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QUALITATIVE STUDY ON SEED PROTEINS OF THYMELAEA HIRSUTA L. POPULATIONS

BY

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ABSTRACT

Thymelaes Hrisuta seeds were shown to contain a major globulin protein of mol. wt. 300000 containing disulphide-linked pairs of subunits of mol. wt 600000 and 55000. These subunits were found to be heterogenous, containing no covalently bound carbohydrate. On reduction, each subunit was cleavaged into four components with mol. wt. ranged from 37000 to 35500, and from 24000 to 19500 . Analysis of seed protin in the first dimension under non-reducing conditions and in the second dimension under reducing conditions displayed four subunit pairs of disulphide-linked pairs. A major albumin protein was consisted of two subunits with mol. wt. 75000, and 70000, other albumin proteins present in significant amount had lower mol. wt.

Qualitative study of the seed population of T. hirsuta proved no significant variation in the seed proteins in each, and indicated that T.hirsuta is genetically stable.

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INTRODUCTION

Despite the economic importance of Thymelaea hirsuta [13,1], no work has been carried out on its seed proteins.

The present study therefore is intened to provide a more thorough characterization of the major seed proteins. Indetifaction of the protein species present would allow an understanding of the qualitative variation in seed proteins of diffefent populations of T. hirsuta and to set a comparison between T. hirsuta proteins with those of other Thymelaeaceae species, therefore relationships between species can be deduced.

MATERIALS AND METHODS

Materials:

T. hirsuta seed were collected from the coastal dunes, non-saline, saline, inland ridge and plateau habitats in mid of 1986 .

Sephadex G-150 was botained from Pharmacia (G.B.) Ltd, london W5 5 SS, U.K., Ultrogel AcA 22 from LKB instruments Ltd, South Croydon Surrey, CR2 8YD, U.K. All other chemicals were botained from BDH Chemicals Ltd. Poole, Dorset, BH12 4 NN, U.K. and were of " Analar" garde or the

purest avaliable.

Methods:

Extraction procedures:

The seed of T. hirsuta seed was eleminated after soaking seeds in 95% H_2SO_4 for 10 minutes and washing thoroughly by running water for 10 minutes. The uncoated seeds were dried under vacuum for 1h before milling for 30s in a ball mill. Meal was extracted for 2h at room temperature at a concentration of 100 mg m^{-1} buffer with 0.1 M potassium phosphate buffer, pH 8.0, containing 0.4 M NaCl, with or without 2-mercaptoethanol (2% v/v) or with 0.2 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) sodium dodecyl sulphate (SDS) again with or without the addition of 2-mercaptoethanol (2%, v/v). Total seed proteins were also extracted with H_2O and the water extracted residue was re-extracted with 0.125 M tris/ borate buffer, pH 8.9. The extracted samples were analysed under dissociating conditions. Potassium phosphate buffer without 2-mercapto-ethanol was used as extractant in the preparation of globlins and albumins which were separated by dialysis of the extractant against 33mM sodium acetate buffer, pH 4.8 at 4°C for 20 min, at 4°C resuspended in distilled water, and lyophilized. The supernatant solution of albumins was lyophilized.

Gel electrophoresis:

Non-dissociating polyacylamide gel electrophoresis (PAGE) and SDS-PAGE were carried out in gel slabs according to the method Laemmli [8] . Gel slabs were calibrated with the following protein subunits as mol. wt. standards: transferrin (76600): bovine albumin (67000); ovalbumin (43000). chymotrypsinogen (25700); myoglobin (17200); cytochrome C (12400). 10% and 17% polyacrylamide gels were used. The presence of disulphide bonded polypeptides was confirmed by cutting stained track from a first gel run under non-reducing conditions and incubating for 1h at room temperature in electrophoretic sample buffer containing 2%(v/v) 2- mercaptoethanol before loading the gel slice into the well of the second dimension gel.

Gel were stained for protein-linked carbohydrate with thymol-sulphuric acid [11] . Phaseolin was used as a positive control and pea legumin as a negative control .

