SEVERAL PROPERTIES OF THE PARTIALLY PURIFIED PROTEINASE INHIBITOR IN EGGPLANT EXOCARP

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(Received August 20, 1975)

Summary A proteinase inhibitor was isolated and partially purified from the exocarp of eggplant, Solanum melongena L., by means of acetate buffer extraction, heat treatment, salting-out and column chromatography on DEAE-cellulose. This preparation showed inhibitory activities on various proteinases; trypsin [EC 3.4.4.4] and Pronase were strongly inhibited while α-chymotrypsin [EC 3.4.4.5] and Nagarse were weakly inhibited. The inhibitor was a protein substance, and, therefore, it was gradually inactivated by the long-time incubation with Pronase. The inhibition mode was non-competitive on trypsin and competitive on Pronase on the basis of Lineweaver-Burk plots. The investigations on the inhibition behavior in the co-existence of two kinds of proteinases suggested that the inhibitor was not of multi-headed type.

The widespread distribution of a number of natural proteinase inhibitors has been proved in plant, animal tissues and certain bacteria (1-4). Especially, the trypsin inhibitors from soybean and mammalian pancreas have been extensively studied from the point of view of their physiological roles, inhibition mechanism, structure and other problems, and also shown to be excellent models for the research on protein-protein interaction. The proteinase inhibitors from different sources have distinct inhibitory specificities on various proteinases and other characteristic properties. The physiological functions of the inhibitors and their nutritional significances have been discussed on the basis of their inhibitory specificities and distribution. Although the proteinase inhibitors in plant materials have been mainly found among the legumes, they have also been isolated from other plants such as, Solanum tuberosa (5), Gramineae (6,7) and Chenopodiaceae (8). In a previous paper (9), we also reported the presence of a proteinase inhibitor in
the exocarp of eggplant, *Solanum melongena* L. This communication describes the inhibitory activities on various proteinases, mode of the inhibition and some properties of the partially purified eggplant proteinase inhibitor.

**MATERIALS AND METHODS**

*Materials.* Commercially obtained eggplant was used. Hammersten’s bovine casein was purchased from E. Merck, Darmstadt, Germany. α-N-benzoyl-D,L-arginine-p-nitroanilide (BApNA) was obtained from the Institute for Protein Research, Osaka University, Osaka, Japan. Proteinases were obtained from the following companies; trypsin (2× cryst., from bovine pancreas, 10,000 BAEE units/mg) and α-chymotrypsin (3× cryst., from bovine pancreas, 50 BTEE units/mg) from the Sigma Chemical Co., Mo., U.S.A., Pronase-P (45,000 p.u.k./g) from the Kaken Kagaku Co., Ltd., Tokyo, Japan, and Nagarse (cryst., 1,500,000 p.u.n./g) from the Nagase Sangyo Co., Ltd., Osaka, Japan.

All other chemicals were of reagent grade.

**METHODS**

*Preparation of proteinase inhibitor from eggplant exocarp.* Eggplant exocarp was homogenized in a mixer with 0.1 M acetate buffer, pH 5.5. Dark-brown but clear extract was obtained from the homogenate by centrifugation and it was successively heated at 80°C for 20 min. After cooling, a small amount of precipitate was separated by filtration. The filtrate was treated with the addition of solid ammonium sulfate to attain 80% saturation and allowed to stand overnight. The resulting precipitate was collected by filtration and then dissolved in distilled water. This solution was desalted by dialysis against water in cellulose tubing (Visking Co., U.S.A.) and lyophilized. The lyophilized preparation was redissolved in 0.02 M phosphate buffer, pH 7.0, and applied to a DEAE-cellulose column equilibrated with 0.02 M phosphate buffer, pH 7.0. Elution of proteinase inhibitor was performed with the same buffer to remove color material. Fractions with trypsin inhibitory activity were collected and dialyzed against water, followed by lyophilization. By these procedures, approximately 70.3 mg of the partially purified inhibitor was obtained from 1 kg wet weight of the eggplant exocarp.

*Assay of proteinase activity.* Hydrolytic activity for BApNA was measured by the modified method of Erlanger et al. (10). Enzyme was dissolved with 0.005 N HCl containing 0.005 M CaCl₂. Substrate, BApNA, was dissolved to give a concentration of 5% in dimethylsulfoxide and then diluted 100-fold with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M CaCl₂. Reaction mixture consisted of 0.02 ml of the enzyme solution, 0.38 ml of the Tris-HCl buffer and 2.0 ml of the substrate solution. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 1.0 ml of 10% acetic acid. The released amount of p-nitroaniline was determined by the measurement of the absorbance at 410 nm.
with an Hitachi UV-VIS Spectrophotometer Model 139, Hitachi Ltd., Tokyo, Japan.

