Chemistry of Natural Compounds, Vol. 31, No. 6, 1995

COMPARATIVE STUDY OF THE PHYSICOCHEMICAL PROPERTIES OF THE SPECIES-SPECIFIC PROTEINS OF COTTON PLANTS OF THE SPECIES *Gossypium hirsutum* AND *G. barbadense*

A. M. Érmatov, D. A. Khashimov, and P. Kh. Yuldshev

UDC 547.96:582.796

Four species-specific proteins have been isolated for the first time: H.0.43 and H.0.51 from a G. hirsutum *cotton plant, and B.0.37 and B.0.48 from* G. barbadense. *The conditions are given for their separation on Sephadex G-100 and on DEAE-cellulose and for their preparative electrophoresis in PAAG. Their isoelectric points and molecular masses have been determined and their subunit compositions have been investigated.*

One of the main tasks in the development of accelerated methods of selecting the parental forms of breeding and hybrid materials is the comparative investigation of the protein components of various species and varieties of cotton plant — their relative electrophoretic mobilities, isoelectric points, amino acid compositions, molecular structures, and biosynthesis — and the search for the main molecular markers responsible for particular characteristics.

A comparative analysis of the water-, buffer-, salt-, and acid-soluble fractions of proteins [1] of cotton plants of the species *G. hirsutum* and *G. barbadense* by electrophoresis in polyacrylamide gel (PAAG) by Davis's method [2] permitted the revelation of some differences in the protein spectra of the buffer- and acid-soluble fractions. The buffer-soluble fractions of both cotton plant species each contained two distinct proteins, the relative electrophoretic mobilities of which were 0.43 and 0.51 for *G. hirsutum* (H.0.43 and H.0.51) and 0.37 and 0.48 for *G. barbadense* (B.0.37 and B.0.48). The protein spectra of all the hybrids and varieties of the cotton plant obtained from the species *G. hirsutum* and *G. barbadense* showed the presence of the corresponding specific proteins. The same proteins were revealed in the protein spectra of the seeds of interspecies hybrids of the first and second generations [3].

The task of the present investigation was a comparative study of these proteins the results of which would show similarities and differences in their physicochemical properties and molecular structures, which, in their turn, would permit an estimate of the degree of their homology and, consequently, the degree of affinity of the two cotton plant species *G. hirsutum* and *G. barbadense* in the phylogenetic respect.

For the preliminary elimination of accompanying proteins and a considerable enrichment of the total proteins with the species-specific representatives, we carried out the fractional salting-out of the buffer-soluble proteins with ammonium sulfate. The seeds of a cotton plant of the variety Tashkent-1 (*G. hirsutum*) and of one of the variety S-6030 (*G. barbadense*) were ground and defatted with acetone as described previously [4]. Analysis of the protein fractions from the precipitate and the supernatant obtained by salting-out showed that, from 60% saturation onwards, trace amounts of the species-specific proteins of *G. hirsutum* and *G. barbadense* appeared in the precipitates. At 70% saturation the amount of species-specific proteins in the precipitate increased, and at 80% saturation, judging from the intensity of the staining of electrophoregrams, the amounts of species-specific proteins in the precipitate and in the solution had become approximately equal. With a further increase in saturation, the amount of species-specific proteins passing into the precipitate increased, but, even at 100% saturation, species-specific proteins remained in the supernatant.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 89 14 75. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 871-877, November-December, 1995. Original article submitted May 15, 1995.

0009-3130/95/3106-0734$12.50

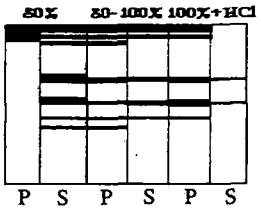

Fig. 1. Sketch of an electrophoregram of the stepwise salting-out of the species-specific proteins of two cotton plant species: P) precipitate; S) solution.

