PHYSICOCHEMICAL PROPERTIES OF PROTEASE A FROM COTTON SEEDS

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It has been found that protease A from dormant seeds of cotton plants of the Tashkent-1 variety consists of two subunits: α and β, differing in molecular weight and carbohydrate content and linked with one another by a disulfide bridge. The amino acid and carbohydrate compositions of the enzyme and its subunits have been determined. A comparative study of peptide maps of protease A and its α- and β-subunits and of their amino acid compositions has permitted the assumption that subunits α and β, in their turn, each consists of two polypeptide chains that are identical or very close in composition.

Continuing a study of the protease A isolated previously from dormant seeds of cotton plants of the Tashkent-1 variety [1], we have determined its amino acid and carbohydrate compositions. The results obtained have permitted certain hypotheses to be put forward concerning the subunit structure of the protease A molecule. On comparing the results of electrophoresis performed in polyacrylamide gel in the presence of the dissociating reagent sodium dodecyl sulfate (NaDDS) and 8 M urea obtained before and after the reduction and carboxymethylation of protease A, we came to the conclusion that the molecule consists of two subunits differing in molecular weight which may be provisionally named α and β and which are linked with one another through a disulfide bridge. For the preparative separation of the subunits we used gel filtration through a column of Sephadex G-50 (Fig. 1).

The purity of the α- and β-subunits obtained was checked by electrophoresis and by the Edman determination of the N-terminal amino acid residues [2]. The amino acid and carbohydrate compositions of the enzyme itself and of the subunits obtained have been determined (Table 1). The N-terminal amino acids were found by Edman's dansyl method: aspartic acid for the α-subunit and glutamic acid for the β-subunit.

The C-terminal amino acids were determined by the use of the hydrazinolysis method with the subsequent identification in an amino acid analyzer of the amino acids split off [5]. Arginine ad histidine were identified as the C-terminal amino acids of protease A.

A comparative study of the amounts of carbohydrates in the whole protease A molecule and the α- and β-subunits by the GLC method showed that the whole amount of carbohydrate detected (10-12%) was present in the α-subunit and there were no carbohydrates in the β-subunit. The qualitative analysis of the TMS derivatives of the methyl glycosides by the GLC method [3] showed the presence in the carbohydrate moiety of arabinose, xylose, mannose, galactose, and glucose in a ratio of 8:4:1:1:3.

The further comparative study of the subunits was carried out by the peptide map method (Fig. 2), the maps being obtained by chromato-electrophoresis. The protease A after reduction and carboxymethylation and also the isolated subunits were hydrolyzed by trypsin (Worthington).

The protease A molecude contains about 50 arginine and lysine residues, but on the peptide maps of protease A itself sixteen peptides were detected, and on those of its α- and β-subunits 12 and 8 peptides, respectively. On analyzing the peptide maps in the light of the amino acid compositions of protease A and its subunits that have been found, it can be assumed that each subunit, in its turn, consists of two polypeptide chains identical or very similar in structure.

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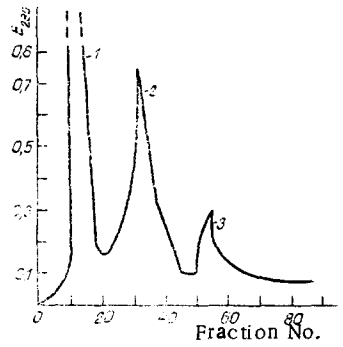

Fig. 1. Separation of the α- and β-subunits of protease A from cotton seeds on a column of Sephadex G-50: 1) uncleaved enzyme; 2 and 3) α- and β-subunits. Column 1.5 × 95.5 cm; rate 36 ml/h; 4-ml fractions.