Proteinase activity for casein was determined by the method of HAGIHARA et al. (11) with a slight modification. Enzyme was principally dissolved with 0.005 N HCl containing 0.005 M CaCl₂. Substrate, casein, was dissolved to give a concentration of 1.2% in 0.05 M phosphate buffer, pH 8.0. Reaction mixture consisted of 0.50 ml of the enzyme solution, 0.50 ml of 0.05 M phosphate buffer, pH 8.0, and 3.0 ml of the casein solution. Incubation was performed at 37°C for 10 min. The reaction was stopped by the addition of 3.0 ml of 0.13 M trichloroacetic acid containing 0.26 M sodium acetate and 0.39 M acetic acid. After incubation at 37°C for 10 min and filtration through filter paper No. 5c (Toyo Roshi Kaisha Ltd., Tokyo, Japan), tyrosine content in 0.50 ml aliquot of the filtrate was determined by the method of FOLIN and CIOCALTEU (12).

Assay of inhibitory activity. The inhibitory activity was expressed as the percent of inhibition (I) to the control activity (T) according to the following equation;

\[ I(\%) = \frac{(T-T*)}{T} \times 100 \]

where \( T^* \) and \( T \) are the activities of proteinase with and without the inhibitor, respectively. Proteinase activity was determined by the method described above.

Chemical analysis of the partially purified inhibitor. Contents of protein, total hexose and amino sugar in the partially purified preparation were determined by the following methods. Protein concentration was calculated on the basis of the nitrogen content determined by the micro-Kjeldahl method (13). Total hexose content was determined according to the phenol-sulfuric acid method of DUBOIS et al. (14) by use of an equiweight mixture of D-galactose and D-mannose as standard hexose. Determination of amino sugar content was performed by the method of CESSI and PILIEGO (15) with D-glucosamine HCl as standard.

RESULTS

Chemical analysis of the partially purified inhibitor

Contents of principal constituents in the partially purified preparation were as follows; protein 62.3% (total nitrogen 10.0%), total hexose 15.4% and amino sugar 2.07%.

Inhibitory activities on various proteinases

Figure 1 shows the inhibitory activities of the partially purified preparation on the hydrolytic activities of trypsin and Pronase. The amount of trypsin in the reaction mixture was 20 μg and that of Pronase was 50 μg. The amount of the inhibitor was increased stepwise up to four-fold of the enzyme amount. Figure 2 shows the inhibitory activities on the proteolytic activities of \( \alpha \)-chymotrypsin and Nagarse. Reaction mixture consisted of \( \alpha \)-chymotrypsin (18.8 μg) or Nagarse
(40 μg) and casein with or without the inhibitor. Inhibitory activity was defined as double the amount of the inhibitor which was required for the 50% inhibition on the activity of 1 μg enzyme. The inhibitor showed significantly stronger inhibition on trypsin and Pronase than that on α-chymotrypsin and Nagarse. The inhibitory activities of each proteinase are summarized in Table 1.

![Graph](image1)

**Fig. 1.** Inhibition of trypsin and Pronase. ●, trypsin 20 μg; ○, Pronase 50 μg.

![Graph](image2)

**Fig. 2.** Inhibition of α-chymotrypsin and Nagarse. ●, α-chymotrypsin 18.8 μg; ○, Nagarse 40 μg.

**Table 1.** Inhibitory activities on various proteinases.

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Inhibitor activity</th>
<th>Substrate</th>
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<tbody>
<tr>
<td>Trypsin</td>
<td>2.7 μg</td>
<td>BApNA</td>
</tr>
<tr>
<td>Pronase</td>
<td>2.4</td>
<td>BApNA</td>
</tr>
<tr>
<td>Nagarse</td>
<td>13.6</td>
<td>casein</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>16.8</td>
<td>casein</td>
</tr>
</tbody>
</table>
Mode of the inhibition

Mode of the inhibition was characterized by Lineweaver-Burk plot method for the hydrolytic activities of trypsin and Pronase. Reaction mixture consisted of trypsin (20 µg) or Pronase (50 µg) and the inhibitor with BApNA substrate ranging in the concentration from 0.12 mM to 2.40 mM. The inhibitor was added to each proteinase at two kinds of doses. The Lineweaver-Burk plot patterns (Fig. 3-a, -b) shows the inhibition is non-competitive on trypsin and competitive on Pronase. In addition, inhibition of Pronase by the substrate was found at higher substrate concentration.

