AN IMPROVED METHOD FOR THE PURIFICATION OF EGGPLANT TRYPsin INHIBITOR

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Summary The trypsin inhibitor in eggplant, Solanum melongena L., was isolated and purified by the improved method with the techniques of dialysis using acetylated cellulose tube and ion-exchange chromatography on DEAE-Sephadex. The final preparation was found to be homogeneous by disc and SDS-polyacrylamide gel electrophoreses. This inhibitor had the molecular weight of about 6,200, the pI value of 4.7, and furthermore characteristic amino acid composition lacking in tryptophan, histidine, valine and methionine. The trypsin inhibition data indicated that the purified inhibitor combined with bovine trypsin [EC 3.4.21.4] in the molar ratio of 1:1. These properties of this inhibitor were in agreement with those of the dialyzable eggplant trypsin inhibitor previously purified, indicating that the dialyzable and non-dialyzable inhibitors in eggplant are identical.

A number of protein proteinase inhibitors have been isolated and purified from the tissues of various plants and animals (1, 2). Especially the inhibitors from leguminous plant seeds have been widely investigated. Whereas, in Solanaceae, a non-leguminous plant, BALLS and RYAN have reported on the purification and characterization of chymotrypsin inhibitor I from potato, Solanum tuberosa (3). IWASAKI et al. have purified and characterized two types of proteinase inhibitors, potato inhibitor II-a and II-b (4, 5). We have also reported on the presence of a trypsin inhibitor in the exocarp of eggplant, Solanum melongena L., and have shown that the inhibitor could be distinguished into dialyzable and non-dialyzable (6). They were at first regarded as distinct inhibitors and their purification studies were therefore progressed individually (7, 8). Recently, the dialy-
zable inhibitor was purified completely (9). On the other hand, a large amount of inhibitory activity was detected in outer solution during the dialysis of the partially purified non-dialyzable inhibitor. This phenomenon suggests that the both inhibitors are identical. In order to elucidate this problem, we attempted to purify the eggplant trypsin inhibitor without separating it into dialyzable and non-dialyzable. In this paper, the improved purification method and some properties of the purified inhibitor are described, and also the identity of both inhibitors is discussed.

**MATERIALS AND METHODS**

**Materials.** Eggplants were obtained commercially. Trypsin (2 × cryst., from bovine pancreas) was purchased from Sigma Chemical Co. Hammarsten’s casein from E. Merck AG, Darmstadt and α-N-benzoyl-D,L-arginine-p-nitroanilide (BAPA) from Peptide Institute Protein Research Foundation, Osaka were used. DEAE-cellulose was obtained from Brown. DEAE-Sephadex A-25 and Sephadex G-50 (fine) were Phamacia products. Cellulose tubing was a product of Visking Co. and it was acetylated by treatment with 7.5% acetic anhydride in pyridine at 70°C for 20 hr in our laboratory. The acetylated cellulose tube was rinsed in 0.1 M acetic acid and water, and stored in 50% ethanol at 4°C until use. Carrier ampholyte was purchased from LKB. As marker proteins for the determination of molecular weight, bovine insulin from Sigma, and horse cytochrome c, whale myoglobin and bovine chymotrypsinogen A from Mann Research Laboratories were used.

All other chemicals used were of the special or reagent grade.

**Determination of the protein concentration.** Protein concentration was calculated from the nitrogen content determined by the micro-Kjeldahl method (10). It was also estimated from the absorbance at 280 nm with a Hitachi UV-VIS spectrophotometer, model 139.

**Assay of trypsin and trypsin inhibitory activities.** The amidase and proteolytic activities of trypsin were determined by the respective methods of Erlanger et al. (11) and Haghara et al. (12), with slight modifications as described in our previous paper (6).

