The effective rate constant of the reaction (K") was determined from the formula $K'' = K/K_2$, where $K = V/C$ and K_2 is the rate constant of the splitting out of p-nitrophenol and C is the molar concentration of a monomeric unit of the polypeptide in the reaction medium, mole/liter.

SUMMARY

The hydrolytic properties of polypeptides of regular structure and containing polyfunctional amino acids with respect to the hydrolysis of p-NPA have been determined as functions of the pH, the temperature, and the concentration of the substrate.

Calculations have been made of V_{max}, K_m, and K" — the effective rate constant of the hydrolysis of p-NPA — and of K_2 — the rate constant of the splitting out of p-nitrophenol.

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ISOLATION OF PROTEASE B FROM COTTON SEEDS

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Protease B has been isolated from dormant cotton seeds by fractionation with ammonium sulfate, ion-exchange chromatography on CM-cellulose, and gel filtration through Acrilex P-10 and Sephadex G-75, with 128-fold purification. The enzyme exists in dimeric and monomeric forms. According to the results of gel filtration, their molecular weights are 72,000 and 36,000, respectively. The enzyme consists of a single polypeptide chain including sugars. The N-terminal amino acid of protease B is alanine. The enzyme possesses proteolytic activity in the pH range from 4 to 6.

The recently increasing interest in plant enzymes is due to their unique properties, the study of which is opening up broad possibilities for the fine regulation of the processes involved in the preparation and storage of plant foodstuffs for animal husbandry. In addition, the proteolytic enzymes of plant seeds fulfill an important function in the growth process. They cleave the globulin components and provide the developing germ with low-molecular-weight nitrogen compounds [1, 2].

Dormant seeds of the cotton plant of the Tashkent 1 variety contain proteolytic enzymes acting over a wide pH range with three maxima: 2.8-3, 4.5-5, and 9-9.3 [3, 4]. The aim of the present investigation was to determine their role in the metabolism of the cell and to study the catalytic action of the cotton proteases.

It is impossible to elucidate the chemical essence of the action of proteolytic enzymes without a detailed study of their primary and spatial structures. We have developed a method for isolating a proteolytic enzyme, which we have called protease B, from dormant

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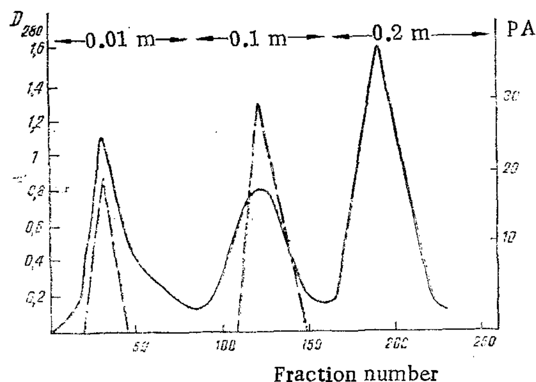


Fig. 1. Chromatography of the active fraction on CM-cellulose: 1) protein (D_{280}); 2) proteolytic activity.

cotton seeds. The stages of purification included fractionation with ammonium sulfate, ion-exchange chromatography, and gel filtration on Acrilex and Sephadex — all this permitted us to achieve a 128-fold purification of the enzyme:

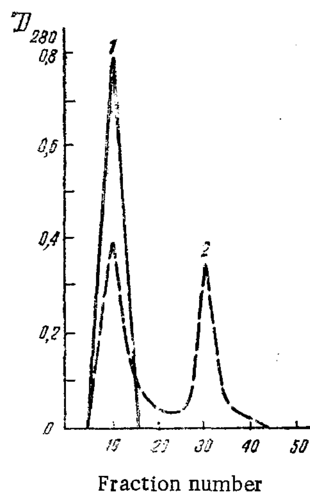
Stage of purification	Volume, ml	Total protein, mg	Total activity, act. units	Specific activity, units/g	Degree of purification	Yield, %
Extraction with 0.1 M phosphate buffer, pH 7.4	500	5400	5400	1	—	—
Precipitation with ammonium sulfate at 60% saturation	175	675	2308	3,42	3,42	100
Chromatography on CM-cellulose	75	63	2091.6	33.2	33,2	10
Gel filtration						
on Acrilex P-10	50	50	2000	40	40	7
on Sephadex G-75	5	10	1280	128	128	1.5

An extract of defatted cottonseed powder was fractionated with ammonium sulfate (60% saturation). The solution of the enzyme in 0.01 M acetate buffer, pH 5.4, was deposited on a column of CM-cellulose. The peak of protease B was eluted with 0.1 M acetate buffer (Fig. 1). The solution contained pigments and low-molecular-weight impurities, the partial elimination of which was achieved by gel filtration through Acriflex P-10 [5].

On gel filtration through a column of Sephadex G-75 equilibrated with water, protease B was eluted as a sharp peak the maximum activity of which coincided with the maximum of the protein peak (Fig. 2). The homogeneity of the enzyme was confirmed by the results of electrophoresis in polyacrylamide gel, the finding of a single terminal amino acid — alanine — and by the presence of a single peak on sedimentation.

In the case of the gel filtration of protease B on Sephadex G-75 in the presence of sodium dodecyl sulfate and 6 M urea, a second peak with a smaller molecular weight appeared on the elution profile (see Fig. 2). The quantitative ratio of the peaks was not constant and depended on the time of incubation of the enzyme solution. We observed a similar pattern on electrophoresis in 7.5% polyacrylamide gel. The preparation gave a single band in the central part of the gel when using Tris buffer, pH 8.2, but when a gel containing sodium dodecyl sulfate was used a band in the lower part of the gel and a less intense one in the center were observed on the electrophoretogram.

