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ISOLATION OF IMMUNOSIMILAR PROTEINS FROM COTTON SEEDS BY IMMUNOAFFINITY CHROMATOGRAPH

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A sorbent highly specific for Verticillium *proteins has been obtained from BrCN-Sepharose and rabbit immunoglobulins. By affinity chromatography using this sorbent a protein immunologically similar to the proteins of the mycelium of the fungus* V. dahliae *has been isolated from cotton seeds of the Tashkent-1 variety. The molecular mass of the protein has been determined, and its proteolytic activity has been established.*

At the present time, numerous facts have accumulated in animal and plant immunology indicating that, in the process of extracellular and intracellular parasitism, microorganisms acquire characteristics enabling them to remain and live in the host organismy for a long time. As early as 1937, T. I. Fedotova expressed the hypothesis that the parasitism of phytopathogenic organisms is possible only when they contain proteins serological similar to the proteins of the host plant [1]. This hypothesis has found confirmation in studies showing the appearance in pathogens of of molecules having similar antigenic determinants to the molecules of the host's tissues [2].

According to Vanderplank, similar mechanisms of the mutual recognition of proteins operate in the animal and vegetable kingdoms. The only difference is that an animal host uses the mutual recognition of proteins in order to render the pathogen harmless — i.e., as a means of immune protection. In the vegetable kingdom, pathogens of the group causing diseases of the gene-for-gene type use recognition of the same type in order to obtain nutriment from the host organism — i.e., as a means of attack [2]. According to this hypothesis, recognition of the host by the pathogen in the gene-for-gene" system amounts the recognition of an antibody by an antigen on the basis of a complementaruty of their surfaces.

The isolation and study of proteins of the host plant proteins immunologically similar to the proteins of the pathogen amounts to finding the pathways and mechanisms of the interaction of the pathogen and the host at the molecular level, which, in its turn, will permit an immunologically based approach to the choice of sources and donors of resistance.

In order to isolate and prepare cottonseed proteins of the Tashkent-1 variety immunologically similar to *Verticillium* proteins (ISPs) we have obtained an affinity sorbent from BrCN-Sepharose 4B and fractions of immunoglobulins G (IgGs) from an antiserum against *Verticillium* proteins. The preparation of the immunoaffinity sorbent, on the one hand, permitted a shortening of the scheme for the isolation of ISPs and, on the other hand, provided the possibility of the repeated use of the sorbent for isolating these proteins under identical conditions.

The water-soluble proteins of the pathogen *V. dahliae* were isolated from the frozen mycelium [3]. Rabbits were immunized and reimmunized with these proteins by a scheme modified by ourselves, and an antiserum with a titer of 1:64 was obtained [4]. Preliminary purification of the immunoglobulin fraction was achieved by fractionating the antiserum by four ammonium sulfate precipitations of the proteins. Further purification and the isolation of the IgGs were conducted on the anion-exchange resin DEAE-Sephadex A-50 [5].

The immunoaffinity sorbent was obtained by incubating BrCN-activated Sepharose 4B and the IgGs by Howen's method [6].

The water-soluble cottonseed proteins were deposited on a column with the immunosorbent and this was left overnight at 4°C. The nonbound proteins were eluted with 0.1 M phosphate buffer containing 0.15 M NaCl, pH 7.4. Protein concentrations were monitored spectrophotometrically by the Warburg–Christian method [7].

The immunosimilar proteins were eluted with 0.15 M NaCl acidified to pH 2.2 with 0.2 N HCl. The proteins then issued as a single peak.

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Electrophoretic analysis of the eluate showed the presence of one protein with a REM of 0.86. The molecular mass of the protein was determined as 12,000 Da by electrophoresis in 7% PAAG in the presence of 0.1% sodium dodecyl sulfate.

In a study of the functional properties of the isolated protein it was found that it possessed proteolytic activity, which was determined with the aid of a modified Anson method, using as substrate a 1% solution of azocasein [9]. The specific proteolytic activity of the ISPs was 1.24 units/mg of protein.

The amino acid composition of the isolated protein was determined. We give below the percentage yields of amino acids calculated on the weight of the protein sample: Asp — 8.25; Thr — 3.77; Ser — 4.88; Glu — 8.47; Pro — 5.6; Gly — 5.56; Ala — 2.5; Val — 3.1; Met — 0.35; Ile — 1.38; Tyr — 5.5; Phe — 3.87; His — 5.47; Lys — 3.4; Arg — 3.7.

