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ISOLATION AND CHARACTERIZATION OF PROTEASE INHIBITORS FROM COTTON SEEDS

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Protease inhibitors of protein nature have been isolated from dormant cotton seeds. The participation of protease inhibitors in the mechanism of protecting the plant from wilt damage is discussed.

The functions of inhibitors of proteolytic enzymes in plant seeds are extremely diverse. Some authors consider that they fulfill the role of proteolysis regulators during growth [1]. Recently, more attention has been devoted to a point of view according to which one of the main functions of inhibitors is the protection of a higher plant from the proteolytic enzymes secreted by some pathogenic microorganisms and insects on contact with plant cells [2, 3].

We have investigated healthy and wilt-affected seeds of a cotton plant of the variety Tashkent-1. It had been shown previously that the proteolytic activities of enzymes in healthy and diseased cotton seeds differ sharply [4]. In seeds gathered from a plant 50% attacked by wilt, only 10-20% of the activity of proteolytic enzymes was retained, while the protein concentration in the extracts was unchanged. Apparently, in response to the intrusion of the pathogen into the plant, there is an increase in the amount of protease inhibitors which primarily inhibit their own proteases, and this explains the sharp decrease in proteolytic activity.

Our aim was to isolate the inhibitors from dormant cotton seeds and to study their properties. In order to isolate from cotton seeds individual protein inhibitors of trypsin and of papain we have developed a scheme of isolation somewhat differing from the generally adopted scheme for isolating inhibitors from seeds [5] by the fact that in the last stages of purification we used gel filtration on Acrilex, which permits the elimination of pigments and substances of low molecular weight, while the use of affinity sorbents enabled the inhibitors to be obtained with a high degree of purity.

The coats were removed from the cotton seeds, which were then ground and defatted with acetone and ether. The cottonseed flour obtained was extracted with phosphate buffer, pH 7.3, the mixture was centrifuged, and the proteins in the extract were precipitated with ammonium sulfate (80% saturation) and centrifuged off. The deposit was redissolved in distilled water, and the solution was dialyzed against 0.05 M acetate buffer, pH 5.5. This led to the isoelectric precipitation of proteins. Proteins with their isoelectric point in the acid pH region remained in solution. The precipitate contained globulins and, together with them, coprecipitated proteins having an inhibiting activity. The precipitate was dissolved in 0.05 M phosphate buffer, pH 7.3, and the solution was deposited on a column of Acrilex P-10. Elution was carried out with the same buffer at the rate of 90 ml/h.

Figure 1 shows an elution diagram of the separation of an inhibitor preparation. Fractions with a volume of 15 ml were collected and analyzed for protein content and inhibitory activity.

Protein inhibitors of proteases, particularly those of plant origin, have an extremely high affinity for the enzymes, and therefore the following stage of effective and selective purification was conducted on an affinity sorbent. Sorbents were synthesized by the addition of ligands (trypsin and papain) to Sepharose 4B by the cyanogen bromide method [6]. A highly purified preparation of the papain inhibitor was obtained with the aid of affinity chromatography on a column with the sorbent papain—Sepharose 4B, to which a solution containing the total inhibitors in 0.1 M $NaHCO_3$ was added. Elution was conducted with carbonate buffer, pH 6.6, with increasing ionic strength of the solution by the addition of NaCl. The nonspecifically sorbed proteins were eluted by a buffer containing 0.05 M NaCl (Fig. 2).

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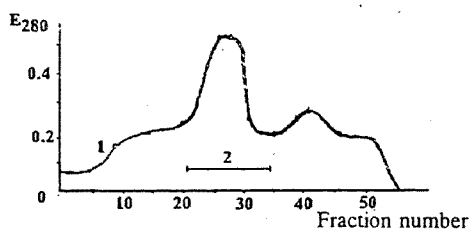

Fig. 1. Gel filtration of an inhibitor preparation on an Acrilex P-10 column: 1) optical density at 280 nm; 2) trypsin-inhibiting activity.

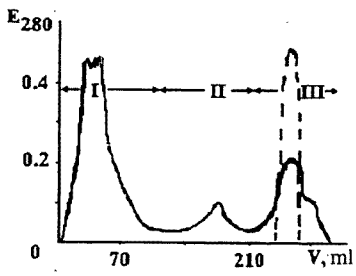

Fig. 2

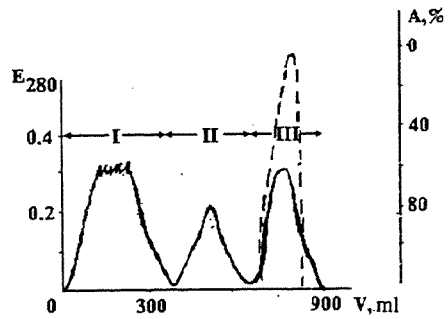

Fig. 3

Fig. 2. Affinity chromatography of the papain inhibitor from cotton seeds. Elution conditions: I) 0.01 M carbonate buffer, pH 6.6, and 0.05 M NaCl; II) 0.01 M carbonate buffer and 0.1 M NaCl; III) 0.01 M NaOH containing 0.1 M NaCl, pH 12.

Fig. 3. Affinity chromatography of the trypsin inhibitor from cotton seeds. Elution conditions: I) 0.1 M ammonium acetate, pH 7.5; II) 0.1 M ammonium acetate and 0.3 M NaCl, pH 7.3; III) 0.1 M ammonium acetate containing 0.5 M NaCl and CH_3COOH, pH of the solution 2.5.

