# PHYSICOCHEMICAL PROPERTIES AND SUBSTRATE SPECIFICITY OF PROTEASE C FROM DORMANT COTTON SEEDS

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The substrate specificity of a proteolytic enzyme – protease C – isolated from cotton seeds has been studied. The activity of protease C is suppressed completely under the action of diisopropyl phosphorofluoridate. Protein inhibitors – duck ovomucoid, soybean inhibitor, and also TPCK – suppressed the activity of protease C to different degrees. On the basis of results obtained in the hydrolysis of the cottonseed reserve proteins, 7S and 11S globulins, and the B chain of insulin, protease C has been assigned to the serine group of endopeptidases. The optimum conditions – pH, time, and temperature – at which protease C exhibits its maximum activity has been determined.

Proteolytic enzymes fulfill various functions in the animal cell, and in plant seeds they acquire a specific function – the cleavage of reserve proteins. In order to determine the properties of the proteases of cotton seeds, we have isolated the enzyme protease C [1] and have studied its capacity for cleaving the reserve proteins of cotton seeds.

The enzyme is fairly heat-stable, retaining its activity on heating to 80°C; its maximum activity when azocasein was used as substrate was observed at 45°C (Fig. 1, a). In the determination of pH optima, acetate (pH 3.6-5.8) and phosphate (pH 4.0-8.2) buffers were used. It can be seen from a graph of the dependence of the activity of protease C on the pH (Fig. 1, b) that the enzyme exhibits its maximum degrees of hydrolysis of the substrates hemoglobin and azocasein at the same pH, 5.8. Fig. 1, c, shows the linear dependence of the rate of hydrolysis of azocasein by protease C on the reaction time.

In order to study the substrate specificity of the enzyme, we investigated the influence of inhibitors and activators on the activity of protease C. The activity of protease C was completely inhibited by diisopropyl phosphorofluoridate – a reagent blocking the serine residue in the active center of serine proteinases. p-Chloromercuribenzoate and other mercury compounds did not affect the activity of the enzyme. Protein inhibitors of trypsin and chymotrypsin – ovomucoid and soybean inhibitor – and also the irreversible inhibitor TPCK specific for these enzymes, after incubation with protease C for 30 min, suppressed its activity by 20-30%. An inhibitor from potatoes, which is an inhibitor with a broad action spectrum [2], suppressing not only trypsin and chemotrypsin but also subtilisin, suppressed the activity of protease C by 70%. EDTA did not inhibit the enzyme, but in an investigation of the influence of $Fe^{3+}$, $Cu^{+}$, $Ca^{2+}$, $Ag^{+}$, and $Zn^{2+}$ ions it was found that in the presence of $Ca^{2+}$ ions the activity of protease C in 0.5% DDS and 8 M urea was retained for a long time (4 h). It is possible that $Ca^{2+}$ ions exert a stabilizing action on the native structure of the enzyme. All the results obtained on the action of inhibitors on protease C are given below:

| Reagent | Concentration in Mixture, M | Residual Activity,% |
|---|---|---|
| Diisopropylphosphorofluoridate | $1\times10^{-2}$ | 0 |
| EDTA | $1{,}5\times10^{-4}$ | 100 |
| Dithiothreitol | $1{,}2\times10^{-3}$ | 105 |
| p-Chloromercuribenzoate | $2{,}6\times10^{-4}$ | 97 |
| $Hg(CH_3COO)_2$ | $2{,}1\times10^{-3}$ | 100 |
| Cysteine | $5\times10^{-2}$ | 104 |
| Glutathione | $1\times10^{-2}$ | 106 |
| TPCK | $3{,}8\times10^{-6}$ | 75 |
| Ovomucoid (duck) | $1\times10^{-6}$ | 80 |

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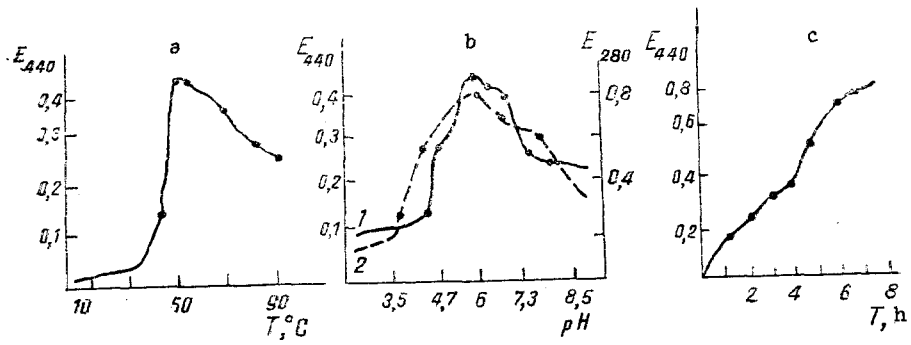


Fig. 1

| Reagent | Concentration in Mixture, M | Residual Activity, % |
|---|---|---|
| Soybean trypsin inhibitor | $5\times10^{-6}$ | 70 |
| Inhibitor from potatoes | $3\times10^{-6}$ | 30 |
| $Fe^{3+}$, $Cu^{+}$, $Ca^{2+}$, $Ag^{+}$, $Zn^{2+}$ | $3\times10^{-2}$ | 100 |

