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ISOLATION AND STUDY OF AN INHIBITOR OF THE INTRINSIC PROTEOLYTIC ENZYME OF COTTON SEEDS

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A scheme has been developed for the isolation of an inhibitor of cottonseed protease A using affinity chromatography on protease-A—Sepharose 4B followed by gel filtration on Sephadex G-150. The molecular mass of the inhibitor is 20 kDa. The protein molecule consists of two subunits with different molecular masses.

It is known that some trypsin and chymotrypsin inhibitors from higher plants possess the ability to suppress the activity of the proteases of phytopathogenic microorganisms, and also that of insect proteases [1, 2]. This fact has served as a basis for the assumption that these proteins may play a definite role in the protection of the plant from pathogens. Recently, reports have appeared on the interaction of inhibitors with the intrinsic proteolytic enzymes of plants [3, 4].

We have previously isolated a serine proteolytic enzyme — protease A — from dormant cotton seeds and have characterized it completely [5]. On studying its functional properties, we found that it hydrolyzes the native reserve proteins of the cotton plant — the 7S and 11S globulins. This enzyme has also been found in the germination of cotton seeds [6]. Continuing these investigations, we have attempted to obtain an inhibitor of serine proteinase A. In the present paper we report information on the development of a method for the isolation from dormant cotton seeds of a protein inhibitor of protease A, and some of its properties.

We isolated from dormant seeds of a cotton plant of the T-1 variety the total protein acting as an inhibitor of trypsin and chymotrypsin and also of the intrinsic protease A. The protease A inhibitor was isolated from the total protein mentioned above by affinity chromatography on synthesized protease-A—Sepharose 4B. The protein obtained with the aid of affinity chromatography was subjected to gel filtration on a column of Sephadex G-150. The protein was eluted in the form of a single main peak the elution volume of which corresponded to a molecular mass of 19.5 kDa. The yield of the protease A inhibitor was about 0.3%. Thus, the scheme of isolating the inhibitor includes: extraction with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl; affinity chromatography on protease-A—Sepharose 4B; and gel filtration on Sephadex G-150.

According to the results of ultracentrifugation, the molecular mass of the inhibitor was 20 kDa. The results of electrophoresis in 10% polyacrylamide gel at pH 8.3 showed that the protein isolated was homogeneous. The calculated value of the molecular mass on electrophoresis in polyacrylamide gel was again 20 kDa.

Experiments of the inactivation of the inhibitor by pepsin showed that it had a protein structure.

We studied the amino acid composition of the inhibitor isolated (Table 1). For comparison we give the amino acid composition of the Kunitz soybean inhibitor. Each of these inhibitors contains two methionine residues and no cysteine residues. The protease A inhibitor is distinguished by somewhat higher content of histidine, threonine, glutamic acid, and proline. The Kunitz inhibitor contains more glycine, valine, and isoleucine. The molecular mass of the protease inhibitor calculated from its amino acid composition is 19 kDa.

To study its subunit structure, the protein was subjected to electrophoresis in polyacrylamide gel in the presence of 8 M urea, sodium dodecyl sulfate (Na-SDS) and β-mercaptoethanol. The resulting electrophoretogram showed two bands, with molecular masses of 8 and 12 kDa. It follows from these facts that the molecule of the inhibitor consists of two subunits with different molecular masses. No cysteine residues were detected in an analysis of the amino acid composition, which indicated

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TABLE 1. Amino Acid Composition of the Protease A Inhibitor from Cotton Seeds

Amino acid, mols/ mole of protein	Protease A inhibitor	The Kunitz inhibitor
Lysine	6.32 (6)	10
Arginine	8.1 (8)	9
Histidine	4.9 (5)	2
Cystine	-	-
Aspartic acid	26.62 (27)	26
Threonine	10.7 (11)	11
Serine	-	-
Glutamic acid	36.3 (36)	18
Proline	12.9 (13)	10
Glycine	10.7 (11)	10
Alanine	8.7 (9)	8
Valine	8.9 (9)	14
Methionine	2	2
Isoleucine	4.05 (4)	14
Leucine	10.5 (11)	14
Tyrosine	4.5 (5)	4
Phenylalanine	10.6 (11)	9

that the separate subunits in the protein molecule could not be linked by disulfide bonds The high level of aspartic and glutamic acids, and also of aromatic and hydrophobic amino acids, permitted the assumption that the interaction between the individual subunits was effected as the result of ionic and hydrophobic interactions.

Peptide maps of the native and CM-molecules served as additional proof of the hypothesis expressed. The peptide maps were identical, with the exception of one peptide. In the amino acid composition of the inhibitor molecule we found 14 arginine and lysine residues and on the peptide map 13 peptides (Fig. 1), which confirmed the absence of S–S bridges in the molecule.