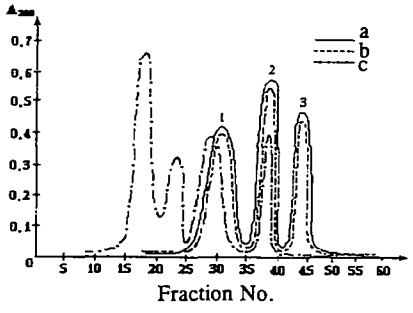

Fig. 2. Elution profiles on a column containing Sephadex G-100 of the proteins from a) *G. hirsutum*; b) *G. barbadense*; c) calibrating standards.

With an increase in the concentration of the Tris-HCl, pH 8.9, buffer for extraction from 0.05 to 0.1 M, the species-specific proteins did not pass into the precipitate at 80% saturation, but only at 90 and 100% saturation. When the concentration of ammonium sulfate was brought up to 100% saturation, the amounts of the H.0.43 and H.0.51 proteins (*G. hirsutum*) and the B.0.37 and B.0.48 proteins (*G. barbadense*) in the solution were still comparable with their amounts in the precipitate, and only after the pH of this solution had been brought to 6.0 (isoelectric precipitation) did the amount of species-specific proteins in the supernatant fall sharply (Fig. 1).

The optimum conditions for salting-out are the use for extraction of 0.1 M Tris-HCl, buffer, pH 8.9 (1:10 weight/volume), 80% saturation of a solution of the buffer-soluble fraction with ammonium sulfate and elimination of the bulk of the accompanying proteins, and further saturation of the solution from 80% to 100% followed by acidification to pH 6.0 in order to obtain a protein fraction considerably enriched with the species-specific proteins.

The next stage in the purification of the species-specific proteins was gel filtration on a column of Sephadex G-100 (Fig. 2).

Chromatography was conducted in 0.05 M NH_4HCO_3 solution, pH 8.3, and the proteins were separated into three fractions. From the results of electrophoresis in PAAG it was found that the species-specific proteins of both *G. hirsutum* and *G. barbadense* were eluted from the column with the second peak and that the distinctive proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) were not separated. It must be mentioned that the elution profiles of the proteins of *G. hirsutum* and of *G. barbadense* were completely identical, and on comparison with suitable calibrating proteins the peak of the species-specific protein fraction coincided with that of lactalbumin (18,000 kDa).

The species-specific proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) that had been enriched by salting-out were separated by chromatography on a column of DEAE-cellulose (Cl^- form) in a NaCl gradient (0 → 0.3 M). As can be seen from the profile of elution from the column, the behaviors of these proteins were very similar and they were clearly separated from other proteins but were not separated from one another — i.e., H.0.43 from H.0.51, and B.0.37 from B.0.48. Electrophoresis of the fractions obtained showed that these proteins were eluted from the column between the 150th and 200th fractions in the case of *G. hirsutum* and between the 160th and 210th fractions in the case of *G. barbadense* (Fig. 3).

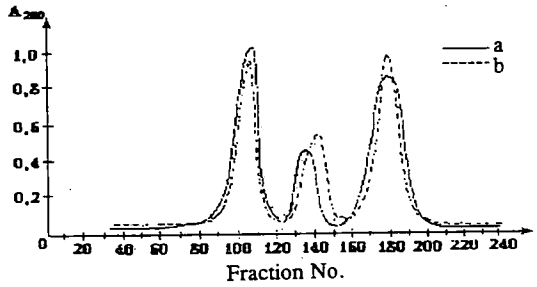

Fig. 3. Chromatography on a column containing DEAE-cellulose of the proteins from: a) *G. hirsutum*; b) *G. barbadense*.

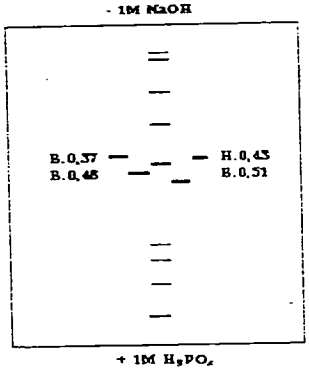

Fig. 4. Isofocusing of the species-specific proteins of *G. hirsutum* and *G. barbadense* on a PAAG plate in the presence of a standard set of proteins: cytochrome C (pI 10.65); ribonuclease (pI 9.45); whale myoglobin (pI 8.3); horse myoglobin (pI 7.3); conalbumin (pI 5.9); β-lactoglobulin (pI 5.34); bovine albumin (pI 4.7); ferritin (pI 4.4); aminoglucosidase (pI 3.5).