An increase in the time of incubation in 1% dodecyl sulfate before electrophoresis led to a fall in the intensity in the band in the central part (sometimes to its complete disappearance) and to a rise of the intensity in the lower part. Proteolytic activity, determined after the careful elimination of sodium dodecyl sulfate from the solution, was present in both peaks obtained from the Sephadex G-75. Prolonged incubation (3 days) led to the complete inactivation of the enzyme. The unusual results obtained in working with protease B in the presence of dissociating agents can be explained by the tendency of proteins and enzymes of plant origin to undergo mutual aggregation and association with other proteins



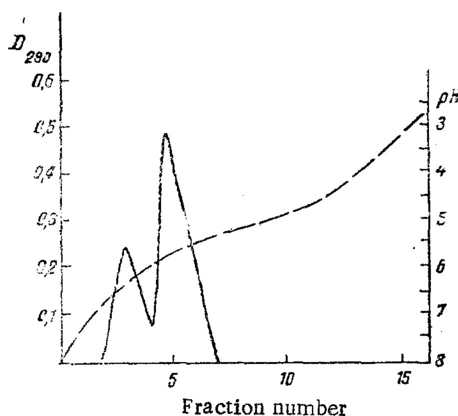


Fig. 2 Fig. 3

Fig. 2. Gel filtration on a column of Sephadex G-75: 1) elution with water; 2) elution with 1% SDS solution.

Fig. 3. Isoelectric focusing of protease B in a borate-polylol system in a pH 3.5-8 gradient.

and pigments [6, 7]. However, in our case we tend to assume the possibility of the existence of protease B in two active forms, which is not exceptional.

K. Titani et al. observed the existence of an enzyme in several forms which, however, after inactivation showed a single band on electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate [8]. K. M. Pangburn et al. isolated two forms of a metalloendopeptidase having the same N-terminal sequence of 15 amino acid residues. V. M. Stepanov [10] explained the presence of multiple forms of enzymes either by genetic changes in the cell or as the result of limited proteolysis.

We assumed that protease B exists in monomeric and dimeric forms with molecular masses, according to the results of gel filtration, of 36,000 and 72,000, respectively. The addition of 2-mercaptoethanol to a solution of the enzyme followed by electrophoresis in the presence of dissociating agents did not change the electrophoretogram, which shows the absence of disulfide bonds between individual polypeptide chains in the molecule.

The subsequent structural investigations of protease B were performed on the monomeric form. The isoelectric point of the protease, pI 5.7, was determined by the IEF method in a borate-polyol system [11] (Fig. 3). The polypeptide chain consists of approximately 283 amino acid residues and covalently attached sugars, the amount of which is 10-12% of the molecular weight:

Amino acid	Number of residues
Aspartic acid	28
Threonine	13
Serine	21
Glutamic acid	60
Proline	8
Glycine	25
Alanine	19
Valine	14
Methionine	1
Isoleucine	10
Leucine	18
Tyrosine	9
Phenylalanine	8
Histidine	7
Lysine	16
Arginine	25

The enzyme preparation possessed activity in the pH range of 4-6 with an ill-defined maximum at pH 5. The proteolytic activity was determined by a modified Anson method [12] using urea-denatured hemoglobin as substrate.

EXPERIMENTAL

An acetone powder of cotton seeds of the variety Tashkent 1 (100 g) was extracted with 3 liters of 0.1 M phosphate buffer, pH 7.4, for 3 h at 2-6°C. The extract was clarified by centrifugation at 6000 rpm. The supernatant solution was brought to 60% saturation with ammonium sulfate by the slow addition of the salt, and it was left for 1 h. The precipitate was collected by centrifugation (15,000 rpm), and it was dissolved in 100 ml of distilled water and dialyzed for 28 h against three 4-liter portions of 0.01 M acetate buffer, pH 5.4.

Ion-Exchange Chromatography. The dialyzed solution of the enzyme (200 ml) was deposited on a column of CM-cellulose (6 × 36 cm). Elution was carried out with acetate buffers of increasing concentration: 0.01 M, 0.1 M, 0.2 M, and 0.5 M. The fraction containing the protease B was eluted with 0.1 M acetate buffer. The solution was concentrated on a FMO2 membrane filter using a UPM-100 membrane (Vladipor).

The gel filtration of the enzyme was performed on a column of Acrilex P-10 (Reanal, Hungary) with dimensions of 4 × 100 cm by the method of Kovaleva et al. [5], and it was then rechromatographed on a column of Sephadex G-75 (1 × 70 cm).

The concentration of protein in the solution was determined spectrophotometrically by the Warburg–Christiani method [13], and the proteolytic activity by a modified Anson method [12].

The electrophoretic studies were performed by Davies' method [14] on a "Reanal" instrument, and isoelectric focusing by Troitskii's method [15] in a borate–polyol system using a pH gradient from 3 to 8.

The amino acid composition was determined on a LKB-4101 amino acid analyzer (Sweden) after acid hydrolysis (5.7 N HCl, 110°C, 24 h).

SUMMARY

Protease B, which exists in two forms, has been isolated from dormant cotton seeds in the homogeneous state. The monomeric form has a molecular weight of 36,000 and consists of a single polypeptide chain including sugars; the N-terminal amino acid is alanine.

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