Ammonium sulphate fractionation :

Solid ammonium sulphate was added to the desired concentration of protein extracts in phosphate buffer without 2-mercaptoethanol, prepared as above. After stirring at 4°C for at least 2h, precipitate and supernatant were separated by centrifugation at 23000x g at 4°C for 20 min.

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Gel filteration and purification of major globulin:

Gel filteration of a protein extract in potassium phosphate buffer without 2-mercaptoethanol was performed on a column of sephadex G-150 (32 mm dia., 350 ml. vol.) equilibrated with extraction buffer. The column was eluted at a flow rate of 23 ml h^{-1} and 10 ml fractions were collected. Fractions were pooled , dialysed against water, and hyophilized for analysis by SDS-PAGE .

The major globulin protein was purified by gel filtration of a solution of globulin protein on the sephadex G-150 column under the above conditions. Appropriate fractions were pooled , dialysed against water, and lyophilized.

Molecular weight determination:

A column of Ultrogel AcA 22 (LKB) 75 cm x 2.2 cm, in 0.1 M Tris / HCl, pH 8.0. , containing 0.25 M sodium chloride and o.o5% (w/v) sodium azide was calibrated with the following mol. wt. markers: rabbit immunoglobulins (150000), aldolase (160000), catalase (240000) pea legumin (400000), ferritin (480000). Aproximately 15 mg purified major protein (from the G-150 column in 5 ml buffer) was applied to the column and eluted by upward flow at the rate of 10 ml h^{-1}. A protein extract as above was also chromatographed on this column .

RESULTS

[illegible]alysis of total extratable proteins

Mature dry *Thymelaea hirsuta* seeds were extracted [illegible] four different [illegible] of conditions, Tris/HCl, pH [illegible] buffer containing [illegible] SDS, without 2-mercaptoethanol [illegible] [illegible] phosphate buffer, pH [illegible].0, containing [illegible] with or without 2-mercaptoethanol (2[illegible] [illegible] were [illegible] by SDS-PAGE. The [illegible] were [illegible]tially [illegible] (results [illegible] shown), but [illegible] the [illegible] or absence [illegible]

[illegible]

fr[illegible] into globulins and albumins by [illegible]

3 3 mM sodium acetate buffer, pH 4.8. The precipitated (globulins) and suspernatant (albumins) were separated, and analysed by SDS-PAGE. Results were shown in Fig. 2A. The major subunits at 60000 and 55000 mol.wt. in the globuin fraction, as were those of 36500, 21000, and 20000 mol. wt. subunits. Albumin subunits were present in two groups of bands; one with mol. wt. around 75000 and the other with mol. wt. around 12000.

Protein extractable by phosphate buffer/saline were fractionated by ammonium sulphate precipitation. Albumin fractions were largely precipitated at 70% saturation, whereas the major globulins were only found in the precipitate and supernatant at 90% , being soluble at 80% saturation.

Gel filtration of phosphate buffer/saline soluble protein on Sephadex-G-150 was carried out. The elution profile is shown in Fig. 2B. The eluted proteins were divided into several fractions on the basis of order of elution as shown in Fig. 2B, and each fraction was analysed by SDS-PAGE (Fig.2A). This showed that (i) the initial peak of protein eluted in the void volume contained relatively large amounts of the subunits of mol. wt. 75000, 70000 as well as the major globulin. (ii) the major globulin subunits of mol. wt. 60000, 55000 eluted predominantly after the

initial peak of the protein (fractions). (iii) the majority of the albumin proteins (subunits of mol. wt. 12000 and 10000), eluted in fraction 4. Similar results were obtained when an extract of Thymelaea hirsuta as above was chromatographed on a column of Ultrogel ACA 22 which has been calibrated with a standard protein.

2. Purification and some properties of the major globulin protein :

The major globulin proteins of Thymelaea hirsuta were purified by precipitation from an extract of Thymelaea hirsuta seed, redissolution of the precipitate, and chromatographing on Sephadex G-150. The resultant material contained subunits of mol. wt. 60000, 55000.

Molecular weight determination of Thymelaea hirsuta globulins was carried out by gel filtration on a calibrated column of Ultrogel AcA 22 at pH 8. The proteins were not resolved this system, but ran as a major peak of mol. wt. 300000 ± 20000.