The inhibitory activity was estimated from the residual trypsin activity in the presence of the inhibitor and expressed as per cent inhibition (I) for the control assay using the following equation;

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

where \( T^* \) and \( T \) are the respective activities of trypsin with and without the inhibitor. The inhibitor unit was defined as the amount of inhibitor required for the complete inhibition of 1 mg of active trypsin. The concentration of trypsin solution used was calculated from the optical factor of 0.67 at 280 nm (13). The purity of the trypsin used was determined by active-site titration using p-nitrophenyl
Purification method of eggplant trypsin inhibitor

$\text{p'}$-guanidinobenzoate hydrochloride (14) and a value of 72% was obtained.

Electrophoretic analyses. Disc electrophoresis for the estimation of protein purity was performed with 7.5% polyacrylamide gel at pH 9.4 by the method of Davis (15). The molecular weight of the inhibitor was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to the procedure of Weber and Osborn (16). Isoelectric focusing of the inhibitor was carried out essentially as described by Vesterberg and Svensson (17).

Amino acid analyses. Amino acid analyses were performed with a Hitachi KLA-5 autoanalyzer. The samples containing norleucine (0.1 µmole/ml) as an internal standard were hydrolyzed at 110°C in 6 N HCl in evacuated and sealed ampules. The times of hydrolysis were 24, 48 and 72 hr. Tryptophan content was determined by the method of Goodwin and Morton (18).

RESULTS

Purification of the eggplant trypsin inhibitor

Twenty-eight kilograms of eggplant exocarps was ground in a homogenizer with 28 liters of 0.1 M sodium acetate buffer, pH 5.5. The extract was heated at 80°C for 10 min after insoluble material had been removed by centrifugation, quickly cooled and filtered. Solid ammonium sulfate was added to the filtrate to give 80% saturation. After standing overnight, the resultant precipitate was collected by filtration and dissolved in a minimum volume of distilled water. This solution was then dialyzed against distilled water using acetylated cellulose tube. The dialyze which was dark-brown in color retained more than 90% of the trypsin inhibitory activity of the original extract. Subsequently, this solution was lyophilized and the lyophilized preparation was dissolved in 1 liter of 0.02 M N-ethylmorpholine acetic acid buffer, pH 7.0. After centrifugation to remove insoluble material, the supernatant solution was applied to a DEAE-cellulose column (5.0 × 65 cm) equilibrated with the N-ethylmorpholine acetic acid buffer and eluted with the same buffer. The elution profile of this chromatography is shown in Fig. 1. The inhibitor was eluted with the buffer and separated from the colored material which was strongly adsorbed on to the DEAE-cellulose. The non-adsorbed fractions with anti-tryptic activity were collected and concentrated with a rotary evaporator at 40°C to obtain the volatile buffer free inhibitor solution. After adjustment to pH 5.4 with dilute pyridine and acetic acid, the concentrated solution was applied to a DEAE-Sephadex A–25 column (5.0 × 63 cm) equilibrated with 0.05 M pyridine acetic acid buffer, pH 5.4. The column was washed with the pyridine acetic acid buffer, then the inhibitor was eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer. Figure 2 shows the elution pattern of the DEAE-Sephadex column chromatography. Two inhibitory activity peaks were observed and the main active peak was associated with the main protein peak. The main peak was collected, subjected to dialysis against distilled
water using the acetylated tube and lyophilized. On the other hand, the minor inhibitor peak was negligibly small. The lyophilized sample was then dissolved in 30 ml of 0.1 M acetic acid and the solution was chromatographed on a Sephadex G-50 column (4.0 × 96 cm) with the use of 0.1 M acetic acid as eluent. As shown in Fig. 3, a symmetrical protein peak was obtained and completely associated with the inhibitory activity peak. This peak was collected, lyophilized and regarded as a final purified inhibitor.

By these procedures, about 200 mg of the purified inhibitor was obtained from the 28 kg of eggplant exocarps. The purification of the eggplant trypsin inhibitor is summarized in Table 1.

**Homogeneity of the purified inhibitor**

The homogeneity of the purified inhibitor was checked by disc and SDS-polyacrylamide gel electrophoreses. As shown in Fig. 4, the inhibitor exhibited homogeneous behaviour as a single protein band in these analyses.

