Metalloenzyme Inhibitor from Kidney Beans

PARTIAL PURIFICATION AND CHARACTERIZATION

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ABSTRACT

Inhibitory activity directed against metalloenzymes has been highly purified from extracts of red kidney beans (Phaseolus vulgaris). The inhibitor is a substance of small molecular weight and appears to be a chelator of Zn²⁺. One milligram of the preparation inhibited 23 milligrams carboxypeptidase A. The inhibitor also strongly inhibited carboxypeptidase B and alkaline phosphatase and could activate phosphoglomutase that had previously been inactivated with Zn²⁺. The isoelectric point of the inhibitor is 4.7. The inhibitor activity was abolished by preincubation with Zn²⁺, Ni²⁺, Co²⁺, or Cu²⁺. The mechanism of inhibition of carboxypeptidases and alkaline phosphatase by the bean inhibitor is apparently due to the complexing and complete removal of Zn²⁺ from the enzymes.

During our studies of the function of plant protease inhibitors we found in several members of the Leguminosae family strong inhibitory activity against CPase A. The CPase inhibitory activity from red kidney bean (Phaseolus vulgaris) was investigated in detail and the purification and some properties of this inhibitor are described. This inhibitor acts by chelating the zinc of the CPase, as well as some other metalloenzymes, and thus differs from the naturally occurring CPase inhibitors previously reported from potato (14, 15), yeast (9), roundworm (7), and a fungus Aspergillus ochraceus (12).

MATERIALS AND METHODS

Materials. Various species and varieties of bean seeds and vegetables were obtained from local grocery stores: CPase A (bovine, crystalline) and CPase B (hog, chromatographically purified) were from Worthington Biochemical Corp. and Sigma Chemical Co., respectively; carbobenzoxy-glycyl-L-phenylalanine and benzoyl-glycyl-L-arginine, from Protein Research Foundation, Osaka; benzoyl-glycyl-L-β-phenyllactate, from Seikagaku Kogyo Co.; alkaline phosphatase (EC 3.1.3.1) (calf intestinal mucosa) and p-nitrophenylphosphate, from Sigma and Tokyo Chemical Industry Co., respectively; phosphoglomutase (EC 2.7.5.1) (rabbit muscle, crystalline), glucose-6-P dehydrogenase (Baker’s yeast, crystalline), α-d-glucose-1-P, α-d-glucose-1,6-diP and NADP⁺, from Sigma; Sephadex G-10, G-15, G-25, and G-100, from Pharmacia Fine Chemicals; Ampholine (pH 3.5–10, batch 17), from LKB-Produkter AB.

Enzyme Assays. Peptidase activity of CPase A was determined spectrophotometrically with carbobenzoxy-glycyl-L-phenylalanne as substrate by modifying the method of Whitaker et al. (18). To 2.9 ml of the buffered substrate solution (1.03 mm, in 0.05 m Tris-HCl containing 0.15 m NaCl, pH 7.5) was added 0.1 ml enzyme solution, and the decrease of A at 223 nm was recorded for 0.5 to 1 min at 25 °C with a Hitachi model 181 spectrophotometer. In this assay method, 1 mg of CPase A hydrolyzed 23.6 µmol substrate/min. Esterase activity of CPase A was determined with benzoyl-glycyl-L-β-phenyllactate as substrate by modifying the method of McClure et al. (10). To 2.9 ml of the substrate solution (1.03 mm, in 0.05 m Tris-HCl containing 0.15 m NaCl, pH 7.5) were added 0.075 ml of the above buffer and 0.025 ml of enzyme solution and the increase of A at 254 nm was measured for 15 s at 25 °C. The specific activity of CPase A in this assay was 684 µmol substrate hydrolyzed/min·mg enzyme. CPase B activity was determined with the substrate benzoyl-glycyl-L-arginine. The assay was monitored spectrophotometrically at 254 nm at 25 °C and pH 7.5 according to Folk et al. (4). The specific activity was 51 µmol substrate hydrolyzed/min·mg enzyme. Alkaline phosphatase activity (specific activity 0.33 µmol/min·mg solid) was determined with p-nitrophenylphosphate at 25 °C and pH 8.0 by the spectrophotometric method (410 nm) of Garen and Leventhal (5). Phosphoglomutase activity (specific activity 184 µmol/min·mg) was determined with α-d-glucose-1-P at 25 °C and pH 7.4 (in 0.05 m Tris-HCl) with the spectrophotometric method (340 nm) of Gustafson and Gander (6).