Inhibition behavior of the inhibitor in the presence of two kinds of proteinases

Changes of free trypsin activity was measured to investigate the influence of the co-existence of another proteinase on the trypsin-inhibitor complex formation. The rate of inhibition on trypsin was shown in Fig. 4, in relation to the presence of the other proteinase. It was calculated according to the following equation:

\[
\text{the rate of inhibition on trypsin} (\%) = \frac{(T_1 - T_3)}{(T_1 - T_2)} \times 100
\]

where \(T_1\) was trypsin activity without the inhibitor and the other proteinase. \(T_2\) and \(T_3\) were trypsin activities in the presence of the inhibitor with and without the other proteinase, respectively. The amounts of trypsin and the inhibitor were 20 µg and 30 µg, respectively. Each of Pronase, Nagarse and \(\alpha\)-chymotrypsin was added at the amount ranging from 10 µg to 200 µg as the other proteinase. In the experiment with \(\alpha\)-chymotrypsin, 10 µg of the inhibitor was used. In the absence of the other proteinase, most of the trypsin was inhibited by the inhibitor. Trypsin activity was represented by the hydrolytic activity for BApNA determined as described in methods. In the case of the combination with Pronase which was also
Fig. 4. Influence of the presence of another proteinase on formation of the trypsin-inhibitor complex. ●, Pronase; ○, α-chymotrypsin; △, Nagarse.

Fig. 5. Pronase digestion of the inhibitor. The reaction mixture consisted of 2.0 mg of the inhibitor and 0.20 mg of Pronase in 1.0 ml of 0.1 M Tris-HCl buffer, pH 8.0.

capable of hydrolyzing BApNA, net trypsin activity was calculated by subtracting the original activity of Pronase from the total activity. The rate of inhibition on trypsin was obviously reduced in the presence of the other proteinase. This phenomenon indicated that trypsin and the other proteinase competed for the inhibitor with each other. Namely, the inhibitor was not considered to be of multi-headed type.

Pronase digestion of the inhibitor

If the proteinase inhibitor is composed of a protein or peptide as an essential component, it is expected to be inactivated by proteinase digestion at prolonged incubation. To 2.0 mg of the inhibitor, 0.20 mg of Pronase was added in 1.0 ml
of 0.1 M Tris-HCl buffer, pH 8.0. At certain intervals during 24 hr incubation at 37°C, the remaining inhibitory activity in 0.02 ml aliquot of the reaction mixture was measured by the method described previously using trypsin and BApNA (Fig. 5). Until the first 5 hr, the inhibitory activity was hardly lost and the inhibitor completely depressed the proteinase activity. Thereafter, the inhibitory activity was, however, linearly reduced to less than one-tenth of the original activity within 24 hr.

DISCUSSION

In the partially purified inhibitor preparation, nitrogen, hexose and amino sugar were detected as its components. These data indicated that the inhibitor might be a glycoprotein or glycopeptide. However, the content of total hexose varied decreasingly in contrast to the increase of the specific activity during the purification. On the other hand, the total nitrogen content increased. Consequently, it was suggested that sugar might not be necessarily an essential component of the inhibitor. Inactivation of the inhibitor by Pronase digestion (Fig. 5) supported that protein was an essential component of the inhibitor molecule. In dialysis step during the preparation, some trypsin inhibitory activities were detected in the outer medium. The inhibitor seems to be of relatively low molecular weight. The dialyzable inhibitor was not used in this study. The inhibitor had such characteristic amino acid composition as follows; glycine, aspartic acid and half-cystine were contained at relatively high contents, but methionine and histidine at very low contents. This feature was similar to that of the Kunitz's soybean trypsin inhibitor (16), except for cystine. The extensive cross-linking due to the high half-cystine content in the inhibitor molecule is an effective factor to the structural rigidity which will afford resistivity to the denaturation.

The present inhibitor was found active on all of the used proteinases; trypsin, α-chymotrypsin, Pronase and Nagarse. Especially, trypsin and Pronase were strongly inhibited. However, on the evaluation of the inhibitory activity, we must consider carefully the assay method, the kind of substrate, purity of proteinase and so on. In fact, the inhibitory activity on trypsin was usually estimated higher by use of BApNA as substrate than by use of casein. The modes of inhibition on trypsin and Pronase were found to be non-competitive and competitive, respectively, on the basis of Lineweaver-Burk plot method. Nevertheless, many workers have already pointed out some problems in the use of Lineweaver-Burk plot method for the determination of inhibition mode. It was shown to derive incompatible results depending upon the conditions in the assay. Therefore, it may be unable to conclude the inhibition mode from the results obtained in this study. Isoelectrofocusing analysis showed that this inhibitor consisted of three kinds of inhibitors having isoelectric points of pH 4.2, 4.7 and 6.0, respectively. The most potential inhibitor for BApNA hydrolytic activity of trypsin was the
inhibitor of pI 4.7. Interestingly, this isoelectric point is similar to that of the Kunitz's soybean trypsin inhibitor (17,18). But, other two inhibitor molecules and a few of non-active proteins were also contained at non-negligible content. This heterogeneity should be always considered at the interpretation of these results.

REFERENCES