TABLE 1

Composition	Protease A	Subunits	
		A	B
Amino Acids			
Lysine	18	9	7
Arginine	30	16	12
Histidine	5	2	2
Cysteine	2	1	1
Aspartic acid	37	18	18
Threonine	36	21	15
Serine	49	31	15
Glutamic acid	98	54	25
Proline	46	26	18
Glycine	39	18	20
Alanine	60	34	25
Valine	25	12	15
Methionine	6	2	4
Isoleucine	22	12	8
Leucine	40	27	15
Tyrosine	19	11	9
Phenylalanine	27	17	9
Carbohydrates	0—12%	+	—
Arabinose	8		
Xylose	4		
Mannose	1		
Galactose	1		
Glucose	3		

EXPERIMENTAL

Electrophoresis in PAAG. The enzyme was first incubated in 8 M urea and 1% NaDDS at 50°C for 1 h. In the process of preparing the gels, 6 M urea and 0.1% NaDDS were added to the microporous (2.5%) and macroporous (7.5%) gels. Electrophoresis was carried out at a current strength of 4 mA per tube for 3 h. The dye Bromophenol Blue was used as marker. Before staining the gel was fixed with 20% TCA for 20 min and it was then treated with 0.25% Coomassie in methanol—water—acetic acid (5:5:1). Dye residues were washed out with a 7% solution of acetic acid.

Reduction and Carboxymethylation. To 150 mg of the enzyme in 25 ml of 8 N urea solution were added 6 ml of Tris-HCl buffer, pH 8.6, 0.6 ml of EDTA solution (50 mg/ml), and 0.2 ml of mercaptoethanol. The reaction was carried out in the dark with a magnetic stirrer in a current of nitrogen at room temperature. After 4 h, 0.5 g of monoiodoacetic acid that had been neutralized with 1 N NaOH was added to the mixture. After 45 min, the reaction was stopped by acidification to pH 3.1 with glacial acetic acid. Then the mixture was dialyzed and freeze-dried. Yield 70 mg.

Separation of the α- and β-subunits of Protease A on a Column of Sephadex G-50. A solution of 8.7 mg of carboxymethylated protease A in 5 ml of double-distilled water was deposited

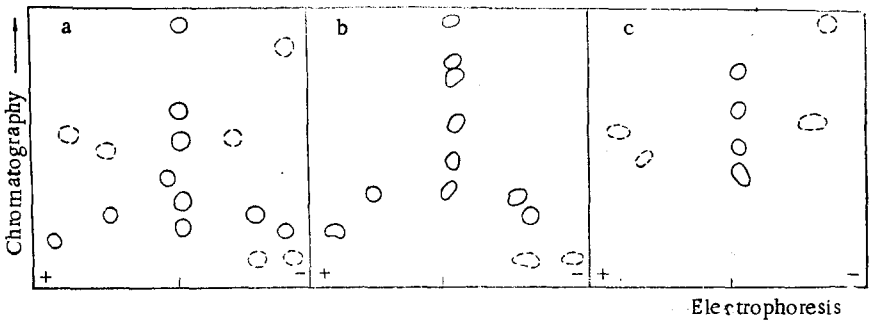

Fig. 2. Peptide maps of reduced and carboxymethylated protease A (a) and of its α- and β-subunits (b and c).

on a column (1.5 × 95.5 cm) of Sephadex G-50. The rate of elution was 36 ml/h. The yield of the α-chain was 2.1 mg and of the β-chain 1.05 mg.

Determination of Amino Acid Compositions. Samples (from 1 to 5 mg) of the α- and β-subunits and of protease A were dissolved in 2-5 ml of 6 N HCl. Hydrolysis was carried out in vacuum-sealed tubes at 110°C for 24 h. The hydrolysates were evaporated with the addition of fresh portions of water and the residues were dried in a vacuum desiccator over alkali. The amino acids were analyzed on a AAA-881 amino acid analyzer.

Enzymatic Hydrolysis of Protease A and of the α- and β-Subunits by Trypsin. After reduction and carboxymethylation, 20 mg of protease A and 10 mg of each of the α- and β-subunits were each dissolved in 2 ml of ammonium bicarbonate, pH 8.8, 0.2 ml of a 0.5 mg/ml solution of trypsin (Worthington) was added, and the enzyme and the subunits were digested at 37°C. After an hour, another 0.2 ml of trypsin solution was added. Digestion was continued for 8 h, the pH being maintained with ammonia. After the end of the reaction, the mixture was centrifuged and was freeze-dried. The chromatography of 10-15 µl of hydrolysate was performed on a 20 × 20 cm plate with a thin layer of FND cellulose (GDR) in the butanol—acetic acid—pyridine—water (15:3:10:12) system. Then the paper was dried and electrophoresis was performed in pyridine-acetate buffer, pH 6.6, at a voltage of 1000 V and a current strength of 40 A was carried out for 35 min. The chromatogram was stained with a 0.1% solution of ninhydrin in ethanol.