Inhibitor Assays. CPase was utilized routinely to assay for the inhibitor. To a solution of CPase A (0.5 mg/ml, in 0.1 m Tris-HCl containing 1 m NaCl, pH 7.5) was added an equal volume of water or inhibitor solution, incubated for 5 h at 25 °C, and then cooled to 2 °C in an ice bath. The inhibition was usually determined with the peptidase assay for CPase A. Increasing inhibitor concentrations were added, and the inhibitory activity was calculated from 50% inhibition. One-half of an inhibitor unit is expressed as the quantity of inhibitor required to inhibit 50% 1 mg of CPase A. The experimental conditions for the other enzymes are described in the legends of figures.

Extraction of Inhibitor from Plants and Fungi. The following procedures were employed to obtain extracts from various plants and fungi for testing inhibitory activities for common vegetables and mushrooms. Five to 10 g of tissue was homogenized in 2 volumes of 0.05 m Tris-HCl containing 0.15 m NaCl (pH 7.5) for 3 min at room temperature, and centrifuged to clarify. The super-

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2 To whom reprint requests should be addressed.
3 Abbreviation: CPase: carboxypeptidase.

 natants were assayed for inhibitory activities against CPase A. For the beans, 5 to 10 g of pulverized beans were stirred in 10 volumes of the buffer for 5 h and clarified by centrifugation. Aliquots of the supernatants were incubated with CPase A for 2 to 3 h at 25 C, and the inhibitor contents were calculated from the inhibition profile obtained with each material. Values reported are the averages of two to four extractions.

Preparative Disc Electrophoresis. The "Prep-Disc" equipment of Canal Industrial Corp. was employed, using the polyacrylamide gel electrophoresis method of Davis (17).

Isoelectric Focusing. Ampholine isoelectric focusing was performed with carrier ampholies with pH 3.5 to 10 on an LKB Ampholine column (110 ml) according to the method of Vesterberg and Svensson (17).

Atomic Absorption Spectroscopy. Various metals were determined with a Hitachi model 207 atomic absorption spectrometer using standard cathode lamps (at 2,139 Å for zinc). Standard metal solutions were prepared in 1% HCl and were diluted with the appropriate buffers.

RESULTS

Inhibitor Contents of Several Common Plants and Fungi. The CPase A inhibitor contents of several common vegetables (potato tubers, tomato fruit, beets) were in the range of 0.04 to 0.8 unit/g of wet weight, and those of Japanese mushrooms (four varieties: tsukuri-take, shimeji, shi-itake, and enokikake) were about 0.3 unit/g. On the other hand, the inhibitor contents of beans of several varieties (red kidney bean, white kidney bean, mottled kidney bean, soybean, adzuki, sasage, green pea, and broad bean) were significantly higher and in the range of 3 to 15 units/g dry weight.

Because of the high activities in beans, a representative species (red kidney bean) was chosen as the plant from which to purify and characterize the inhibitory substance.

Partial Purification of Inhibitor from Red Kidney Bean. The following purification steps were performed at 4 C unless otherwise noted. All gel filtrations employed flow rates of 30 to 60 ml/h.

Pulverized red kidney beans (360 g) were stirred vigorously in 3 liters of 0.1 M Tris-HCl containing 0.15 M NaCl (pH 8.0) for 10 h, and centrifuged to remove insoluble materials. Acetone was added to the stirring supernatant (3 liters) at room temperature to give a 25% concentration and after standing for 30 min the precipitate was removed by centrifugation and discarded. Additional acetone was added to give a final concentration of 70%, and stirred for 30 min. The resulting precipitate was collected and dissolved in 900 ml 0.05 M Tris-HCl (pH 7.5). The dissolved precipitate was again fractionated with acetone at room temperature. After removal of a precipitate at 40% acetone, the concentration was brought to 80%, and the mixture was further stirred for 1 h. The precipitate, which was very adhesive to glass, was collected and dissolved in 70 ml 0.05 M Tris-HCl (pH 7.5).

