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Qualitative Study on Seed Proteins of *Thymelaea hirsuta* Populations

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With 4 Figures

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Summary

SAMMOUR R. H. & SHARAF EL-DIN A. 1989. Qualitative study on seed proteins of *Thymelaea hirsuta* populations. – Phyton (Austria) 29 (1): 83–92, with 4 figures. – English with German summary.

Thymelaea hirsuta L. seeds were shown to contain a major globulin protein of mol. wt. 300,000 containing disulphide-linked pairs of subunits of mol. wt. 60,000 and 55,000. 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 37,000 to 35,500, and from 24,000 to 19,500. Analysis of seed protein 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. 75,000 and 70,000, other albumin proteins present in significant amount had lower mol. wt. Qualitative study of the total seed proteins of the five populations of *Th. hirsuta* proved no significant variation in the seed proteins in eachs, and indicated that *Th. hirsuta* is genetically stable.

Zusammenfassung

SAMMOUR R. H. & SHARAF EL-DIN A. 1989. Qualitative Untersuchungen am Samenprotein verschiedener *Thymelaea hirsuta*-Populationen. – Phyton (Austria) 29 (1): 83–92, mit 4 Figuren. – Englisch mit deutscher Zusammenfassung.

Das in *Thymelaea hirsuta* L. – Samen hauptsächlich vorkommende Globulin (MG = 300.000) besteht aus zwei durch Disulfidbrücken paarweise verbundenen Untereinheiten von MG = 60.000 und 55.000. Diese heterogenen Untereinheiten

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enthalten kovalent gebundenes Kohlenhydrat. Unter reduzierenden Bedingungen wird jede Untereinheit in vier Komponenten von MG 37.000–35.500 bzw. 24.00–19.500 gespalten. Analyse unter nicht-reduzierenden Bedingungen in der ersten Dimension und reduzierenden in der zweiten ergaben vier Paar von Untereinheiten von disulfid-verbundenen Paaren. Ein größerer Anteil von Albumin setzt sich aus zwei Untereinheiten von MG 75.000 und 70.000 zusammen. Andere in nennenswerter Menge vorkommende Albumine weisen niedrigere MG auf. Die qualitative Untersuchung der Gesamtproteine der Samen von *Th. hirsuta* aus 5 Populationen ergaben keine signifikanten Unterschiede, womit sich *Th. hirsuta* als genetisch stabil erweist.

1. Introduction

Despite the economic importance of *Thymelaea hirsuta* (SARG 1965, EL-GHONEMY & al. 1979, AYYAD & EL-KADI 1982), no work has been carried out on its seed proteins.

The present study therefore is intended to provide a more thorough characterization of the major seed proteins. Identification of the protein species present would allow an understanding of the qualitative variations in seed proteins of different populations of *Th. hirsuta* and to set a comparison between *Th. hirsuta* proteins with those of other *Thymelaeaceae* species, therefore relationships between species can be deduced.

2. Materials and Methods

2.1. Materials

Seeds of *Thymelaea hirsuta* L. were collected from the coastal dunes, non-saline, saline, inland ridge and inland plateau habitats in Egypt in mid of 1986.

Sephadex G-150 was obtained 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 obtainend from BDH Chemicals Ltd. Poole, Dorset, BH12 4 NN, U. K. and were of „Analar" grade or the purest avaliable.

2.2. Extraction procedures

The seed coat of *Th. hirsuta* seeds 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 1 h before milling for 30 s in a ball mill. Meal was extracted for 2 h 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 withouth 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-mercaptoethanol was used as extractant in the preparation of globulins and albumins which were separated by dialysis of the extractant against

33 mM 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.

2.3. Gel electrophoresis

Non-dissociating polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE were carried out in gel slabs according to the method of LAEMMLI 1970. Gel slabs were calibrated with the following protein subunits as mol. wt. standards: transferrin (76,600); bovine serum albumin (67,000); ovalbumin (43,000), chymotrypsinogen (25,700); myoglobin (17,200); cytochrome C (12,400). 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 1 h at room temperature in electrophoretic sample buffer containing 2% (v/v) 2-mercapto-ethanol before loading the gel slice into the well of the second dimension gel.

Gel were stained for protein-linked carbohydrate with thymol-sulphuric acid (RACUSEN 1979). Phaseolin was used as a positive control and pea legumin as a negative control.

2.4. 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 2 h, precipitate and supernantant were separated by centrifugation at 23,000× g at 4°C for 20 min.

2.5. 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 m 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 hypophilized 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.

