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PROTEASE A FROM COTTON SEEDS.

ISOLATION AND PURIFICATION OF THE ENZYME

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Protease A has been isolated in the homogeneous state from dormant seeds of cotton plants of the Tashkent-I variety. A scheme is proposed for the isolation and purification of the enzyme which includes the following stages: extraction of the defatted seeds with 0.1 M phosphate buffer, pH 7.4; precipitation of the protein with ammonium sulfate at 60% saturation; desalting by dialysis; and ion-exchange chromatography on a column containing CM- and DEAE-celluloses. The molecular weight of the enzyme has been determined as 60,000. The enzyme efficiently hydrolyzes azocasein and the 7S and 11S reserve proteins of cotton seeds. Its maximum activity appears at pH 6.4-7.4 and a temperature of 35-40°C; it is not activated by sulfhydryl reagents and loses its activity in the presence of diisopropyl phosphorofluoridate. The assumption is made that protease A belongs to the serine type of trypsin-like proteases.

The presence of proteolytic enzymes in cotton seeds was first reported by Rossi-Fanelli et al. [1]. Then Gatsu and Jacks detected the presence of a proteinase with an acid pH optimum of its action in cotton seeds in a form associated with the aleurone grains [2]. On the basis of this fact, it was assumed that these enzymes played an important role in the breakdown of the reserve protein during the growth of the seeds.

In investigations of the proteolytic enzymes of dormant cotton seeds performed previously, we showed that 75% of the total activity is due to acid proteases [3] and their molecular weights are between 30,000 and 140,000 [4]. In the present paper we give the results of the isolation and purification of a homogeneous proteolytic enzyme of cotton seeds — protease A [5]. A scheme has been developed for purifying protease A which includes the following steps: extraction of the defatted seeds with 0.1 M phosphate buffer, pH 7.4; precipitation of the protein with ammonium sulfate at 60% saturation; desalting by dialysis; and ion-exchange chromatography on a column containing CM- and DEAE-celluloses. Information on the isolation and purification procedure is given below: (following page, below Fig. 1).

The homogeneity of the purified enzyme was established by electrophoresis in polyacrylamide gel and by the method of isoelectric focussing in a borate-polyol system by the method described in [6]. The isoelectric point of protease A is located in the region of pH 4.9. Graphs of the purification of protease A on columns containing CM- and DEAE-celluloses are given in Figs. 1, 2, and 3. The molecular weight of the enzyme was determined by its gel filtration through a column of Sephadex G-200 that had been calibrated with protein markers

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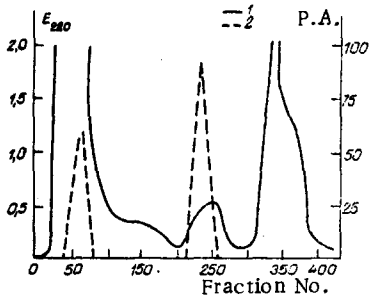

Fig. 1

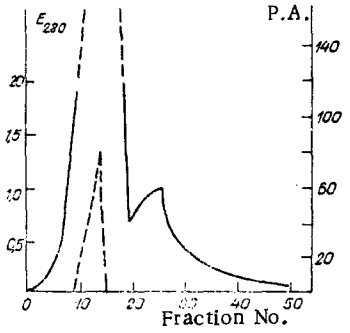

Fig. 2

Fig. 1. Purification of protease A from cotton seeds on a column of CM-cellulose (5 × 35 cm column, rate 60 ml/h, 10-ml fractions): 1) protein; 2) proteolytic activity.

Fig. 2. Rechromatography of protease A on a column of CM-cellulose (5 × 35 cm column, rate 60 ml/h, 10 ml fractions).

Stage of purification	Volume, ml	Total protein, mg	Activity, units total	Activity, units specific	Yield, % on protein	Yield, % on activity
1. Extraction with 0.1 M phosphate buffer pH 7.4	1500	6000	24000	4	100	100
2. Precipitation with ammonium sulfate (60%)	150	1748	20970	12	29	87
3. Chromatography on CM-cellulose	100	200	11200	56	3,3	46
4. Rechromatography on CM-cellulose	60	52,5	4305	82	0 87	18
5. Fractionation of on DEAE-cellulose	20	12	1224	102	0,2	5,0

having known molecular weights. The molecular weight in the region of 60,000 was found for protease A.

To establish the biochemical characteristics of the enzyme isolated, we determined the temperature optimum (35-40°C) and the pH optimum (6.4-7.4) of the action of protease A (Figs. 4 and 5).

The enzyme was not activated by sulfhydryl reagents (cysteine and β-mercaptoethanol) and was not inhibited by p-mercuribenzoate, which shows that it does not belong to the group of thiol proteases. Protease A was partially inhibited by EDTA and lost its activity in the presence of diisopropyl phosphorofluoridate, which gives grounds for assigning it to the group of trypsin-like serine proteases.

The action of protease A on the characteristic 7S and 11S proteins of cotton seeds was studied, and a good degree of their hydrolysis was found.

EXPERIMENTAL

Isolation. A defatted powder (50 g) of seeds of cotton plants of the Tashkent-K variety was extracted with 1.5 liter of 0.1 M phosphate buffer, pH 7.4 at 4-6°C for 3 h. The mixture was centrifuged at 6000 rpm for 20 min, and the supernatant liquid (P.A. = 4 units) was treated with ammonium sulfate to 60% saturation. The resulting precipitate was centrifuged off at 15,000 rpm and was then dissolved in the minimum amount of distilled water and was dialyzed against 0.01 M acetate buffer, pH 5.4 (P.A. = 12 units).