Carbohydrate residues covalently attached to the polypeptide chain were not shown to be present in the 60000 and 55000 mol. wt. subunits by treatment of an SDS-PAGE analysis with phaseolin and bovine serum albumin (run on the same gel slab) were used as positive and negative controls.

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3. Variation in seed proteins of different populations of Thymelaea hirsuta L.

Defatted meals of T. hirsuta seeds, collected from a number of shrubs in each habitat, were separately extracted with Tris/HCl buffer and analyzed on 10% PAGE. The electrophoretic pattern of the seed protein of each individual showed four bands. The broadest band was the second one from the top of the gel. Analysis of the total seed proteins by PAGE technique indicated that there is no interpopulation or intrapopulation variations (data not shown).

The defatted meals of T. hirsuta seed of the five habitates were separately extracted with Tris/HCl buffer containing 2% SDS and analyzed on 17% SDA-PAGE under non-reduction and reducing conditions. Under non-reducing condition, the electrophoetic pattern of the seed proteins of non-saline habitat varies from the other habitats in the Rm of 28000 mol. wt. band, see Fig. 3A. However, under reducting condition the electrophoretic patterns of inland ridge and inland plateau habitats, in addition to the previous variation display a unique low mol. wt. band (Fig. 3B).

The total seed proteins of the five habitats were separately extracted with distilled water and the water

extracted residues were re-extracted with Tris/ borate buffer. This protocol of extraction was the same as that of Stegemann [15], who was able to differentiate among a number of cultivars of same species. The electrophoretic patterns of the water extracted proteins of the five habitats showed no variations (data not presented). The extracted residue was re-extracted with tris/ borate buffer, pH 8.9, and analysed on 17% SDS-PAGE (Fig. 4). Data exhibits that (i) there is an intrapopulation variations among T. hirsuta populations, (ii) The relative mobility of the major globulin is the same, whether extracted with Tris/HCl or Tris-borate buffers; (iii) The relative mobility of a number of bands of the globulin fraction varies from their comparable bands extracted with Tris/HCl buffer.

DISCUSSION

The major T. hirsuta globulin protein is mainly composed of two major bands; one with 60000 mol. wt. and the other with 55000 mol. wt. However, the use of two dimensional SDS-polyacrylamide gel electrophoresis, with non-reducing conditions in the first dimension and reducing conditions in the second dimension has proved that each major subunit is actually consisted of two subunits with the same mol. wt. therefore, each band was cleavaged by the addition of 2-mercoptoethanol into two acidic subunits with mol. wt. ranged from 37000 to 35500 and two basic

subunits with mol. wt. ranged from 24000 to 19500.

On the basis of the mol. wt. of the protein a hexameric structure seems likely, analogous to that proposed for Pisum legumin [16].

The major globulin of T. hirsuta is clearly similar to legume globulins of the Pisum sativum legumin type (i.e., 300000- 400000 mol. wt. (11S), subunit mol. wt. 60000- 55000 heterogeneous disulphide- subunits conaining no covalently bound carbohydrate).

Analysis of the total seed proteins of T. hirsuta L. populations (extracted with buffer) on SDS-PAGE under non-reducing and reducing conditions exhibited a limited intrapopulation variation. This variation might be attributed to the dioecious nature of T. hirsuta L. populations which enforces cross fertilization [3,2,5]. It has been reported that cross fertilization is biologically very important in providing a large number of gene pre-mutations [7,9,17]. These gene premutations enable the plant to overcome abrupt changes environmental conditions. Although, it might be speculated that intrapopulation variation in T. hirsuta L. populations is due the dioecious nature of the species, it can not be ruled out that this variation might be also attributed to somatic instability recorded

in T. hirsuta [5] , such somatic instability was found to have an effect on the morphological features in a number of species [14, 16, 6], and on the electrophoretic pattern of the total bulb proteins and acid phosphatase isozymes of the leaves of Urginia maritima (R.Sammour, unpulished data).