The inhibitor was eluted with a 0.01 M NaOH solution containing 0.1 M NaCl, pH 12. The purity of the papain inhibitor obtained was determined by electrophoresis in 7% polyacrylamide gel in Tris buffer containing 0.1% SDS. On the electrophoretogram, the papain inhibitor was revealed in the form of a colored band the electrophoretic mobility of which in the presence of protein markers corresponded to a molecular mass of 12,000 Da.

The isolation of the trypsin inhibitor from cotton seeds on the affinity sorbent trypsin—Sepharose 4B is shown in Fig. 3 and has been described in [7].

A study of the action spectra of the inhibitor preparations isolated showed that in the presence of the papain inhibitor the rates of hydrolysis of casein and of hemoglobin by cottonseed protease B fell sharply. The proteolytic activity of protease B, determined by a modification of the Anson method [8], was 10% retained, while the activity of serine protease C isolated from dormant cotton seeds was suppressed completely by the trypsin inhibitor likewise isolated from cotton seeds. In addition, both inhibitors effectively suppressed the proteolytic activity of proteases of the mycelium of the fungus *Verticillium dahliae* — the causative agent of verticillium wilt in the cotton plant. The maximum effect of the suppression of the proteolytic activity of a mycelium extract with a protein concentration of 10 mg/ml was observed on the addition of an inhibitor preparation having the same concentration in a ratio of 10:1. On the addition of a solution of the papain inhibitor to a solution of the fungal mycelium extract its proteolytic activity decreasd by 30%, while the trypsin inhibitor suppressed proteolytic activity by 40%.

It is known that the nature of pathogen—plant interrelationships is determined mainly by cell proteins, and when there is no compatibility the pathogen cannot develop in the host plant [9]. The active influence of the papain and trypsin inhibitors

isolated from cotton seeds on the proteases of the mycelium of the verticillium fungus permits us to assume that cottonseed inhibitors take part in the mechanism of protection of a wilt-affected plant.

EXPERIMENTAL

Defatted and ground cotton seeds were extracted with phosphate buffer, pH 7.4. The protease inhibitors were isolated from the supernatant by salting-out at 80% saturation with ammonium sulfate. Then the inhibitors were purified with the aid of isoelectric precipitation at pH 5.5 and by gel filtration on Acrilex P-10. The concluding stage of purification was performed on an affinity sorbent (the elution conditions have been described above).

The activity of the inhibitors was estimated from the degree of suppression of the proteolytic activities of papain and trypsin, and also of proteases B and C from cotton seeds and the proteolytic enzymes of the fungus *Verticillium dahliae*. The activities of the enzymes were determined from their action on the substrate casein [8]. The concentration of the enzymes in the initial solutions for activity determination was about 10 mg/ml.

To determine the activities of the inhibitors, 0.2 ml of inhibitor solution was added to 1.8 ml of enzyme solution, and the mixture was kept for 10 min. Then 2 ml of the casein substrate was added and the test-tube was shaken and was incubated at 40°C for 10 min. After 10 min, 4 ml of trichloroacetic acid was added to stop the enzyme reaction and to precipitate the proteins and high-molecular-mass hydrolysis products. After this, the mixture was filtered into dry test-tubes.

A 1-ml sample of the filtrate was taken, and 5 ml of a 0.5 M solution of sodium acetate and 1 ml of the Folin reagent were added. After 20 min the absorption of the solution at 660 nm was determined on a SF-16 spectrophotometer. In a control test-tube, 4 ml of trichloroacetic acid was added immediately to a mixture of enzyme and inhibitor solutions and the further procedure was as described above.

Proteolytic activity was calculated from the formula

$$\text{ПЕ} = \frac{\text{Д} \cdot 4 \cdot 1000}{\text{ТЭ} \cdot 10 \cdot \text{М}} \quad *$$

The proteolytic activity of the enzyme was taken as 100%, and the degrees of suppression of the activity by the cottonseed trypsin and papain inhibitors were calculated correspondingly.

REFERENCES

1. S. N. Pokrovskii, N. Yu. Lobazina, and M. A. Belozerskii, Bioorg. Khim., **4**, No. 2, 269 (1978).
2. V. V. Mosolov, Vopr. Med. Khim., No. 5, 52 (1987).
3. Hara Saburo, Protein, Nucl. Acid and Enzyme, No. 8, 920 (1989).
4. M. I. Gartsbein, T. D. Kasymova, M. A. Kuchenkova, P. Kh. Yuldashev, K. Kh. Akhmedov, R. G. Kim, and Yu. T. Uzakov, Khim. Prir. Soedin., 704 (1987).
5. S. N. Pokrovskii and M. A. Belozerskii, Biokhimiya, **45**, 2104 (1980).
6. E. V. Lushnikova, N. V. Fedurkina, E. V. Sokolov, L. I. Krotova, and V. V. Mosolov, Methods of Modern Biochemistry [in Russian], Nauka, Moscow (1975).
7. T. D. Kasymova, K. T. Abdurakhmanova, and P. Kh. Yuldashev, Khim. Prir. Soedin., 134 (1990).
8. E. D. Kaverzneva, Prikl. Biokhim. Mikrobiol., **7**, No. 2, 225 (1971).
9. G. I. Borodin and V. I. Runov, The Physiological–Biochemical Principles of the Pathogenicity of the Causative Agent of Verticillium Wilt of the Cotton Plant [in Russian], Fan, Tashkent (1983).

*The symbols are not explained in Russian original — Translator.