Ion-Exchange Chromatography on a Column of CM-Cellulose. The enzyme (2 g) in 100 ml of buffer solution was deposited on a column (5 × 35)cm, filled with CM-cellulose equilibrated with 0.01 M acetate buffer, pH 5.4. Elution was performed stepwise with 0.01 M, 0.1 M, and

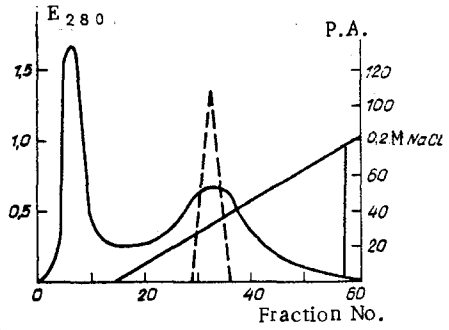

Fig. 3

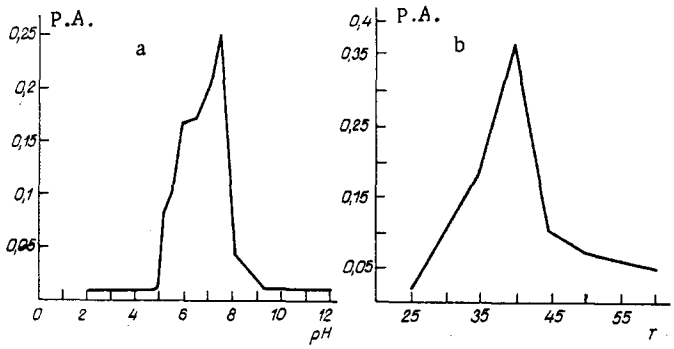

Fig. 4

Fig. 3. Purification of protease A on a column of DEAE-cellulose (1.5 × 14 cm column, rate 30 ml/h, 4-ml fractions).

Fig. 4. Dependence of the activity of protease A: a) on the pH; b) on the temperature.

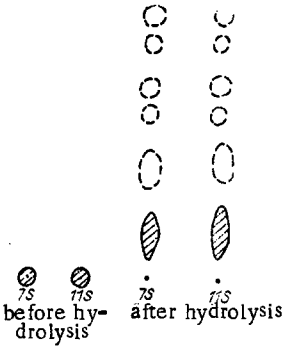

Fig. 5. Chromatograms of hydrolysates of the 7S and 11S proteins.

0.2 M acetate buffer. Fractions containing protease A were eluted with the 0.01 M acetate buffer. The rate of elution was 60 ml/h, fractions with a volume of 10 ml each being collected. The solution was concentrated on a FM02 membrane apparatus using IMM membranes (Vladipor). The yield was 200-250 mg or 3.3% in terms of protein, P.A. = 56 units. The enzyme was rechromatographed under the same conditions. The yield was about 1% in terms of protein, with P. A. = 82 units.

Ion-Exchange Chromatography on a Column of DEAE-Cellulose. A solution of 50 mg of protease A in 20 ml of 0.01 M phosphate buffer, pH 7.4, was deposited on a column (1.5 × 14 cm) filled with Reanal DEAE-cellulose equilibrated with the same buffer. The rate of elution was 30 ml/h, and fractions with a volume of 4 ml each were collected. The protease A was eluted in the second fraction when a gradient of 0.2 M NaCl was applied, and this fraction was dialyzed and freeze-dried. The yield was 0.2% in terms of protein; P.A. = 102 units.

The concentration of protein in solution was determined spectrophotometrically by the Warburg–Christian method [7].

Disk electrophoresis in polyacrylamide gel was performed by Davis's method [8]. Proteolytic activity was determined by Anson's method [9].

Determination of the pH Optimum. The following buffer solutions were prepared for determining the pH optimum dependence of activity: 1) pH 2-6.5 — citrate-phosphate; 2) pH 6.5-7.5 — phosphate; 3) pH 8.6-12 glycolic.

Activity was determined by Anson's method using as substrate azocasein or, in the region of acid pH values, hemoglobin.

Determination of the Temperature Optimum. To 2 ml of azocasein in phosphate buffer were added 1-ml portions of enzyme solution (1 mg/ml). The mixtures were incubated for 30 minutes at temperatures of from 20 to 55°C with an interval of 5°C. A maximum of activity was observed at 35-40°C.

Action of Protease A on the Reserve Proteins of Cotton Seeds. In each case, 3 mg of the 7S and 11S reserve proteins of cotton seeds was suspended in 3 ml of phosphate buffer solution, pH 7.4, and protease A was added in a ratio of 1:30. Hydrolysis was carried out at 37°C, pH 7.4, for 18 h. After the end of the reaction, the mixture was centrifuged and the hydrolysates were deposited on 10 × 20 cm plates with a thin layer of FND cellulose (GDR) and were chromatographed in a butan-1-ol—acetic acid—water (5:1:2) system. After drying, the chromatogram was stained with a 1% solution of ninhydrin in acetone.

SUMMARY

1. A homogeneous preparation of protease A has been isolated from the seeds of a cotton plant of the Tashkent-1 variety, and a scheme for the isolation and purification of the enzyme has been developed. Its molecular weight has been determined as 60,000.

2. Protease A is a typsin-like enzyme with a pH optimum of 6.4-7.4 and a temperature optimum of 35-40°C.

3. Protease A hydrolyzes the 7S and 11S reserve proteins present in cotton seeds.

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