In 1983, Stegman found that using buffer ions that interfers with carbohydrate like borate ions governs part of the migration rate of proteins in PAG slabs. Data of the electrophoretic analysis of the residue of T. hirsuta L. populations, extracted with tris,borate buffer is found to be in good agreement with Stegemann finding. However, there is a methodological difference between the work reported here and that of Stegemann. Whereas Stegemann used tris/borate buffer as gel buffer , it was used here as an extractant buffer. It is of interest to find out the presence of borate ion, either in the extractant buffer [12] or in SDS-polyacrylamide gel slabs [15] , has an obvious effect on the migration rate of the glucoproteins. The constancy of the migration of major globulin of T. hirsuta L. populations, extracted either with Tris/HCl or Tris/borate buffers, supports the finding (presented in this work) that major globulin of t. hirsuta contains no covalently bound carbohydrate.

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FIGURE LEGENDS

Fig. 1A : SDS-PAGE of T. hirsuta proteins extracted in 0.1 M potassium phosphate buffer, pH 8.0 , containing 0.4 M NaCl. Track a: without addition of 2-mercaptoethanol; track b: with addition of 2% 2-mercaptoethanol. B. Two-dimensional SDS-PAGE analysis of total T. hirsuta proteins. First dimension (horizonal) unreduced (-2ME), second dimension (vertical) reduced (+2ME). Scales indicate mol. wt x 10^{-3} .

Fig. 2A : SDS-PAGE of albumin (track a) and globulin (track b) fractions of T. hirsuta proteins: analysis of fractions from gel filtration of T. hirsuta seed proteins on Sephadex-G-150 (Fig. 2B). Track c, fraction 1: track d, fraction 2; track e. fraction 3; track f, fraction 4.

Fig. 2B : Chromatography of T. hirsuta proteins on Sephadex G-150 column , elution profile of column.

Fig. 3 : SDS-PAGE of T. hirsuta proteins extracted in Tris/ HCl buffer, pH 6.8 A, under non reducing condition B, under reducing, condition 1, coastal dunes habitat; 2, non-saline habitat ; 4, inland

ridge habitat; 5, inland platesu habitat.

Fig. 4 : SDS-PAGE of T. hirsuta water residue extracted in tris/borate buffer, ph 8.9 , 1, coastal dunes habitat, 2; non-saline habitat, 3, saline habitat, 4, inland ridge habitat, 5, inland habitat.

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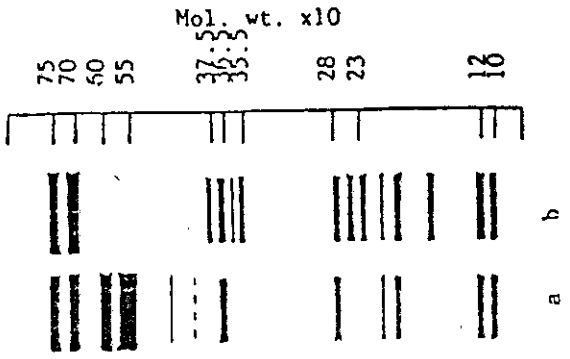