We also studied the action of protease C on synthetic substrates. As substrates we used the p-nitroanilides of peptides which, on hydrolysis, form p-nitroaniline, differing in its absorption spectrum from the substrate [3]

ZL-Ala-L-Ala-L-Leu-pNA
Pyr-LPhe-pNA
Z-L-Ala-L-Gly-L-Leu-pNA
Pyr-L-Ala-L-Ala-L-Leu-pNA
Z-L-Gly-L-Gly-L-Leu-pNA
N-Bz-D-L-Arg-pNA

Protease C hydrolyzed well Pyr-L-Ala-L-Ala-L-Leu-pNA and Z-L-Ala-L-Ala-L-Leu-pNA, which are specific substrates of serine proteinases of the subtilisin type.

When the globulins of cotton seeds were used as substrates, we found that protease C did not hydrolyze the native reserve proteins. However, denaturation of the 7S and 11S globulins by treatment with phenol followed by heating to 100°C for 30 min made the reserve proteins accessible for hydrolysis by protease C. Twenty-four-hour hydrolysates were deposited on plates with thin layers of cellulose and were chromatographed. On the chromatograms obtained, protein spots with $R_f$ 0.71, 0.65, 0.56, 0.35, and 0.21 were detected for the hydrolyzate of the 7S globulin, and spots with $R_f$ 0.65, 0.6, 0.4, and 0.29 for the 11S globulin.

In order to study the specificity of protease C in relation to the nature of the peptide bonds cleaved, we carried out the hydrolysis of the denatured and oxidized chain of insulin. The N-terminal amino acids of the cleaved polypeptide chain of insulin were determined by the dansyl chloride method [4]. On chromatograms, the dansyl derivatives of alanine, glycine, cysteine, threonine, and serin were detected.

On comparing the results of hydrolyses of the 7S and 11S globulins and the B-chain of insulin by protease C, we concluded that protease C belongs to the group of endopeptidases.

## EXPERIMENTAL

Determination of Proteolytic Activity [5]. In 1 ml of a solution of azocasein in 0.1 M phosphate buffer containing $3 \times 10^{-2}$ M KCl, $10^{-4}$ M EDTA, and $3 \times 10^{-3}$ M DTT, pH 6.0, was added 0.5 ml of a solution of the enzyme (1 mg/ml). The mixture was incubated at 40°C for 30 min. The reaction was stopped by the addition of 2 ml of 10% TCA, and samples were placed in an ice bath for 30 min. Then each precipitate was separated off by filtration and the filtrate was treated with a 0.5 M solution of NaOH in a ratio of 1:1. The optical densities of solutions of the samples at 440 nm were determined in a SF-16 spectrophotometer. As a unit of activity was taken that amount of enzyme causing a rise in $E_{440}$ by 1 unit/min under the conditions of the experiment.

Determination of the Dependence of the Activity of Protease C on the Influence of Inhibitors. A reaction mixture of a solution of protease C and the substrate azocasein was treated with 0.5 ml of a solution of inhibitor of definite concentration, and the activity was determined by the procedure given above.

The action of protease C on synthetic substrates was determined by the procedure of [3].

Hydrolysis of the 7S and 11S Globulins of Cotton Seeds. A solution of 200 mg of the 7S globulin in 50 ml of 0.1 M phosphate buffer containing $3 \times 10^{-2}$ KCL and $3 \times 10^{-3}$ M DTT, pH 6.0, was treated with 10 ml of a solution of enzyme containing 10 mg of protease C. The mixture was incubated at 40°C for 24 h, 3 ml aliquots being taken after 3, 7, and 16 h, and these were dried and deposited on plates with a thin layer of FND cellulose (Filtrak; GDR). Chromatography was carried out in the butan-1-ol–acetic acid–pyridine–water (15:3:10:12) system. The amounts of peptide fragments from the 16- and the 24-hour hydrolyses were identical. Hydrolysis of the 11S globulin was carried out under similar conditions.

Hydrolysis of the B-Chain of Insulin. A mixture containing 1 ml of a 0.1% solution of insulin in 0.01 M acetate buffer, pH 5.8, and 1 ml of a 0.01% solution of protease C was incubated at 40°C for 17 h. Hydrolysis was stopped by heating in the water bath at 100°C for 20 min, and the mixture was evaporated to dryness. The N-terminal amino acids of the fragments obtained were determined by the dansyl chloride method [4]. A solution of 0.1 mg of the hydrolysate in 0.2 ml of 0.1 M bicarbonate buffer, pH 8.1, was treated with 0.2 ml of a solution of 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) in acetone (5 mg/ml) and the mixture was kept at 37°C for 1 h. Then it was evaporated to dryness and 2/3 of it was hydrolyzed with 5.7 N HCl. The DNS-amino acids were identified by thin-layer chromatography on silica gel plates [6].

## SUMMARY

1. It has been established that protease C is an endopeptidase and belongs to the serine group of proteases.

2. The optimum conditions for the manifestation of the maximum activity of the enzyme and its substrate specificity have been determined.

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