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PROTEINASE OF GERMINATED COTTON SEEDS

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A scheme has been developed to isolate and purify proteinase D from 3-day germinated cotton seeds. The physicochemical properties and substrate specificity were studied. It is found that cysteinic proteinase D cleaves auxiliary proteins to low-molecular-weight peptides and free amino acids.

Key words: cotton, proteases, auxiliary proteins, germination of seeds.

Seed germination is accompanied by rapid mobilization of auxiliary substances, in particular, proteins, which are the principal source of nitrogen in germinating seeds. The main role in metabolism of auxiliary proteins belongs to proteolytic enzymes. We isolated and fully characterized homogeneous proteases A, B, and C from dormant cotton seeds. A study of the proteolysis of auxiliary proteins of cotton seeds revealed that protease A is the enzyme that induces hydrolysis. In other words, it acts on the native auxiliary proteins 7S and 11S globulins [1] whereas proteases B and C affect only modified auxiliary proteins [2]. It was also found that protease A fulfills yet another criterion for involvement of proteases in the decomposition of auxiliary proteins. It is present in germinating seeds [3]. A protease that disappears during germination certainly cannot play a significant role in the hydrolysis of auxiliary proteins [4].

It was shown earlier that the principal auxiliary cottonseed protein 11S globulin begins to hydrolyze in the very earliest stages of seed germination and finishes in the first 4-5 days of germination. Only limited proteolysis occurs in the first 3 days; extensive hydrolysis, only on the fourth day. Then, this protein was not observed by either immunochemical methods or electrophoresis and analytical ultracentrifugation. Obviously, this is due to the synthesis in the germinating seeds of a new proteinase that actively hydrolyzes modified 7S and 11S globulins. Therefore, we further investigated the development of a scheme for isolating and purifying proteinase D from 3-day germinated cotton seeds. The developed scheme, which is given below, includes extraction of defatted seeds with phosphate buffer (0.1 M, pH 7.4), precipitation of protein by $(NH_4)_2SO_4$ (80%), desalting by dialysis, and gel filtration through a Sephadex G-150 column. The yield was 0.5%; activity, 100 PE units/g.

The homogeneity of the produced enzyme was estimated by electrophoresis in polyacrylamide gel (PAAG). Analysis of the electrophoresis spectra under dissociating conditions with sodium dodecylsulfate (Na-DDS) and β-mercaptoethanol showed that the enzyme does not contain interchain S–S bonds, i.e., it consists of one polypeptide chain with a molecular weight of 18 kDa.

Nonproteinaceous substances (lipids, carbohydrates, etc.) were determined by TLC under various conditions. We established that proteinase D does not contain lipids. Ascending paper chromatography and TMS-methylglycoside analysis by GLC revealed glucose, galactose, and arabinose in a 3:1:4 ratio in the carbohydrate portion.

The amino acid composition was determined (Table 1). We observed a high content of glutamic acid, which is characteristic for plant proteins, and the essential amino acids arginine and lysine.

The nature of the functional groups at the active center was elucidated by inhibition analysis. Proteinase D was not inhibited by EDTA (10^{-3} M) and retained its activity in the presence of diisopropylfluorophosphate and phenylmethylsulfonylfluoride ($2 \cdot 10^{-3}$ M). It was activated by such sulfhydryl reagents as cysteine and β-mercaptoethanol and inhibited by *p*-chloromercuribenzoate ($4 \cdot 10^{-4}$ M). This enabled proteinase D to be assigned as a thiol proteinase. The optimum pH of cysteinic proteinase was 6.0-6.5; the optimum temperature, 37-40 C.

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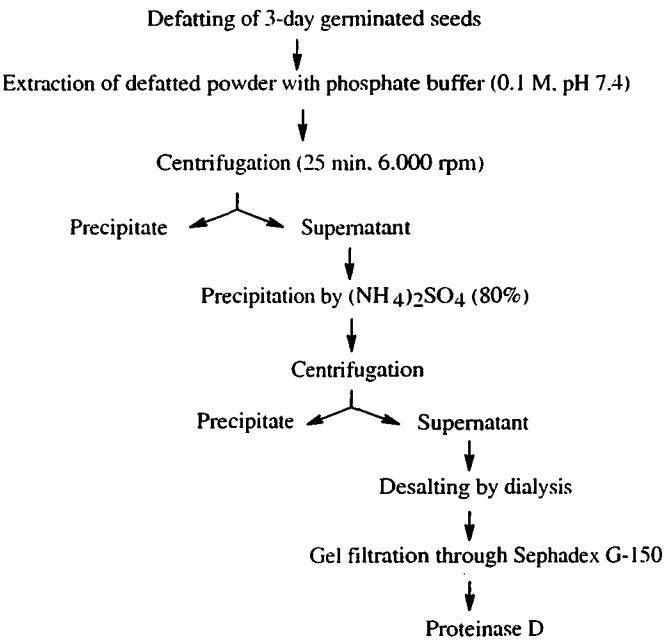
TABLE 1. Amino-acid and Carbohydrate Composition of Proteinase D from 3-Day Germinated Cotton Seeds

