Purification and Some Properties of Trypsin Inhibitors from Buckwheat Seeds†

Toshifumi Kiyohara and Teruo Iwasaki

Laboratory of Biochemistry, Faculty of Agriculture, Kobe University, Nada-ku, Kobe, Hyogo 657, Japan

Received February 29, 1984

Seven proteins which inhibited trypsin were purified from buckwheat seeds by (NH₄)₂SO₄ fractionation, gel-filtration, and DEAE- and CM-cellulose column chromatographies. The homogeneties of the purified inhibitors were established by polyacrylamide gel electrophoresis. These inhibitors were thermostable at acidic and neutral pH's and acted on trypsin more powerfully than on chymotrypsin. Three of them, BTI He, IIIb1, and IIIb2, were typical temporary inhibitors of trypsin and the others, BTI I, IIa, Iib, and IIIa, were permanent ones. These seven inhibitors had essentially no inhibitory activity against subtilisin, Aspergillus sydowi proteinase, neutral subtilopeptidase, papain, or pepsin.

Naturally-occurring protein proteinase inhibitors have been isolated from various plants and characterized in terms of their chemical and physicochemical properties by many investigators.¹⁻³ We have hitherto studied the properties,⁴⁻⁷ the mechanism of inhibition,⁸⁻¹⁰ and the primary structures¹¹⁻¹⁶ of proteinase inhibitors from potato tubers and adzuki beans. Buckwheat, of the genus Fagopyrum, is an important protein supplement for human foodstuffs and animal feedings. Laporte et al.¹⁷ first found proteinase inhibitors in buckwheat. Ikeda et al.¹⁸⁻¹⁹ reported the isolation and the properties of three trypsin inhibitors with molecular weights of about 8000, having very similar amino acid compositions.

We obtained seven trypsin inhibitors from buckwheat seeds which were different from the three trypsin inhibitors of Ikeda et al. in their amino acid compositions and proteinase inhibition profiles.

In this paper, the purification procedure of the seven trypsin inhibitors and some of their properties will be described.

† The abstract of this work was presented at the 55th meeting of the Japanese Biochemical Society, held in Osaka, October, 1982.
0.1 M NaCN at 37°C for 2 hr and preincubation with the inhibitors was at 30°C in 0.1 M sodium phosphate buffer pH 7.6, containing 0.02 M NaCN. The assay was by the casein digestion method as described above. For the assay of neutral subtilopeptidase, incubation was at pH 6.8 in 0.02 M sodium phosphate buffer containing 1 mM CaCl₂. Incubation of the inhibitors with pepsin and the determination of residual pepsin activity were at pH 1.8 in Clark-Lubs buffer by the method of Anson²¹) except for the replacement of hemoglobin by casein as the substrate. The inhibitor activity was expressed as the percent inhibition to the control assay using the following equation:

\[ I (\%) = \frac{(C - R)}{C} \times 100 \]

where \( R \) and \( C \) are enzyme activities with and without the inhibitors, respectively. One unit of inhibitor activity was defined as 50% inhibition of the activity of 2 mg of enzyme.

**RESULTS**

**Purification of inhibitors**

The crude inhibitor preparation mentioned above was dissolved in a sufficient volume of 0.1 M acetic acid and divided into two portions. Each portion was filtered through a Sephadex G-50 column, yielding three peaks. Only the second fraction inhibited trypsin and this was filtered repeatedly through the same column until a single peak was obtained. The active fraction was collected and lyophilized. The preparation was then dissolved in 0.05 M Tris-HCl buffer and applied to a column of DEAE-cellulose. Elution was done as described in EXPERIMENTAL. Three main fractions, I, II, and III, with inhibitory activity were detected (Fig. 1). Each fraction was separately rechromatographed under the same conditions

---

**Preparation of crude inhibitor.** One kilogram of finely ground buckwheat seeds was immersed in 10 liters of 2% NaCl solution for 2 hr at room temperature. The suspension was filtered through cheesecloth and then through a thin layer of Hyflo-supercel under vacuum. The extract obtained was heated at 80°C for 10 min. After removal of the coagulated proteins by filtration through a Hyflo-supercel layer, the filtrate was brought to 30% saturation of \((\text{NH}_4)_2\text{SO}_4\). The precipitate formed was removed by filtration using the same procedure. To the filtrate, \((\text{NH}_4)_2\text{SO}_4\) was added to give 60% saturation. The resulting precipitate was collected by filtration, next dissolved in 0.05 M acetate buffer, pH 4.7. Elution was first with the same buffer and then with a linear gradient to 0.2 M NaCl in the same buffer.