2.6. Molecular weight determination

A column of Ultrogel AcA 22 (LKB) 75 cm×2.2 cm, in 0.1 M Tris/HCl, pH 8.0, containing 0.25 M sodium chloride and 0.05% (w/v) sodium azide was calibrated with the following mol. wt. markers: rabbit immunoglobulins (150,000), aldolase (160,000), catalase (240,000) pea legumin (400,000), ferritin (480,000). Approximately 15 mg purified major protein (from the G-150 column in 5 ml buffer) was applied to the column and eluted by upward flow rate at the rate of 10 ml h^{-1}. A protein extract as above was also chromatographed on this column.

3. Results

3.1. Analysis of total extractable proteins

Mature dry *Thymelaea hirsuta* seeds were extracted under four different sets of conditions, Tris/HCl, pH 6.8 buffer, containing 2% SDS, with or without 2-mercaptoethanol (2%) or potassium phosphate buffer, pH 8.0, containing 0.4 M NaCl, with or without 2-mercaptoethanol (2%). The resulting extracts were analysed by SDS-PAGE. The protein subunit patterns were essentially the same whether or not SDS was present (results not shown), but differed greatly according to the presence or absence of 2-mercaptoethanol (Fig. 1A). Since some subunits were only present if 2-mercaptoethanol, the presence of disulphide bonded subunits was suspected. Two-dimensional gel electrophoretic techniques using non-reducing conditions in the first dimension and reducing conditions in the second dimension were used to show that subunits of mol. wt. 60,000 and 55,000 were cleavaged into four acidic subunits (α-subunits) with mol. wts. of 37,000, 36,500, 36,000, and 35,500, and four basic subunits (β-subunits) with mol. wts. of 24,000, 23,000, 20,000 and 19,500 (Fig. 1B). All subsequent SDS-PAGE was carried out under non-reducing conditions unless otherwise stated.

Protein extractable by phosphate buffer/saline were fractionated into globulins and albumins by dialysis against 33 mM sodium acetate buffer,

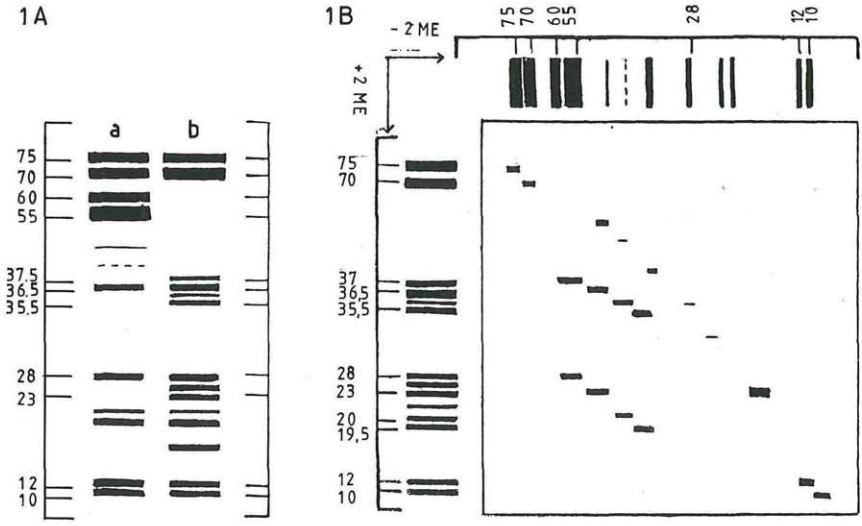

Fig. 1A: SDS-PAGE of *Th. hirsuta* proteins extracted in 0.1 M potassium phosphate buffer, pH 8.0, containing 0.4 M NaCl. Track a: without addition of 2-mercaptoethanol; track b: with addition of 2% 2-mercaptoethanol. 1B: Two-dimensional SDS-PAGE analysis of total *Th. hirsuta* proteins. First dimension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

pH 4.8. The precipitated (globulins) and supernatant (albumins) were separated, lyophilized, and analysed by SDS-PAGE. Results were shown in Fig. 2A. The major subunits at 60,000 and 55,000 mol. wt. found in the globulin fraction, as were those of 36,500, 28,000, 21,000, and 20,000 mol. wt. subuntis. Albumin subunits were present in two groups of bands; one with mol. wt. around 75,000 and the other with mol. wt. around 12,000.