Amino acid	Protein content, mol/mol	Amino acid	Protein content, mol/mol
Asp	5.64	Met	1.88
Thr	1.66	Ile	7.12
Ser	2.52	Leu	11.66
Glu	28.76	Tyr	8.88
Pro	3.50	Phe	14.18
Gly	1.72	His	10.22
Ala	12.86	Lys	18.40
Cys	1.90	Arg	38.92
Val	2.16		

Carbohydrates	Monosaccharide ratio
Glucose	3
Galactose	1
Arabinose	4



Then, the mechanism of action of proteinase D on auxiliary proteins was studied. We previously noted that proteinase A is responsible for the initial stages of hydrolysis (first 3 days of germination) [3]. The cleavage of a limited number of peptide bonds modifies the auxiliary proteins. Then they can be hydrolyzed by other proteases, for example, B and C [2]. Our investigations demonstrated that protease A can modify 7S and 11S globulins and hydrolyze them into peptides [1]. Purified cysteinic proteinase D was used to hydrolyze 11S globulin that was isolated from dormant and germinating cotton seeds. The degree of hydrolysis was monitored by TLC and in an amino-acid analyzer. It was established that only 11S globulin from the germinating seeds was hydrolyzed to free amino acids whereas hydrolysis of auxiliary protein from dormant seeds was incomplete.

The presented data indicate that preliminary modification of 11S globulin by proteases of dormant cotton seeds is important. Such modification increases the hydrolysis of auxiliary protein and facilitates more effective transfer of nutrients to the sprout.

Cysteinic proteinase D from germinated cotton seeds has an optimum pH and activity for preliminarily modified auxiliary protein that are similar to proteinase B from vetch [5], proteinases from germinated pumpkin and sunflower seeds [6], and proteinases from germinated buckwheat seeds [7].

Thus, traditional methods of protein chemistry are used to develop a scheme for isolating and purifying proteinase D from germinating cotton seeds. Their physicochemical and substrate specificity are studied. This enabled the role of proteinase D in the decomposition of auxiliary proteins to be determined. It was established that proteinase D cleaves auxiliary protein into low-molecular-weight peptides and amino acids.

A thorough study of the whole complex of proteinases from cotton seeds makes it possible to unravel the mechanism of one of the basic biochemical processes occuring in cotton during seed germination and to explain the role of the individual proteolytic enzymes. After modification of auxiliary proteins from cotton seeds by protease A from 3-day germinated seeds, further hydrolysis can occur with simultaneous action of protease A and other proteases, for example, B and C, which are found in dormant seeds, and proteinse D, which is also observed during germination. Their mutual effect can guarantee complete hydrolysis of auxiliary proteins.

EXPERIMENTAL

Cotton seeds were germinated in a thermostatted chamber in moist sand for 3 days. Germinated seeds were ground and defatted with cold acetone to give a powder from which protein was isolated.

The protein concentration in solution was determined by the absorption at 280 nm by the Lowry method [8].

The proteolytic activity of the isolated enzyme was determined by the method of Ansen [9].

The activity as a function of pH was determined by preparing the following buffers: pH 2-6.5, citrate—phosphate; pH 6.5-7.5, phosphate; pH 8.6-12, glycolic. The activity was determined by the method of Ansen using azocasein as substrate. The substrate at acidic pH was hemoglobin.

Electrophoresis in polyacrylamide gel was performed by the Davis method [10].

A qualitative reaction for lipids in proteinase D was carried out using TLC on Silufol plates and hexane—ether (7:3) (iodine developer). Carbohydrates in the enzyme were detected using ascending paper chromatography in the presence of standards and butanol—pyridine—water (6:4:3).

Auxiliary proteins 7S and 11S of dormant cotton seeds were hydrolyzed by proteinase D for 24 and 48 h with an enzyme:substrate ratio 1:30 in phosphate buffer at pH 7.0 and 37-40° C.

The amino-acid composition of proteinase D (5 mg) was determined by dissolving it in HCl (2 ml, 6 N) and hydrolyzing it in sealed ampuls for 24 h at 110° C. The hydrolysate was evaporated with fresh portions of water added and dried in a vacuum desiccator over alkali. The analysis was performed on an AT-339 (Czech Republic) analyzer.

The optimum temperature was determined by adding to azocasein (2 ml) in phosphate buffer a solution (1 ml) of enzyme (1 mg/ml). The mixture was incubated for 30 min at 20-55° C. The maximum activity was observed at 37-40° C.

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