Similar behaviors of the species-specific proteins of *G. hirsutum* and *G. barbadense* were observed in chromatography on a column of CM-cellulose in a concentration gradient of acetate buffer, pH 4.5.

In the final stage of the purification and separation of the species-specific proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) we used preparative electrophoresis in PAAG. The proteins were extracted from the gel by electroelution and were then dialyzed and lyophilized. The individuality of the proteins was checked by analytical electrophoresis and isoelectric focusing.

The isoelectric focusing of the species-specific proteins was conducted on commercial PAAG plates with Ampholines having a pH range of 3.5-9.5 in parallel with a standard set of proteins having known isoelectric points (Fig. 4). On comparing the isoelectric points of these proteins it was established that they were very close to one another, that the H.0.51 (*G. hirsutum*) and B.0.48 (*G. barbadense*) proteins each gave a single band, and that their isoelectric points were between pI 5.9 (conalbumin) and 5.34 (β-lactoglobulin). The isoelectric points of B.0.37 (*G. barbadense*) and H.0.43 (*G. hirsutum*) were between pI 5.9 (conalbumin) and 7.3 (horse myoglobin). From the results obtained it was found that the H.0.43 and H.0.51 species-specific proteins of *G. hirsutum* had the isopoints 6.0 and 5.6 respectively, and the B.0.37 and B.0.48 proteins of *G. barbadense* had the isopoints 6.2 and 5.8.

We then investigated the behavior of these proteins on electrophoresis in the presence of sodium dodecyl sulfate (Na-DDS) and β-mercaptoethanol by Laemmli's method. As can be seen from Fig. 5, incubation even in 0.1% Na-DDS greatly changed the electrophoretic mobilities of all the proteins. The molecular masses of the proteins were determined by comparison

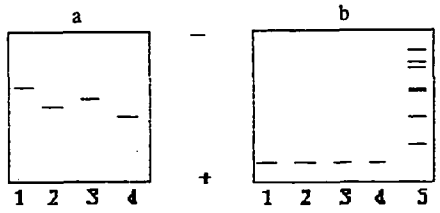

Fig. 5. Sketch of electrophoregrams of the native (a) and the denatured (b) species-specific proteins of *G. hirsutum* and *G. barbadense*: 1) B.0.37; 2) B.0.48; 3) H.0.43; 4) H.0.51; 5) calibrating proteins: phosphorylase B (94,000); albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,000); and α-lactalbumin (18,000).

with standard proteins (9 kDa). Gel filtration and electrophoresis showed that the native proteins each consisted of two polypeptide chains. The electrophoretic mobilities of the proteins did not change after treatment with β-mercaptoethanol. This indicated the absence of disulfide bonds between the polypeptide chains of the native protein molecules of both *G. hirsutum* and *G. barbadense*.

Thus, four species-specific proteins from two cotton plant species — H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) — have been isolated and purified for the first time. The optimum conditions for the isolation of the species-specific proteins have been selected. It has been established that in chromatography on Sephadex G-100, on DEAE-cellulose, and on CM-cellulose, the behaviors of the species-specific *G. hirsutum* and *G. barbadense* proteins are identical. The isoelectric points of the four proteins have been determined: for H.0.43, pI 6.0; for H.0.51, pI 5.6; for B.0.37, pI 6.2; and for B.0.48, pI 5.8. It has been found that the species-specific proteins of cotton plants of the species *G. hirsutum* and *G. barbadense* differ in their isoelectric points and relative electrophoretic mobilities. The molecular masses of the native species-specific proteins have been determined found to be 18,000 Da for each of the four. When the proteins were denatured the electrophoretic mobilities of all of them became the same. It has been found that the native species-specific proteins each consists of two identical polypeptide chains with molecular masses of 9 kDa.

EXPERIMENTAL

Seeds of cotton plants of the species *G. hirsutum*, variety Tashkent-1, and of the species *G. barbadense*, variety S-6030, were ground and defatted with cooled acetone until a flour passing through a 0.25 sieve was obtained.