Fig. 1A

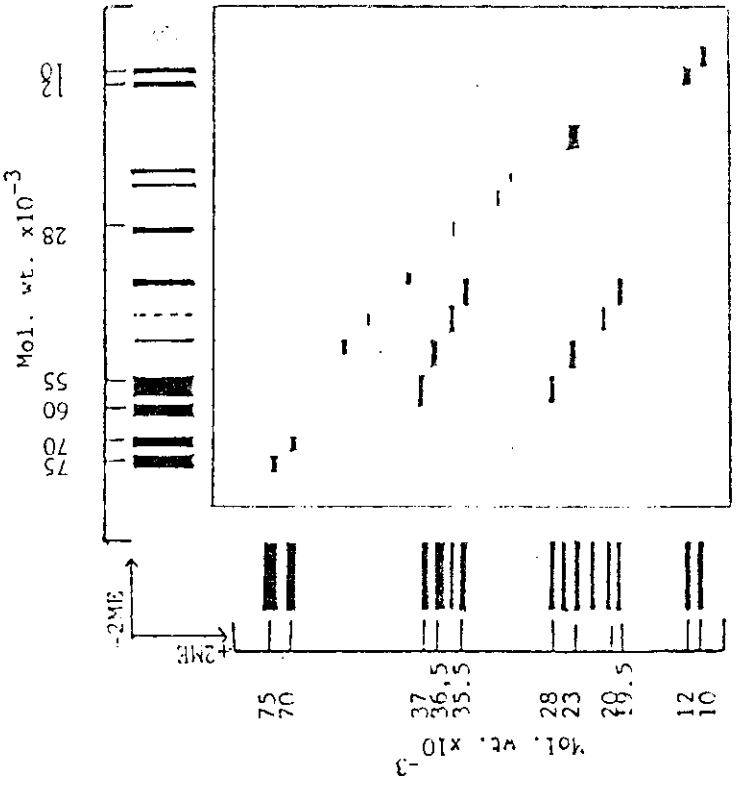

Fig. 1B

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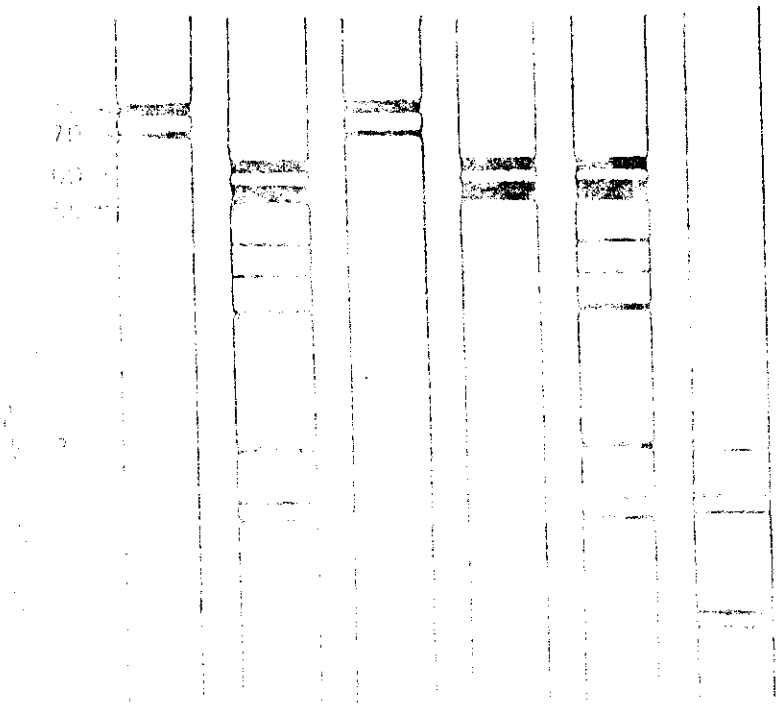