**Ultraviolet absorption spectrum**

The ultraviolet absorption spectrum of the inhibitor was determined in 0.05 M
Fig. 2. DEAE-Sephadex A–25 chromatography of the active fraction obtained by DEAE-cellulose chromatography. The inhibitor solution was applied to a column (5.0 × 63 cm) of DEAE-Sephadex A–25 equilibrated with 0.05 M pyridine acetic acid buffer, pH 5.4. The column was washed with the same buffer. The inhibitor was eluted first with a linear gradient of NaCl (0–1.0 M) in the same buffer and then with 0.05 M pyridine acetic acid buffer, pH 5.4 containing 1.0 M NaCl at a flow rate of 120 ml/hr. The trypsin inhibitory activity of 50 μl of each fraction was assayed by the method used in the experiment shown in Fig. 1. The fractions represented by the bar were collected, dialyzed and lyophilized. ●—●, absorbance at 280 nm; ○—○, trypsin inhibitory activity; ----, NaCl concentration.

Table 1. Summary of the purification procedure for the eggplant trypsin inhibitor.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (liter)</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Ratio</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>43.0</td>
<td>98,900</td>
<td>1,630</td>
<td>0.0165</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment (NH₄)₂SO₄ precipitate (0.8 satn.)</td>
<td>38.4</td>
<td>74,300</td>
<td>1,610</td>
<td>0.0217</td>
<td>1.32</td>
<td>98.8</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>3.10</td>
<td>7,910</td>
<td>1,590</td>
<td>0.201</td>
<td>12.2</td>
<td>97.5</td>
</tr>
<tr>
<td>DEAE-Sephadex A–25 chromatography</td>
<td>5.00</td>
<td>1,310</td>
<td>1,090</td>
<td>0.832</td>
<td>50.4</td>
<td>66.9</td>
</tr>
<tr>
<td>Sephadex G–50 gel filtration</td>
<td>0.670</td>
<td>205</td>
<td>683</td>
<td>3.33</td>
<td>202</td>
<td>41.9</td>
</tr>
<tr>
<td></td>
<td>0.313</td>
<td>202</td>
<td>682</td>
<td>3.38</td>
<td>205</td>
<td>41.8</td>
</tr>
</tbody>
</table>

*a Protein = nitrogen content × 6.25.

*b 1 unit = the amount of inhibitor required for the complete inhibition of 1 mg of active trypsin based on the casein-digestion method.
sodium acetate buffer, pH 5.5 with a Shimadzu double-beam spectrophotometer, UV–200. The inhibitor had a typical UV absorption curve for protein (maximum at 277 nm and minimum at 253 nm). The extinction coefficients, $E_{1%1cm}^{277}$, were determined to be 0.714 at 277 nm and 0.662 at 280 nm for the purified inhibitor dried in vacuo over P$_2$O$_5$ to constant weight. Therefore, the optical factor converting to absorbance at 280 nm to mg of protein per ml was calculated to be 1.51 for this inhibitor.

**Isoelectric point**

Isoelectric focusing was carried out using the carrier ampholyte of pH 3.5–10. Three milligrams of the inhibitor was applied to a column and electrophoresis was performed at 4°C for 72 hr with 1 watt of electric power. Figure 5 represents the result of isoelectric focusing. A single protein and a single activity peak were observed at the same position, pH 4.7. This indicates that the inhibitor is an acidic protein having a pI value of 4.7.
Fig. 4. Electrophoretic patterns of the purified inhibitor. (a) Disc electrophoresis. Electrophoresis was carried out at pH 9.4 with 7.5% polyacrylamide gel. A current of 2 mA per tube was supplied for 120 min at 5°C. (b) SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed with 20% polyacrylamide gel. A current of 8 mA per tube was supplied for 7 hr at room temperature.

