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 (NH₄)₂SO₄ (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 (2·10⁻³ M). It was activated by such sulfhydryl reagents as cysteine and β-mercaptoethanol and inhibited by p-chloromercuribenzoate (4·10⁻⁴ 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.
TABLE 1. Amino-acid and Carbohydrate Composition of Proteinase D from 3-Day Germinated Cotton Seeds

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

Carbohydrates | Monosaccharide ratio
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Glucose | 3
Galactose | 1
Arabinose | 4

Defatting of 3-day germinated seeds

 Extraction of defatted powder with phosphate buffer (0.1 M, pH 7.4)

 Centrifugation (25 min, 6,000 rpm)

 Precipitate | Supernatant

 Precipitation by (NH₄)₂SO₄ (80%)

 Centrifugation

 Precipitate | Supernatant

 Desalting by dialysis

 Gel filtration through Sephadex G-150

 Proteinase D

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 cysteine 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.