [4] contradicted the data obtained in the present study which indicated that albumins represented 41.65% (w/w) and globulins 22.09% (w/w) of the total seed proteins. This contradiction could be due to methodological or varietal differences.

In SDS-PAGE the extraction with distilled water gave few number of bands with MW(s) less than 20 KD (Fig., lane no. 1). The protomer of the buffer extracted proteins contained the major polypeptides (52 KD and 48 KD) which were purified and biochemically characterized by Dure and Chlan [5] (Fig. 1, lane no. 2). Interestingly similar protein patterns were seen in the water or buffer extracted in the presence of 2-ME (Fig. 1, lane no. 12-13). The protomers of the proteins extracted with SDS were characterized with two unique polypeptides with MW(s) weights 45 KD and 39.5 KD (lane no. 4 and 5). The appearance of these polypeptides in SDS extracts may be due to the ability of SDS to break the hydrogen bondings which bind the membrane proteins to phospholipids in cell membranes. Thes polypeptides therefore are likely to be cell membrane-binding proteins. In the presence of 2-ME the polypeptide with MW(s) 45 Kd disappeared and new band with MW(s) 23.5 KD was shown on the gel (lane no. 14 and 15). This suggested that this polypeptide is a disulfide bonded protein. Extraction with urea alone or urea/2-ME in the presence or absence of 2% ampholyte (v/v) gave similar protomers (lane no. 6-10). The presence of the band with MW(s) 23.5 KD in the protomers of urea/ampholyte extract suggested the dissociating and reducing nature of the ampholyte.

Two dimensional SDS-PAGE gel was done for further resolution (especially for the band with MW(s) 45 KD, first by SDS-PAGE, and in the second dimension by subjecting the gel strips before electrophoresis to 2-ME for 20 minutes (Fig. 2). As shown in this figure the polypeptide with MW (s) 45 KD was cleaved under reducing conditions to two subunits with MW(s) 22 KD and 23 KD. These subunits were come off the diagonal confirming the legumin-like protein nature of this protein. By analogue to legumin proteins of Pisum a hexameric structure is suggested to the legumin-like protein of cotton[13].

In SDS-PoroPAGE (Fig. 3) the protomers of all extracts were very similar to those of SDS-PAGE in Fig. 1.

PoroPAGE revealed similar patterns with water and Tris/borate buffer extracts (Fig. 4 lane no. 1-2). After reduction with 2-ME, the pattern changes: the number of bands increase especially the higher MW(s) bands (lane no. 8-10). Meal extracted with urea or urea/2-ME (boiled for 3 minutes) in the presence of ampholyte (lane no. 4-5) gave identical electrophoretic pattern. In the sample extracted with urea with or without 2-ME one strong band with high electrophoretic mobility was seen (lane no. 3 and 7).

PAGIF was carried out between pH 3-10 in cylindrical tubes containing 8 M urea. PAGIF-patterns of water or buffer extract did not allow for good differentiation or clear separation of the band. This may be attributed to the presence of phytic acid in the extracts. Same thing has happened with *Vicia faba* and flax cultivars, and potato tubers [14-15]. the PAGIF-pattern of urea or urea/ampholyte extract showed a wide range of variations in bands distribution (Fig. 5), extending from strong alkali to slight acidic [5]. However, the extraction in the presence of 2-ME had no effect on the major polypeptides, but influence the other bands to accumulate at pI-values between 4 and 7 (lane no. 5-6).

Mappings were done for further resolution, first by PAGIF, and in the second dimension by SDS-PAGE. These experiments (Fig. 6) revealed that polypeptides with MW(s) 52 KD and 48 KD were homogeneous with pI-values between 9-9.5. However the other bands were highly heterogeneous showing a great number of dots (Fig. 4, A,B,C and D). The mappings of the PAGIF-gels of urea/2-ME in the presence or absence of ampholyte showed a slight decrease in the number of dots with the increase in its density.

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