Gel Filtration. The solution was filtered through a column of Sephadex G-25 (4.8 × 65 cm) equilibrated with 0.05 M HCOONH4 (pH 7.5). The eluate containing the inhibitory activity (from 850 to 1,270 ml) was pooled and lyophilized. The lyophilized powder was dissolved in 43 ml of distilled H2O and applied to a column of Sephadex G-15 (5 × 85 cm) equilibrated with 0.02 M HCOONH4 (pH 7.5). Again, eluate containing the inhibitory activity was pooled.

The pooled solution was concentrated to a small volume (about 15 ml) with a rotary evaporator at 50 C, and filtered again on a column of Sephadex G-15 (5 × 84 cm) with the above solvent (Fig. 1). Fractions 118 to 126 were pooled and lyophilized.

Preparative Disc Electrophoresis. The lyophilized powder (700 mg) was dissolved in 5 ml of distilled H2O, and mixed with 2.5 ml 0.49 M Tris-HCl (pH 6.7, containing 0.46 ml N,N,N',N'-tetramethylenediamine per 100 ml) and 2.5 ml 40% sucrose. This mixture was layered on a spacer gel in an upper column (PD2/320). The spacer gel was 3.5% polyacrylamide (2.9 cm) and the separation gel was 9% polyacrylamide (6 cm). As an electrode solution, 25 mm Tris containing 0.19 M glycine (pH 8.3) was employed. Electrophoresis was performed at a constant voltage of 300 v, cooling the column to 15 C with running water. Elution was performed with 0.1 M Tris-HCl (pH 8.3) at a flow rate of about 50 ml/h. The active fractions were pooled and concentrated to 3.6 ml with a rotary evaporator at 50 C.

Sephadex G-10 Filtration. The pooled, concentrated solution was gel-filtered through a column of Sephadex G-10 (2.5 × 94 cm) equilibrated with 0.02 M HCOONH4 (pH 6.0) (Fig. 2). Fractions 39 through 42 were pooled and lyophilized. The resulting white powder weighed 6.5 mg. One mg of this powder contained 23.1 units of CPase inhibitory activity in the peptidase assay. This material, while not fully pure, was utilized to study several of its properties. The purification results are summarized in Table I.

![Fig. 1. Sephadex G-15 gel filtration of metalloenzyme inhibitor. An aliquot of each fraction was diluted 10 times with distilled H2O and mixed with an equal volume of CPase A solution (0.5 mg/ml, dissolved in 0.1 M Tris-HCl containing 1 M NaCl, pH 7.5). Incubation was performed at 5 h at 25 C. Inhibition was determined with the peptidase assay for CPase A as described in the text. Bracketed fractions (6.8 ml each) were pooled and lyophilized. (---): Aabs (●): per cent inhibition.](image1)

![Fig. 2. Sephadex G-10 gel filtration of the metalloenzyme inhibitor. An aliquot of each fraction was diluted 10 or 20 times with distilled H2O, and incubated with CPase A. Bracketed fractions (5.0 ml each) were pooled and lyophilized. (---): Aabs (●): per cent inhibition, 10-fold dilution; (○): per cent inhibition, 20-fold dilution.](image2)
Table 1. Purification of a metalloenzyme inhibitor from red kidney beans

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Total activity (units)</th>
<th>Specific activity (units/(A_{280}))</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extraction</td>
<td>88,800†</td>
<td>2,380</td>
<td>0.027</td>
</tr>
<tr>
<td>2</td>
<td>Acetone, 25–70%</td>
<td>24,200</td>
<td>1,360</td>
<td>0.064</td>
</tr>
<tr>
<td>3</td>
<td>Acetone, 40–80%</td>
<td>9,540</td>
<td>1,110</td>
<td>0.32</td>
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<td>4</td>
<td>Sephadex G–25</td>
<td>3,490</td>
<td>797</td>
<td>0.23</td>
</tr>
<tr>
<td>5</td>
<td>1st Sephadex G–15</td>
<td>287</td>
<td>539</td>
<td>1.88</td>
</tr>
<tr>
<td>6</td>
<td>2nd Sephadex G–15</td>
<td>15.9</td>
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<td>22.5</td>
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<td>40.3</td>
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<tr>
<td>8</td>
<td>Sephadex G–10</td>
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<td>62.6</td>
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<tr>
<td></td>
<td>Lyophilization</td>
<td>6.5 (mg)</td>
<td>150</td>
<td>23.1 (mg)</td>
</tr>
</tbody>
</table>

† Extracted from 360 g of beans.