Isolation of the Buffer-Soluble Proteins. The cottonseed flour (100 g) was extracted three times with 1-liter portions of distilled water to eliminate the bulk of the water-soluble proteins. The extract was separated by centrifugation at 3000 rpm for 30 min. Then the buffer-soluble proteins were extracted from the flour by suspending it in 1 liter of 0.05 M Tris-HCl buffer with pH 8.9 for 1 h. After centrifugation at 3000 rpm for 1 h, the deposit was discarded.

Salting-out of the Species-Specific Proteins. To seven aliquots (50 ml each) of an extract of buffer-soluble proteins was added ammonium sulfate to 40, 50, 60, 70, 80, 90, and 100% saturation, respectively, and the mixtures were left in a refrigerator until precipitates had formed. The precipitates were separated off by centrifugation at 3000 rpm and a temperature of 4-6°C for 20 min and were dissolved in the minimum volumes of distilled water; the solutions obtained were then dialyzed against water, and lyophilized. The supernatant solutions (10 ml portions) were also dialyzed against water and lyophilized. The salting-out process was monitored by analytical electrophoresis, with analysis of the precipitates and supernatants for their levels of species-specific proteins.

The resulting solution of buffer-soluble proteins was treated with 3 M Tris-HCl buffer solution, pH 8.9, to a final concentration of 0.1 M and was then 80% saturated with dry ammonium sulfate and left in the refrigerator until a precipitate had formed. This precipitate of accompanying proteins was separated off by centrifugation at 3000 rpm for 20 min at 4-6°C. The supernatant was 100% saturated with dry ammonium sulfate, and the pH of the solution was brought to 6 with 1 M HCl. The precipitate of species-specific proteins that deposited was collected by centrifugation, dissolved in the minimum amount

of distilled water, and dialyzed against distilled water. In the last change of distilled water, its pH was brought to 8-9 with aqueous ammonia in order to dissolve the proteins that had precipitated. The solution obtained after dialysis was lyophilized and was analyzed by electrophoresis in PAAG by Davis's method. The total yield of proteins amounted to 200 mg (0.2%).

Chromatography on Sephadex G-100. The total proteins enriched with the species-specific proteins obtained by salting-out were fractionated by gel filtration on a column (2.5 × 100 cm) of Sephadex G-100. A solution of 150 mg of the total proteins in 1 ml of 0.05 M NH_4HCO_3, was deposited on the column after it had been equilibrated with the same buffer. The proteins were eluted a rate of 4 ml/cm^2·h, 5-ml fractions being collected and their UV absorption at λ 280 nm being measured on a SF-46 spectrometer. Fractions corresponding to a single peak were combined, lyophilized, and analyzed by electrophoresis in PAAG. The yield of each of the species-specific proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) was 60 mg (40% of the total).

On elution from the Sephadex G-100 column (2.5 × 100 cm), the calibrating proteins formed four peaks. The first peak corresponded to Blue Dextran (2,000,000 Da) and immunoglobulin (160,000 Da) in the free volume because of their high molecular masses, the second peak to bovine serum albumin (BSA, 67,000 Da), and the third and fourth peaks to ovalbumin (43,000 Da) and lactalbumin (18,000 Da). The lactalbumin peak issued in the 35th-40th fractions, where the species-specific proteins of *G. hirsutum* and *G. barbadense* issued.

Chromatography on DEAE-Cellulose. Solutions of 200 mg of the total proteins enriched with the species-specific proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) in 5 ml of 0.05 M Tris-HCl buffer, pH 8.9, were deposited on a column (1.5 × 50 cm) of DEAE-cellulose previously equilibrated with the same buffer. The columns were washed with the initial buffer and the proteins were eluted by a linear concentration gradient of NaCl (0 → 0.3 M) in the same buffer. The total volume of the gradient was 600 ml and the rate of elution 27 ml/h, 9-ml fractions being collected. The fractions corresponding to a single peak were combined, dialyzed against distilled water, and lyophilized. The fractions were analyzed by Davis's method of electrophoresis in PAAG.