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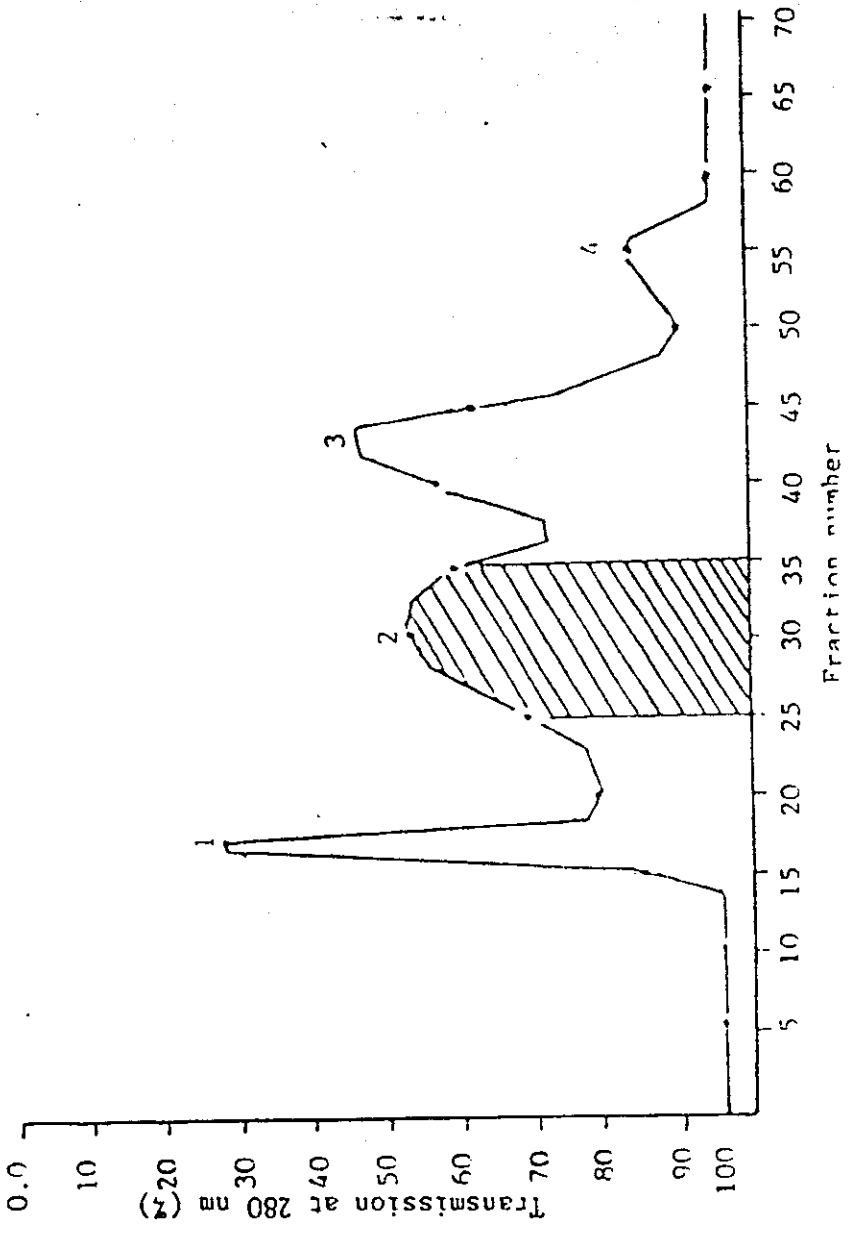

Fig. 2B

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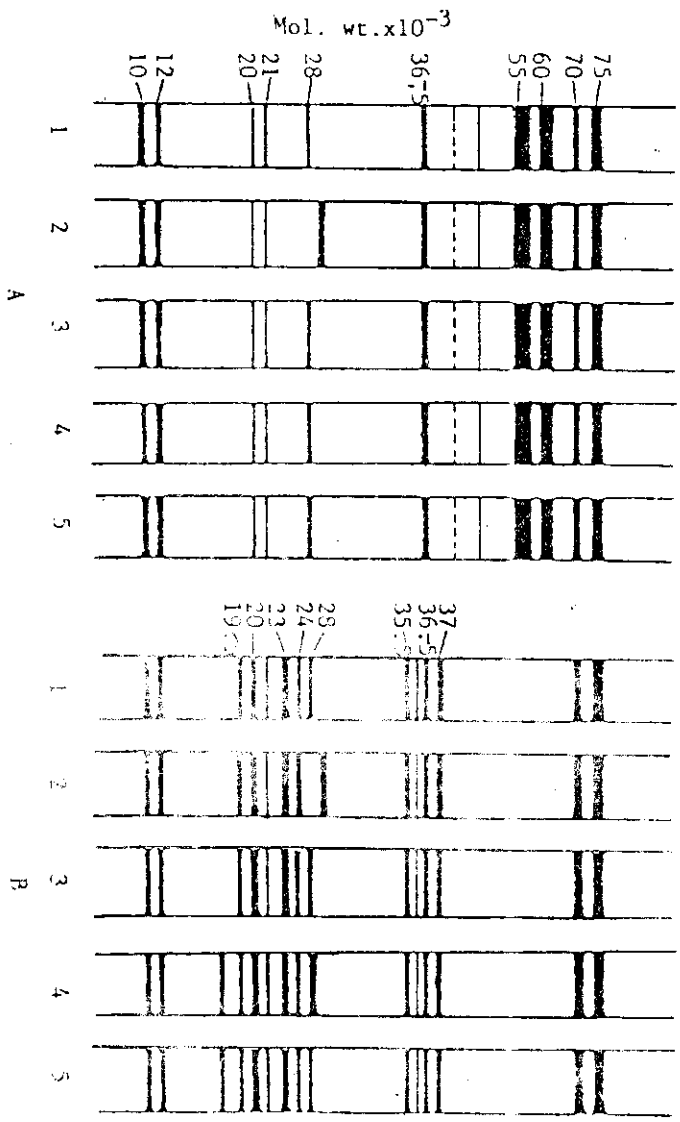