Inhibition Profile of CPase A. Both peptidase and esterase activities of CPase A were inhibited by the inhibitor preparation. From 50% inhibition in the respective inhibition curves, the inhibitory activities were calculated to be 23.1 and 25.5 units/mg. The inhibition curve of CPase A was usually slightly sigmoidal, and this was independent of the purity of the inhibitor.

Effects of Incubation Time and Temperature on CPase A Inhibition. Inhibition of CPase A by the inhibitor was clearly affected by incubation time and temperature. A progressive inhibition was observed during incubation at 25 and 37°C. At 37°C incubation, relatively rapid and maximal inhibition was obtained. At this temperature the control enzyme progressively lost activity, and the residual activity after 5 h was about 50%. At 25°C, inactivation of the control enzyme was negligible. Therefore, routine incubation conditions of 25°C and 5 h were chosen for incubation during further experimentation.

pH Dependence of CPase A Inhibition. The pH dependence of CPase A inhibition was examined, employing two buffer systems, CH₃COONa (pH 5–6.5), Tris-HCl (pH 7–9.2), and Britton-Robinson buffer (pH 5–9.7, H₃PO₄-CH₃COOH-H₂BO₃-NaOH) (1). Inhibitory activity was not found at pH 5 to 6, but was present at higher pH values and progressively increased, even above pH 9. In the Britton-Robinson buffer system, a peak of inhibition was observed at pH 7.9, dropping off at pH 8.7 and again increasing as pH increased. The inhibition of CPase B (in a CH₃COONa-Tris-HCl system) exhibited a profile similar to that of CPase A, continuing to increase in the pH region of 7 to 9.5.

Isoelectric Point of Inhibitor. The isoelectric point (pI) of the inhibitor was estimated to be 4.7, determined by Ampholine isoelectric focusing. Because Ampholine showed considerable inhibitory effect on CPase A, the inhibitor was detected by its activity over that of Ampholine itself, determined in control run without inhibitor.

Inhibition of CPase B and Alkaline Phosphatase. Inhibition of CPase B and calf intestinal alkaline phosphatase (3) by the inhibitor is shown in Figure 3. Both inhibition profiles were different from that of CPase A. The former enzymes were not linearly inhibited but the inhibition was proportional to the logarithm of the inhibitor added. The inhibition exhibited the same effects of incubation time and temperature previously found with CPase A inhibition.

Inactivation of Inhibitor by Divalent Metal Ions. Preincubation of the inhibitor (0.55 unit/ml, final) with the divalent metal ions (1 μM to 1 mM, final) Zn²⁺, Ni²⁺, Co²⁺, or Cu²⁺ destroyed the inhibitor activity toward CPase A. The extrapolated concentration of Zn²⁺ that completely abolished the inhibitory activity was about 26 μM (Fig. 4). Other divalent cations including Ca²⁺, Mg²⁺, Mn²⁺, Cd²⁺, Fe³⁺, or Hg²⁺ had no effect on the inhibitor activity toward CPase A.

Activation of Zn²⁺ Inactivated Phosphoglucomutase by Inhibitor. A further demonstration of the probable metal-binding property of the inhibitor was its ability to activate Zn²⁺-inactivated phosphoglucomutase. The enzyme which had been blocked with Zn²⁺ (16%) was fully activated. The increase in enzyme activity was roughly proportional to the amount of the inhibitor added.

Metal-combining Properties of Inhibitor. The above results supported a hypothesis that the affinity of the bean inhibitor for Zn²⁺ was the basis of its inhibitory activity toward CPase. The binding of Zn²⁺ by the inhibitor was examined by combining Sephadex G-25 gel filtration with atomic absorption spectroscopy.

When chromatographed individually on Sephadex G-25 (Fig. 5A), Zn²⁺ and the bean inhibitor exhibited different elution volumes. When mixed with the bean inhibitor (Fig. 5B), Zn²⁺ was detected at the elution volume region of the bean inhibitor, indicating that the Zn²⁺ had been complexed with the inhibitor.

The capacity of the bean inhibitor to complex with Ni²⁺, Co²⁺, Cu²⁺, Cd²⁺, Mg²⁺, and Hg²⁺ was also examined by the same method. We found that the bean inhibitor could combine with Ni²⁺, Co²⁺, Cu²⁺, and partially with Cd²⁺.