It is known from the literature that a resistant variety is characterized by a comparatively high content of inhibitors, while in a susceptible variety a high proteolytic activity and the absence of inhibiting activity are observed [7].

It was interesting to trace the influence of the protease inhibitor on the proteolytic activity of *Verticillium* proteins. It was found that the proteolytic activity was suppressed to the extent of 95%. These results are in harmony with the hypothesis that protease inhibitors play a not unimportant role in the interrelationships of pathogen and plant, taking part in the protection of the plant from bacterial and fungal diseases.

Thus, the inhibitor of the intrinsic serine protease A of cottonplant seeds plays an important role in the protection of the plant from verticilliosis.

EXPERIMENTAL

The conjugation reaction was performed by the following scheme: 1 g of BrCN-Sepharose 4B was steeped in water for 30 min. After swelling, the volume had increased to 3.5 ml. The swollen gel was transferred to a glass filter and was washed with 0.001 N HCl (200 ml). For neutralization, the gel was washed immediately with 0.1 M $NaHCO_3$ and 0.5 M NaCl buffer. Then it was transferred to a beaker (3.5 ml of swollen gel) and protease A was added in a calculated amount of 35 mg of protease A dissolved in 3.5 ml of buffer (0.1 M $NaHCO_3$ + 0.5 M NaCl) to 3.5 ml of resin, and the mixture was stirred at room temperature for 2 h or at 4°C overnight. The completeness of the conjugation reaction was determined from the optical

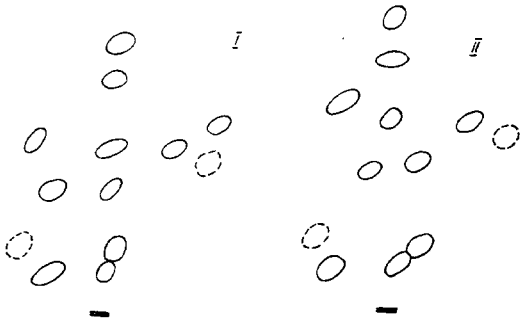

Fig. 1. Peptide maps of the native and carboxymethylated molecules of the protease inhibitor molecule.

density of the filtrate at 280 nm. The unblocked part of the active groups of the Sepharose 4B was blocked by washing with 0.2 M Tris buffer and 0.2 M sodium acetate buffer. The prepared conjugate was charged into a column with dimensions of 1 × 10 cm.

Isolation of the Total Inhibiting Fraction. An acetone powder (5 g) was extracted (1:10) at room temperature with 0.5 M NaCl and 0.05 N Tris-HCl buffer, pH 8.0, for 3 h and then (1:5) overnight at 5°C.

Gel Filtration. A solution of 10 mg of the inhibitor in 5 ml of physiological solution was deposited on a column (2.5 × 5.5 cm) of Sephadex G-100. Rate of elution 24 ml/h.

Electrophoresis in polyacrylamide gel was conducted by Davis's method [8].

Determination of the Amino Acid Composition. A 5-mg sample of protease A inhibitor was dissolved in 2 ml of 6 N HCl and hydrolysis was carried out in vacuum-sealed tubes at 110°C for 24 h. The hydrolysate was evaporated with the addition of fresh portions of water and was dried in a vacuum desiccator over alkali. Analysis was carried out on a AT-339 analyser (Czechoslovakia).

Reduction and Carboxymethylation of the Protease A Inhibitor. To 30 mg of the inhibitor in 5 ml of 8 M urea solution were added 3 ml of Tris-HCl buffer, pH 8.6, 0.3 ml of EDTA solution (50 mg/ml), and 0.1 ml of mercaptoethanol. The reaction was conducted in the dark with a magnetic stirrer in a current of nitrogen at room temperature. After 4 h, 0.2 g of monoiodoacetic acid was added to the mixture and its was neutralized with 1 M NaOH. After 45 min, the reaction was stopped by acidification to pH 3.1 with glacial acetic acid. The reaction product was dialyzed and freeze-dried.

The reduced and carboxymethylated inhibitor (10 mg) was dissolved in 2 ml of ammonium bicarbonate (pH 8.8), 1.2 ml of a solution of trypsin (Wartington [sic] (0.5 mg/ml) was added, and the inhibitor was digested at 37°C. After an hour, another 0.2 ml of trypsin was added. Digestion was continued for 24 h, the pH being maintained with ammonia. After the end of the reaction, the mixture was centrifuged and freeze-dried. The hydrolysate (10 μl) was deposited on a 20 × 20 cm plate with a thin layer of FND cellulose (Germany) and was chromatographed in the butanol–acetic acid–pyridine–water (15:3:10:12) system. After the plate had been dried, electrophoresis was conducted in pyridine acetate buffer, pH 8.6, for 35 min at a voltage of 1000 V and a current strength of 40 mA. The chromatograms were revealed with a 0.1% solution of ninhydrin in ethanol.

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