The use of immunoaffinity chromatography for isolating the ISPs presented some difficulty, since the preparation of an immunoaffinity sorbent generally involves monospecific antisera, i.e., antisera to a definite protein. In the present case we used a heterogeneous antiserum, i.e., an antiserum to the total water-soluble proteins of *V. dahliae* of race II. In these circumstances, of course, the resolving capacity of the column was decreased, but the specificity of the method was retained, since proteins having the same antigenic derminants as the *Verticillium* proteins were sorbed. The amount of these proteins varies according to the concrete pathogen–host plant pair and depends on the vegetative organ from which the host-plant proteins were isolated. Under the given conditions of analysis, 20% of the total proteins deposited was specifically sorbed.

The immunochemical specificity of the protein obtained was checked by Ouchterlony's method of precipitation in an agarose gel [4]. The protein isolated gave a precipitation line with an antiserum against the water-soluble proteins of *Verticillium* mycelium.

The experimental results showing that the immunosimilar protein isolated possessed a proteolytic activity that correlated with existing information on the role of proteolytic enzymes and protease inhibitors in the mechanism of protective reactions in the plant organism [10].

Thus, the use of a heterogeneous antiserum against *Verticillium* proteins has permitted us to obtain from BrCN-Sepharose 4B an immunosorbent for isolating the immunosimilar proteins of cotton seeds. It has been established that the ISP isolated in this way from seeds of the Tashkent-1 variety of cotton possesses proteolytic activity, which serves as a direct indication of the role of proteolytic enzymes in the mechanism of the interaction of pathogen and plant host.

EXPERIMENTAL

The water-soluble proteins of *V. dahliae* were isolated from frozen mycelium of *Verticillium* of race II with a substrate–buffer mixture — 10% NaCl in phosphate buffer, pH 8.0, with the addition of 12% of glucose and 1% of ascorbic acid as antioxidants — in accordance with the methodological instructions of [3].

The proteins from a cotton plant of the Tashkent-1 variety were isolated from healthy and diseased seeds obtained from the G. S. Zaitsev Institute of Selection and Seed Production. After the grinding and defatting of the seeds, an acetone power was obtained, and this was extracted with 10% NaCl in phosphate buffer, pH 8.0, in a proportion of 10 ml per 1 g of sample in accordance with the methodological instructions of [3].

Rabbits were immunized over 6 months by a modified scheme at 20 points subcutaneously in the region of the neck and back and in the lymph nodes in the region of the rear extremities. The initial dose injected was 5 mg/ml in an equal amount of complete Freund's adjuvant.

The titer of the antiserum was determined as 1:64 by Ouchterlony's method in 1% agarose gel [4]. The IgGs were isolated from the antiserum by ion-exchange chromatography on DEAE-Sephadex A-50 [5]. Fourfold fractionation of the antiserum with ammonium sulfate at 50% saturation had been carried out beforehand.

The immunoaffinity sorbent was obtained by binding BrCN-activated Sepharose 4B with the antibodies of rabbit antisera against the *Verticillium* proteins [6]. The BrCN-Sepharose 4B was incubated at 4°C for 18 with the IgGs that had previously been dialyzed against 0.1 M acetate buffer, pH 5.2, in a proportion of 30-40 mg of protein per 1 g of Sepharose. After the binding reaction, the free groups of the Sepharose were blocked with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl. Before the binding of the immunosorbent with the proteins, it was washed with 0.1 M $NaHCO_3$, pH 8.5, and with 0.01 M $NaHCO_3$ containing 0.15 M NaCl. Then the sorbent was equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 0.15 M NaCl.

The freeze-dried water-soluble cottonseed proteins were dissolved in phosphate buffer, pH 7.4, deposited on a column of the immunosorbent, and left at 4°C for 18 h. The immunosimilar protein was eluted with 0.15 M NaCl acidified to pH 2.2 with 0.2 M HCl.

The molecular mass of the protein was determined by electrophoresis in 7.5% PAAG in the presence of 0.1 M sodium dodecyl sulfate in 0.1 M phosphate buffer, pH 7.2, according to Laemmli [8]. The following marker proteins were used: bovine serum albumin, MM 67,000 Da; ovalbumin, MM 45,000 Da; trypsin, MM 24,000 Da; and cytochrome C, MM 12,000 Da. The proteins were pretreated by boiling at 100°C for 5 min in the presence of sodium dodecyl sulfate. The were stained with a 0.2% solution of Coomassie R-250.

Proteolytic activity was determined with the use, as substrate, of a 1% solution of azocasein in 0.1 M phosphate buffer, pH 7.0 [9]

The amino acid analysis of the protein was performed on a T 339 aminoacid analyzer (Czechoslovakia). Acid hydrolysis was conducted with 6 N HCl at 110°C for 18 h.

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