Chromatography on CM-Cellulose. After suitable pretreatment [6], a column of CM-cellulose (2.5 × 20 cm) was equilibrated with 0.05 M acetate buffer, pH 4.5. A solution in 10 ml of the initial buffer of 500 mg of the protein (*G. hirsutum* and *G. barbadense*) obtained on salting-out was deposited slowly (20 ml/h) on the column. The column was washed with the same buffer and the proteins were eluted with a concentration gradient of sodium acetate (0.05 → 0.5 M). The total volume of the gradient was 500 ml and the rate of elution 30 ml/h, 5-ml fractions being collected and their UV absorptions being measured. The fractions corresponding to a single peak were combined, dialyzed against distilled water, and lyophilized. The lyophilized proteins were analyzed by electrophoresis in PAAG according to Davis.

Separation of the Species-Specific Proteins by Preparative Electrophoresis in PAAG. After its purification on Sephadex G-100, the lyophilized fraction of the species-specific proteins H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) was separated by preparative electrophoresis in 7% PAAG by Davis's method. Polyacrylamide gel was prepared in a thickness of 3 mm with one long pocket for the protein solution. A solution of 30 mg of protein in 1 ml of 0.05 M Tris-glycine buffer, pH 8.3, was deposited in the pocket of the gel. Electrophoresis was conducted at a current of 30 mA for 2 h. The edge of the gel was cut off and was stained with a solution of Amido Black B in 7% acetic acid. The colored bands of the gel were applied to the main part of the gel from the two sides, and the bands corresponding to the species-specific proteins H.0.43, H.0.51, B.0.37, and B.0.48 were cut out and were then comminuted.

The proteins were extracted from the gel by electroelution. For this, small pieces of the PAAG corresponding to one protein were transferred to a special chamber filled with 0.05 M Tris-glycine buffer, pH 8.3, and closed at the bottom with a kapron [polycaprolactam] grid. Below, at a distance of 3 mm from the kapron grid a cellophane film retaining protein molecules but passing small ions was fixed. Between the kapron grid and the cellophane film in a frame of transparent plastic there was a side opening through which was poured a solution of the protein eluted from the pieces of gel under the action of a constant current (10 mA) for 24 h. Then the protein solution was dialyzed against distilled water and lyophilized. The species-specific proteins obtained — H.0.43 and H.0.51 (*G. hirsutum*) and B.0.37 and B.0.48 (*G. barbadense*) — were analyzed for homogeneity by electrophoresis in PAAG.

The isoelectric focusing of the proteins was carried out by the method of [4]. A commercial set of proteins with known isoelectric points (pI Marker Proteins; Protein Test Mixture 9, Serva) contained: cytochrome C (pI 10.65), ribonuclease (pI 9.45), whale myoglobin (pI 8.3), horse myoglobin (pI 7.3), conalbumin (pI 5.9), β-lactoglobulin (pI 5.34), bovine albumin (pI 4.7), ferritin (pI 4.4), and aminoglucosidase (pI 3.5).

Electrophoresis under denaturing conditions was conducted by Davis's method. The PAAG was 12% polymerized, and the calibrating proteins were phospholipase B (MM 94,000), albumin (MM 67,000), ovalbumin (MM 43,000), carboanhydrase (MM 30,000), trypsin inhibitor (MM 20,000), and α-lactalbumin (MM 18,000).

REFERENCES

1. D. A. Khashimov, B. D. Dzhalilov, and P. Kh. Yuldashev, Khim. Prir. Soedin., 661 (1986).
2. B. J. Davis, Ann. N.Y. Acad. Sci, **121**, No. 2, 404 (1964).
3. D. A. Khashimov, B. D. Dzhalilov, and P. Kh. Yuldashev, Khim. Prir. Soedin., 310 (1987).
4. A. M. Érmativ, P. Kh, Yuldashev, N. Zh. Sagdiev, and Sh. I. Salikhov, Khim. Prir. Soedin., 563 (1990).
5. U. K. Laemmli, Nature (London), **227**, 680 (1970).
6. L. A. Osterman, Chromatography of Proteins and Nucleic Acids [in Russian], Nauka, Moscow (1985).