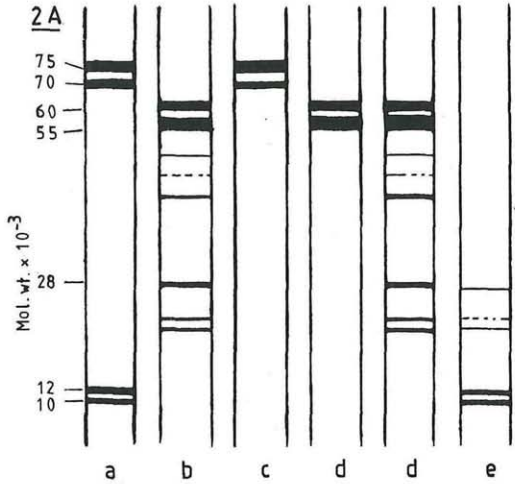

Fig. 2A: SDS-PAGE of albumin (track a) and globulin (track b) fractions of *Th. hirsuta* proteins; analysis of fractions from gel filtration of *Th. hirsuta* seed proteins on Sephadex-G-150 (Fig. 2 B). Track c, fraction 1; track d, fraction 2; track e, fraction 3; track f, fraction 4.

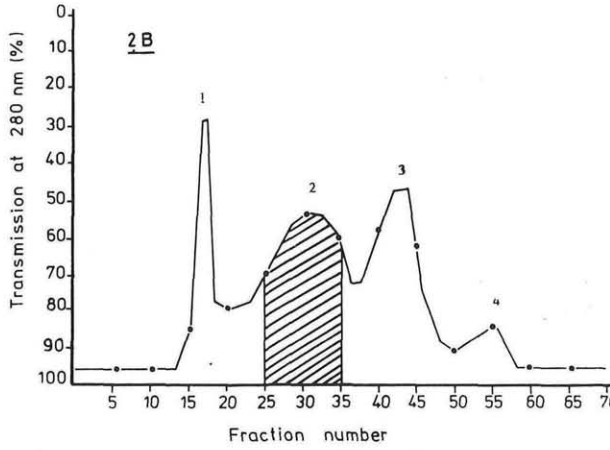

Fig. 2B: Chromatography of *Th. hirsuta* proteins on Sephadex-G-150 column, elution profile of column.

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. 75,000, 70,000 as well as the major globulin. (ii) The major globulin subunits of mol. wt. 60,000, 55,000 eluted predominantly after the initial peak of the protein (fractions). (iii) The majority of the albumin proteins (subunits of mol. wt. 12,000 and 10,000), eluted in fraction 4. Similar results were obtained when an extract of *T. hirsuta* as above was chromatographed on a column of Ultrogel ACA 22 which has been calibrated with a standard protein.

3.2. Purification and some properties of the major globulin proteins

The major globulin proteins of *Th. hirsuta* were purified by precipitation from an extract of *Th. hirsuta* seeds, redissolution of the precipitate, and chromatographing on Sephadex G-150. The resultant material contained subunits of mol. wt. 60,000, 55,000.

Molecular weight determination of *Th. hirsuta* globulins was carried out by gel filtration on a calibrated column of Ultrogel ACA 22 at pH 8. The proteins were not resolved by this system, but ran as a major peak of mol. wt. 300,000 ± 20,000.

Carbohydrate residues covalently attached to the polypeptide chain were not shown to be present in the 60,000 and 55,000 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.

3.3. Variation in seed proteins of different populations of *Thymelaea hirsuta*

Defatted meals of *Th. 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 speed 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 *Th. hirsuta* seeds of the five habitates were separately extracted with Tris/HCl buffer containing 2% SDS and analyzed on 17% SDS-PAGE under non-reducing and reducing conditions. Under non-reducing condition, the electrophoretic pattern of the seed proteins of

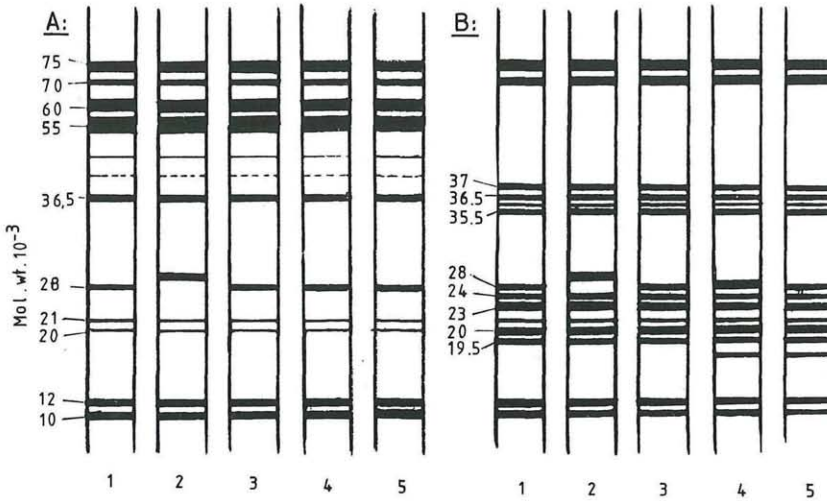

Fig. 3: SDS-PAGE of *Th. hirsuta* proteins extracted in Tris/HCl buffer, pH 6.8. A: under non reducing condition, B: under reducing, condition. 1, coastal dunes habitat; 2, non-saline habitat; 3, saline habitat; 4, inland ridge habitat; 5, inland plateau habitat.

