Isolation of Minor Proteinase Inhibitors from Eggplant Exocarp

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In the exocarp of eggplant, Solanum melongena L., at least four kinds of proteinase inhibitors were found. These inhibitors were isolated by DEAE-Sephadex column chromatography and isoelectrofocusing. Main inhibitor and one of minor inhibitors showed stronger inhibitory activity for trypsin [EC 3.4.21.4] than chymotrypsin [EC 3.4.21.1]. The pI values were 4.66 for the main inhibitor and 4.06 for the minor inhibitor.

The other minor inhibitors of pI 4.36 and pI 5.98 inhibited chymotrypsin preferably and they had different molecular weights determined by gel filtration from that of the main inhibitor, although these values were very close to each other.

Generally, naturally occurring proteinase inhibitor exists in several different forms in one material. For example, at least four or more inhibitors have been isolated from soybean.1) All these inhibitors in soybean have been found to show different inhibitory specificities against proteinases and to have different chemical compositions. Occurrence of some different inhibitors in the same material have been shown in other sources; lima bean,2) potato,3) wheat,4) barley5) and so on. The reason of the existence of inhibitor in different forms is not clear. We reported that trypsin inhibitor in the exocarp of eggplant existed in multiple forms which showed different isoelectric points.6−9) This paper describes the isolation of minor inhibitors from the eggplant exocarp and their properties.

MATERIALS AND METHODS

Materials. The eggplant used was obtained commercially. Bovine trypsin (2× crystallized, from bovine pancreas), bovine α-chymotrypsin (3× crystallized, from bovine pancreas) and Kunitz bovine pancreatic trypsin inhibitor were purchased from Sigma Chemical Co., Mo., U. S. A. α-N-Benzoyl-L-arginine-p-nitroanilide (BAPNA), p-tosyl-L-arginine methyl ester (TAME), acetyl tyrosine ethyl ester (ATEE) and bacitracin were obtained from Nakarai Chemical Ltd., Kyoto, Japan. Carrier ampholyte was purchased from LKB Produkter AB, Sweden. All other chemicals were of reagent grade.

Inhibitory activity assay. Inhibitory activity against trypsin was determined by the spectrophotometric method with a BAPNA substrate or by the method using a pH-stat, RTS 622, Radiometer, Copenhagen, Denmark, as reported previously.10) Inhibitory activity against chymotrypsin was determined with the pH-stat as follows; the assay was done at 25°C under flushing of N2 gas. The incubation mixture consisted of 0.20 ml of 1 M CaCl2, 25 μl of chymotrypsin solution (200 μg/ml) and an appropriate amount of the inhibitor in a total volume of 1.925 ml. This mixture was adjusted to pH 8.0. After preincubation for 2 min, the reaction was started by the addition of 0.1 ml of 2 M ATEE solution (0.2 M in 50% ethanol). The residual chymotrypsin activity was titrated by the pH-stat using 0.01 M NaOH. One unit of inhibitory activity is defined as the amount of inhibitor required for the complete inhibition of 1 μg of proteinase.

Isoelectrofocusing. Isoelectrofocusing was performed essentially by the method of Vesterberg and Svensson.31) Electrophoresis was carried out at 1 watt for 72 hr at 5°C. The solution in the column was fractionated into 1.2 ml portions to measure the pH at 25°C with a pH meter, PHM 62 Standard, Radiometer, the absorbance at 280 nm and the inhibitory activity against trypsin.

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RESULTS

Isolation of the main and minor inhibitors from eggplant exocarp

The decolored crude inhibitor solution was obtained from the extract of eggplant exocarp by heat treatment, ammonium sulfate precipitation and DEAE-cellulose chromatography as reported recently in our laboratory. To the concentrated decolorized inhibitor solution was added pyridine to a final molarity of 0.05 M and the solution was adjusted to pH 5.4 with acetic acid. This solution was applied to a DEAE-Sephadex A-25 column equilibrated with 0.05 M pyridine acetic acid buffer, pH 5.4. During washing of the column with the same buffer, the minor inhibitor [I] was eluted in the non-absorbable fraction. For the further elution, the increasing molarity of NaCl from zero to 1 was employed. The fraction with strong trypsin inhibitory activity was pooled as the main inhibitor fraction, but the first several tubes containing impure non-active protein were omitted. These results are shown in Fig. 1.

The main inhibitor fraction shown in Fig. 1, was concentrated, desalted by gel filtration on Sephadex G-25 and lyophilized. The lyophilized sample, dissolved in 0.05 M pyridine acetic acid buffer, pH 5.4, was rechromatographed on a DEAE-Sephadex A-25 column as mentioned above (Fig. 2).

The main inhibitor fraction obtained in the first DEAE-Sephadex A-25 column chromatography was separated by rechromatography into two fractions: main inhibitor and minor inhibitor [II]. Each of these three inhibitor fractions was lyophilized after desalting by gel filtration.

Summary of the purification of these inhibitors is shown in Table I. Minor inhibitors, [I] and [II], were only 1.2% and 0.8% of the total trypsin inhibitory activity, respectively. About 5.3 mg of the main inhibitor was obtained from 1 kg of wet eggplant exocarp. The amount of starting eggplant exocarp was 12.86 kg in wet weight in Table I.