Fig. 3

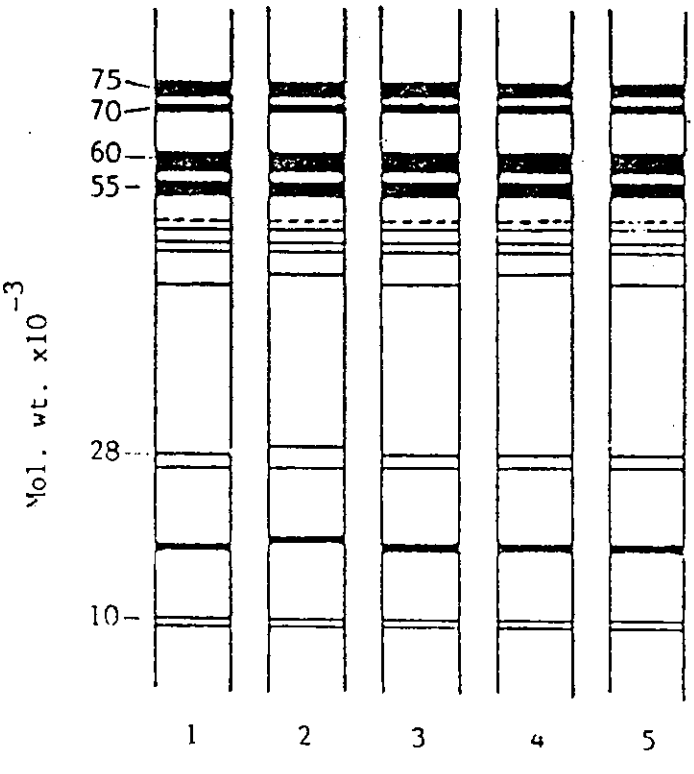

Fig. 4

دراسة نوعية على البروتينات البذرية لنبات المثنان

رضا حلمى أحمد سمور ، أحمد شرف الدين
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وجد أن البروتينات البذرية لنبات المثنان تحتوى على جلوبيلن بروتين ذو وزن جزيئ ٣٠٠٠٠٠ ويتركب من أزواج من التحت المرتبطة برابطة كبرتين مزدوجة وذات أوزان جزيئية ٦٠٠٠٠و٥٥٠٠٠ لقد وجد أن هذه التحت وحدات غير متجانسة كما أنها لا تحتوى على كربوهيدرات ٠ بالأختزال انقسمت كل تحت وحده الى أربع مكونات بأوزان جزيئية تتراوح من ٣٧٠٠٠ الى ٣٥٥٠٠ أو من ٢٤٠٠٠ الى ١٩٥٠٠ ٠ تحليل البروتينات البذرية فى الاتجاه الأول تحت ظروف غير أختزالية وفى الأتجاه الثانى تحت ظروف أختزالية أظهر أربع أزواج من تحت الوحدات المرتبطة بروابط كبريتية مزدوجة ٠ كما أظهرت الدراسة أن البروتينات البذرية لنبات المثنان تحتوى على البومين بروتين متكون من تحت وحدتين ذو وزن جزيئ ٧٥٠٠٠ ، ٧٠٠٠٠ على التوالى كما يتكون من عدد من التحت وحدات ذو أوزان جزيئية صغيرة ٠

الدراسة النوعية للبروتينات البذرية لنبات المثنان المجمعة من خمس أماكن متباينة بيئيا (المستخلصة بعدد من المحاليل المنظمه والمحلله تحت Dissociating and non-dissociating conditions) أوضحت عدم وجود تباين فى الطرز البروتينية بين المجاميع المختلفة كما أظهرت التبات الوراثى لنبات المثنان ٠