non-saline habitat varies from the other habitats in the Rm of 28,000 mol. wt. band, see Fig. 3A. However, under reducing 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).

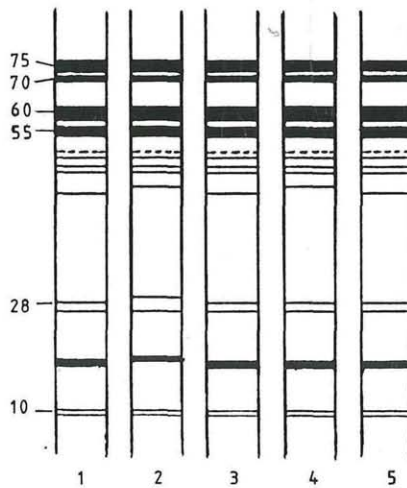

Fig. 4: SDS-PAGE of *Th. hirsuta* water residue extracted in Tris/borate buffer, pH 8.9, 1, coastal dunes habitat; 2, non-saline habitat; 3, saline habitat; 4, inland ridge habitat; 5, inland habitat.

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 1983, who was able to differentiate among a number of cultivars of the same species. The electrophoretic patterns of the water extracted proteins of the five habitats showed no variations (data not presented). The water extracted residue was re-extracted with tris/borate buffer, pH 8.9, and analysed on 17% SDS-PAGE (Fig. 4). Data exhibit 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.

4. Discussion

The major *Th. hirsuta* globulin protein is mainly composed of two major bands; one with 60,000 mol. wt. and the other with 55,000 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 37,000 to 35,500 and two basic subunits with mol. wt. ranged from 24,000 to 19,500.

On the basis of the mol. wt. of the protein a hexameric structure seems likely, analogous to that proposed for Pisum legumin (MATTA & al. 1981). The major globulin of *Th. hirsuta* is clearly similar to legume globulins of the *Pisum sativum* legumin type (i. e., 300,000–400,000 mol. wt. (11S), subunit mol. wt. 60,000–55,000 heterogeneous disulphide-linked subunits containing no covalently bound carbohydrate).

Analysis of the total seed proteins of *Th. hirsuta* populations (extracted with buffers) on SDS-PAGE under non-reducing and reducing conditions exhibited a limited intrapopulation variation. This variation might be attributed to the dioecious nature of *Th. hirsuta* populations which enforces cross fertilization (DONEE & al. 1984, DENELLE 1985 EL-KEBLAWY 1987). It has been reported that cross fertilization is biologically very important in providing a large number of gene pre-mutations (KALMUS & SMITH 1960, LEVIN 1975, WILSON 1979). These gene premutations enable the plant to overcome abrupt changes in environmental conditions. Although, it might be speculated that intrapopulation variation in *Th. hirsuta* populations is due to the dioecious nature of the species, it can not be ruled out that this variation might be also attributed to somatic instability recorded in *Th. hirsuta* (EL-KEBLAWY 1987). Such somatic instability was found to have an

effect on the morphological features in a number of species (SHIMAMOTS & HAYWARD 1975, TAN & DUNN 1977, EL-SADEK & ABD-EL-WAHID 1980), and on the electrophoretic pattern of the total bulb proteins and acid phosphatase isozymes of the leaves of *Urginea maritima* (SAMMOUR, unpublished data).

In 1983, STEGEMANN found that using buffer ions that interfere with carbohydrate like borate ions governs part of the migration rate of proteins in PAG slabs. Data of the electrophoretic analysis of the water residue of *Th. hirsuta* 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 that the presence of borate ion, either in the extractant buffer (SAMMOUR 1988) or in SDS-polyacrylamide gel slabs (STEGEMANN 1983), has an obvious effect on the migration rate of the glucoproteins. The constancy of the migration of major globulin of *Th. hirsuta* populations, extracted either with Tris/HCl or Tris/borate buffers, supports the finding (presented in this work) that major globulin of *Th. hirsuta* contains no covalently bound carbohydrate.

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