Figures 3, 4 and 5 show the respective
TABLE I. SUMMARY OF PURIFICATION OF INHIBITORS FROM EGGPLANT EXOCARP

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total inhibitory unit x 10^6 TIU</th>
<th>Specific inhibitory unit TIU/μg protein</th>
<th>Recovery %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>776.0</td>
<td>0.017</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>715.0</td>
<td>0.020</td>
<td>92.1</td>
<td>1.15</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation &amp; dialysis</td>
<td>472.8</td>
<td>0.065</td>
<td>60.9</td>
<td>3.75</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
<td>303.2</td>
<td>0.488</td>
<td>39.1</td>
<td>28.2</td>
</tr>
<tr>
<td>DEAE-Sephadex A–25 chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main inhibitor</td>
<td>272.5</td>
<td>2.635</td>
<td>35.1</td>
<td>152.0</td>
</tr>
<tr>
<td>Minor inhibitor [I]</td>
<td>2.59</td>
<td>0.202</td>
<td>0.33</td>
<td>11.7</td>
</tr>
<tr>
<td>DEAE-Sephadex A–25 rechromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main inhibitor</td>
<td>207.6</td>
<td>3.070</td>
<td>26.8</td>
<td>177.1</td>
</tr>
<tr>
<td>Minor inhibitor [II]</td>
<td>1.74</td>
<td>1.640</td>
<td>0.22</td>
<td>94.6</td>
</tr>
</tbody>
</table>

a) TIU; Trypsin Inhibitory Unit. One unit is defined as the amount required for the complete inhibition of 1 μg of trypsin.

Inhibitory specificity

Inhibitory activity of each inhibitor for trypsin or chymotrypsin was shown by the inhibitory unit per 0.01 ml of each sample solution obtained by isoelectrofocusing (Table II). The main inhibitor and two minor inhibitors obtained from the minor inhibitor [II] showed stronger inhibitory activity for trypsin than for chymotrypsin. However, the two minor inhibitors from minor inhibitor [I], especially the inhibitor with a pI value of 5.98, showed obviously stronger inhibitory activity for chymotrypsin than for trypsin.

Molecular weight

The molecular weights of the main inhibitor and two minor inhibitors which showed stronger inhibitory activity for chymotrypsin than for trypsin, were measured by gel filtration on Sephadex G–50. As shown in Fig. 6, the elution positions of these three inhibitors were very close to one another and their molecular weights were calculated to be 6250 for the pI 4.36 inhibitor, 6450 for the pI 5.98 inhibitor and 6950 for the main inhibitor. To confirm the difference of the molecular weights between the main and minor inhibitors, each minor inhibitor was subjected to gel filtration with
TABLE II. INHIBITORY SPECIFICITIES OF THE INHIBITORS

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pH</th>
<th>Inhibitory activity unit</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main inhibitor</td>
<td>4.66</td>
<td>3.056</td>
<td>1.949</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>Minor inhibitor [I]</td>
<td>4.36</td>
<td>0.658</td>
<td>0.923</td>
<td></td>
<td>1.40</td>
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<tr>
<td></td>
<td>5.98</td>
<td>0.586</td>
<td>1.124</td>
<td></td>
<td>1.92</td>
</tr>
<tr>
<td>Minor inhibitor [II]</td>
<td>4.06</td>
<td>0.264</td>
<td>0.187</td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>4.51</td>
<td>0.574</td>
<td>0.287</td>
<td></td>
<td>0.50</td>
</tr>
</tbody>
</table>

a) The ratio value was calculated by the following equation; chymotrypsin inhibitory activity/trypsin inhibitory activity.

Fig. 6. Molecular Weight Determination by Gel Filtration on a Sephadex G-50 Column.
The gel filtration of each standard protein or sample was carried out individually. Ten % of acetic acid was used as the eluent. The elution points were estimated by the measurement of the absorbance at 280 nm or trypsin inhibitory activity of each fraction. Column size, 2.5 × 44.5 cm; fraction volume, 1.5 ml; flow rate, 18.8 ml/hr. [A], bacitracin (MW 1450); [B], Kunitz bovine pancreatic trypsin inhibitor (MW 6513); [C], cytochrome c (MW 12,400); [D], whale myoglobin (MW 17,500); △, main inhibitor; ●, minor inhibitor of pI 5.98; ×, minor inhibitor of pI 4.36.

The main inhibitor under the same condition as mentioned above. Each of the minor inhibitors was clearly separated from the main inhibitor as shown in Fig. 7-a and 7-b. These results indicate that the molecular weights of the minor inhibitors were very close to that of the main inhibitor, but not identical.

DISCUSSION

In the eggplant exocarp, five kinds of proteinase inhibitors were found. The inhibitor of pI 4.51 separated from the minor inhibitor [II] may be identical with the main inhibitor because their pI values and inhibitory specificities are similar. Probably, the main inhibitor would contaminate the minor inhibitor [II] fraction at the rechromatography step. At least, four kinds of proteinase inhibitors are confirmed to be present in the eggplant exocarp. These inhibitors have different pI values and inhibitory specificities. Also, they have different molecular weights, although their values are very close to one another. In previous papers,9–12) we reported the trypsin inhibitor having the pI value of 4.7 and molecular weight of 6200. This inhibitor corresponds to the main inhibitor having the molecular weight of 6950 by the gel filtration method described in this paper. The discrepancy of the values of the molecular weights may be due to the variation in the experimental conditions.
It is interesting that the inhibitors, which show stronger inhibitory activity for chymotrypsin than for trypsin, are detected in the eggplant exocarp, although their contents are very low. Any functions of these inhibitors are not understood yet. However, the existence of inhibitors having different inhibitory specificities suggests that these inhibitors play some roles in the growing of eggplant fruit.

REFERENCES