The concentration of protein in solution was determined spectrophotometrically by the Warburg—Christian method [4].

N-Terminal amino acids were determined by the dansyl Edman method [2].

C-Terminal amino acids were determined by the hydrazinolysis method [5]. A weighed sample (3 mg) of enzyme was dissolved in 0.5 ml of anhydrous hydrazine, and the solution was evaporated in vacuum and was thermostated at 110°C for 16 h. After the end of the reaction the solution was evaporated in a rotary evaporator and the last traces of hydrazine were eliminated by keeping the product in a vacuum desiccator over concentrated H_2SO_4. Then it was dissolved in 1 ml of distilled water, an equal amount of benzaldehyde was added, and the mixture was centrifuged (3 thousand rpm, 2-5 min), and the benzaldehyde layer was centrifuged off. This was repeated 2-3 times. The aqueous fraction was freeze-dried and investigated on an amino acid analyzer.

SUMMARY

1. It has been established that protease A isolated from dormant cotton seeds consists of two subunits — α and β — linked by a disulfide bridge.

2. The amino acid and carbohydrate compositions and the N- and C-terminal amino acids of protease A and its subunits have been determined.

3. On the basis of a comparative study of the peptide maps and the amino acid compositions of protease A and its subunits the hypothesis has been made that each subunit, in its turn, consists of two polypeptide chains identical or very similar in composition.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTAMINES

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The protamines from the gonads of the sturgeon *Acipenser stellatus* have been separated by high-performance liquid chromatography. The proteins were eluted with mixtures of water and ethanol having a gradient of ethanol concentrations in the presence of trifluoroacetic acid (TFA). The influence of the concentration of TFA and the temperature of the column on separation was studied. The quantitative (95-98%) isolation of the protamines from the column was achieved at a temperature of 30°C and a 0.15% concentration of TFA.

Protamines are the main protein components of the chromatin of the sex cells and are highly basic low-molecular-weight proteins rich in arginine residues. In contrast to the basic nuclear proteins of the somatic cells (histones) which largely have a constant composition, the set of protamines in spermatozoa depends on the species affinity of the organism, and, as a rule, includes 2-4 components.

The study of protamines is of considerable interest from the point of view of elucidating their function in the process of spermatogenesis, and also in connection with their use in medicine — for example, as heparin antagonists and as prolonging agents for certain antibiotics.

The methods of isolating protamines used at the present time are lengthy and laborious and therefore the development of a fast and reliable method for their isolation, separation, and quantitative determination is of great importance. Ion-exchange chromatography on cation-exchangers of medium strength, which is usually used for the preparative isolation of protamines, with elution of the proteins by a gradient of sodium chloride involves the necessity for an additional stage — the desalting of the proteins. The quantitative analysis of the protamines is carried out with the aid of electrophoresis in polyacrylamide gel, but because of the nonproportionality of the binding of dyes with protamines having different amino acid compositions, the determination has only a semiquantitative nature.

We have previously reported on the possibility of separating protamines by high-performance liquid chromatography (HPLC) [1]. In the present paper we describe in detail an experiment and an analysis of the influence of various factors on the separation.

Stellin, a protamine from sturgeon gonads, consisting of two components, A and B, which are tri- and diprotamines, respectively [2, 3], was investigated.

For the reversed-phase chromatography of the stellin we used a sorbent based on silica gel modified with octadecylsilane, Zorbax ODS, with water as the mobile phase. The elution of the protein was carried out with a concentration gradient of ethanol (0-100%). Under these conditions it was impossible to achieve the desorption of the protamines from the column. The reason for the irreversible binding of the stellins is apparently the interaction of the basic amino acids (which make up more than 70% of the amino acid composition of the proteins) with sorbent that had not been modified with silanol groups. A similar phenomenon has been observed in the separation of histones on a μBondapak C_{18} column [4] using an acetonitrile gradient. To decrease the ionic interaction of the stellins with the sorbent we used an ion-

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