Removal of Zn²⁺ from CPase A molecule by the bean inhibitor was detected similarly by column chromatography. The elution volumes of Zn²⁺-CPase A from Sephadex G-100, as judged by assays of Zn²⁺ and peptidase activity coincided, as expected, and their elution volumes differed from that of the bean inhibitor chromatographed separately. When CPase A was incubated with the bean inhibitor (92% inhibited) and filtered through Sephadex G-100, Zn²⁺ now eluted with the bean inhibitor. In the same manner, similar results have been obtained with EDTA, a well-known Zn²⁺ chelator and inactivator of CPase A, and with Ampholine (pH 3.5–10).
comitate by the inhibitor were approximately proportional to the concentration of inhibitor. The curves for inhibition and activation were slightly sigmoidal, perhaps due to inactivation of a small part of the inhibitor by excess divalent metal ions which might be contaminants in the assay reagents.

The inhibitor activity toward CPase A was reversed with Zn$^{2+}$ (Fig. 4) and also with Ni$^{2+}$, Co$^{2+}$, or Cu$^{2+}$. Moreover, we showed (Fig. 5) that the inhibitor complexes with Zn$^{2+}$ and apparently inhibits CPase A by removing the Zn$^{2+}$ from the active site of the enzyme. The inhibition of CPase B and alkaline phosphatase and the activation of Zn$^{2+}$-inactivated phosphoglucomutase are apparently due to the same chelating property of the inhibitor. The bean inhibitor thus differs from the potato (14, 15), yeast (9), and roundworm CPase inhibitors (7) which form stable complexes with the carboxypeptidases they inhibit. The bean inhibitor mechanism also apparently differs from that of ochratoxin A, a CPase A inhibitor, which is also a substrate of CPase A (12).

Metal-binding constituents have been reported previously in legume seeds (8). It seems that the metal-binding substance in soybean seeds is phytic acid (11). The substance reported herein cannot be phytic acid since only trace amounts of phosphate were detected in our purified samples and some other properties, reported below, differ from those of phytic acid.

The chemical nature of the bean inhibitor remains to be determined. The following data have been obtained about this inhibitory substance: (a) the inhibitor was dialyzable, and its elution volume from gel filtration with Sephadex G-15 (0.1 M Tris-HCl, pH 7.5) was found to be below mol wt 500; (b) although inhibitory activity against CPase A was lost after heat treatment at 97°C for 30 min in 1 M HCl, it was stable at 25°C in 1 M HCl or at 97°C in 1 M NaOH for 30 min; (c) 1 mg of the purified inhibitor (23 units/mg) was equivalent to 0.54 mg of l-glutamic acid when reacted with ninhydrin, but only equivalent to 0.08 mg of D-glucose when reacted with phenol-sulfuric acid; (d) the inhibitor was completely inactivated by treatment with diazomethane or acetic anhydride. From these results and the isoelectric point of 4.7, the inhibitor can be described as a slightly acidic substance of small molecular weight. The further purification and characterization of this inhibitor and further studies of its physiological role in plant tissues remain questions of interest.

**DISCUSSION**

Strong inhibitory activity against CPase A was found in beans from eight species of legume plants. Among the species, 1 g of dried beans exhibited from 3 to 15 inhibitor units of activity against CPase A. These values were much higher than those of other vegetable or fungal tissues tested and over 10-fold higher than potato (0.3 unit/g) (15) from which the carboxypeptidase class of inhibitors was first discovered (13).

One mg of the highly purified inhibitor from red kidney beans inhibited 23 to 26 mg CPase A in the peptide and esterase assays, respectively. The above preparation was not fully pure and the inhibitor may inhibit considerably more enzyme per mg than reported here (Fig. 2). Inasmuch as the inhibitor lost activity when incubated with Zn$^{2+}$, Ni$^{2+}$, or Cu$^{2+}$, additional activity may have been lost due to the presence of metals during purification.

The inhibition of CPase A and the activation of phosphoglucomutase by the inhibitor were approximately proportional to the concentration of inhibitor. The curves for inhibition and activation were slightly sigmoidal, perhaps due to inactivation of a small part of the inhibitor by excess divalent metal ions which might be contaminants in the assay reagents.

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