Fig. 5. Isoelectric focusing of the purified inhibitor. Experimental procedures are described in the text. The trypsin inhibitory activity of 10 μl of each fraction was measured by the method used in the experiment shown in Fig. 1. ●—●, absorbance at 280 nm; ○—○, trypsin inhibitory activity; ---, pH.
Molecular weight
The molecular weight of the purified inhibitor was determined by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 6, the molecular weight was estimated to be about 6,300. This value agreed well with the molecular weight of the dialyzable eggplant trypsin inhibitor determined by gel filtration, SDS-polyacrylamide gel electrophoresis and equilibrium ultracentrifugation (9).

Amino acid composition
The results of the amino acid analyses are shown in Table 2. They were computed based on the molecular weight of 6,300. The inhibitor was composed of 58 amino acid residues, from which the molecular weight was calculated to be 6,203. In addition, this table indicates that the protein is rich in aspartic acid, glycine and half-cystine, and lacking in tryptophan, histidine, methionine and valine. This amino acid composition was almost identical to that of the dialyzable inhibitor (9).

Trypsin inhibitory activities
The purified inhibitor showed stoichiometric inhibition on the amidase and proteolytic activities of trypsin. One µg of the inhibitor completely inactivated 3.7 µg of active trypsin when BAPA was used as the substrate and also inactivated 3.3 µg of the trypsin in the case of casein as the substrate. From these results, if it is assumed that 1 mole of trypsin is inhibited by 1 mole of the inhibitor, the
Table 2. Amino acid composition of the eggplant trypsin inhibitor.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per molecule</th>
<th>Integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Ammonia</td>
<td>6.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Serine</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Proline</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>8.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Valine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

| Total            | —         | —   | —   | 58 |
| Molecular weight | —         | —   | —   | 6,203 |

\[ ^a \text{Values obtained by extrapolation to zero hydrolysis time.} \]
\[ ^b \text{Determined spectrophotometrically.} \]

The molecular weight of the eggplant inhibitor is calculated to be 6,300–7,000 on the basis of the molecular weight of 23,300 for trypsin (19). This value is in good agreement with the molecular weight estimated by SDS-polyacrylamide gel electrophoresis. Therefore, it is concluded that the inhibitor combined with trypsin in the molar ratio 1:1.

DISCUSSION

The present paper describes the improved purification method and some properties of the eggplant trypsin inhibitor.

The eggplant inhibitor was fractionated by extraction with 0.1 M sodium acetate buffer, pH 5.5, heat treatment and salting-out, and further purified by column chromatographies on DEAE-cellulose, DEAE-Sephadex A-25 and Sephadex G-50 (Table 1). These procedures, especially the initial steps, are similar to those used in the purification of the dialyzable eggplant inhibitor (9), but two procedures which were not utilized in our previous investigation were employed in this study. One is the use of the acetylated cellulose tube, which was useful for the desalting of 80% ammonium sulfate fraction without separating the inhibitor into dialyzable
and non-dialyzable, and the other is the technique of ion-exchange chromatography on DEAE-Sephadex A-25, on to which the inhibitor was adsorbed after decolorization by DEAE-cellulose chromatography. By the introduction of two procedures, the inhibitor could be purified 205-fold over the original extract. The purified eggplant inhibitor obtained had the following characteristics: the molecular weight of about 6,200 (Table 2); the pl value of 4.7 (Fig. 5); the optical factor at 280 nm of 1.51; the trypsin inhibition of 1:1 molar ratio and the amino acid composition lacking in tryptophan, histidine, valine and methionine (Table 2). These properties were in good or near agreement with those of the dialyzable inhibitor (8, 9). This indicates that the dialyzable inhibitor is apparently identical to the inhibitor prepared by the improved method in this paper.

In the previous paper (6), it is found that the crude eggplant trypsin inhibitor contained three components with pl values of 4.2, 4.7 and 6.0, respectively. The components with pl values of 4.2 and 6.0 were minor inhibitor fractions. The studies on these minor inhibitor fractions in eggplant are now in progress.

REFERENCES

5) IWASAKI, T., KIYOHARA, T., and YOSHIKAWA, M. (1972): Chemical and physicochemical characterization of two different types of proteinase inhibitors (inhibitors II-a and II-b) from potatoes. J. Biochem. (Tokyo), 72, 1029-1035.