**Disc-electrophoresis.** This was by the method of Davis²⁴) on 7.5% polyacrylamide gel at pH 8.9 and 4 mA per column to examine the homogeneity of the purified preparations.

**Dissociation constants for enzyme-inhibitor complexes.** These were estimated by the method of Green and Work²⁵)

---

**Fig. 1. DEAE-Cellulose Column Chromatography of Crude Inhibitor.**

- , absorbance at 280 nm; --, trypsin inhibitory activity; ---, NaCl concentration.

Conditions are described in EXPERIMENTAL.
and lyophilized after desalting by filtration through a column of sephadex G-50.

For further purification, the three inhibitor fractions obtained above were dissolved in 0.05 M acetate buffer, pH 4.7, and separately put on a CM-cellulose column. Fraction I was divided into two main fractions, one of which inhibited trypsin. The active fraction, I, was further purified by crystallization. A lyophilized preparation was dissolved in a minimum volume of water and cold acetone was added drop by drop to the solution until a cloudy precipitate appeared. After standing for a few minutes at room temperature, the crystals of inhibitor I appeared (Fig. 2a).

Chromatography of inhibitor fraction II on the CM-cellulose column revealed that the fraction contained several inhibitors (Fig. 3a). Among them, two main active fractions, IIa and IIb, were tested for homogeneity without further purification. Another active main fraction, IIc, was re-chromatographed under the same conditions, yielding a single peak. The minor active fractions could not be purified further.

Inhibitor fraction III was also separated on the column into three active fractions, IIIa, IIIb1, and IIIb2, as shown in Fig. 3b. Fraction IIIa was crystallized by dissolving the lyophilized preparation with a minimum volume of
Fig. 3. CM-Cellulose Column Chromatographies of Inhibitor Fractions II and III.
(a), Fraction II; (b), Fraction III.
—, absorbance at 280 nm; ——, trypsin inhibitory activity; ———, NaCl concentration.
Conditions are described in EXPERIMENTAL.

Table I. Purification of Trypsin Inhibitors from Buckwheat Seeds (1000 g)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg protein)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline extract</td>
<td>38,000</td>
<td>1,036</td>
<td>0.027</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Crude preparation</td>
<td>6,200</td>
<td>889</td>
<td>0.14</td>
<td>86</td>
<td>5</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>1,200</td>
<td>808</td>
<td>0.67</td>
<td>78</td>
<td>25</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chromatographya</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>78</td>
<td>180</td>
<td>2.31</td>
<td>17</td>
<td>86</td>
</tr>
<tr>
<td>II</td>
<td>97</td>
<td>150</td>
<td>1.55</td>
<td>14</td>
<td>57</td>
</tr>
<tr>
<td>III</td>
<td>175</td>
<td>323</td>
<td>1.85</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chromatographya</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>36</td>
<td>120</td>
<td>3.33</td>
<td>12</td>
<td>123</td>
</tr>
<tr>
<td>IIa</td>
<td>16</td>
<td>58</td>
<td>3.63</td>
<td>6</td>
<td>134</td>
</tr>
<tr>
<td>IIb</td>
<td>12</td>
<td>41</td>
<td>3.42</td>
<td>4</td>
<td>127</td>
</tr>
<tr>
<td>IIIa</td>
<td>29</td>
<td>110</td>
<td>3.79</td>
<td>11</td>
<td>140</td>
</tr>
<tr>
<td>IIC</td>
<td>17</td>
<td>32</td>
<td>1.88</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>IIIb</td>
<td>48</td>
<td>94</td>
<td>1.96</td>
<td>9</td>
<td>73</td>
</tr>
<tr>
<td>IIIb2</td>
<td>51</td>
<td>98</td>
<td>1.92</td>
<td>9</td>
<td>71</td>
</tr>
</tbody>
</table>

a After re-chromatography.
b After crystallization.
c Activity against trypsin.

0.05 M acetate buffer, pH 4.7 (Fig. 2b). Fractions IIIb1 and IIIb2 were re-chromatographed under the same conditions and obtained as single peaks.

All of the seven preparations thus purified were lyophilized after desalting by gel filtration as mentioned above and called buckwheat trypsin inhibitors (BTI) I, IIa, IIb, IIC, IIIa, IIIb1, and IIIb2. The purification steps and yields of the inhibitors are summarized in Table I.

Homogeneity of inhibitor preparations
The seven BTI's obtained above were tested for homogeneity by polyacrylamide gel electrophoresis. Only a single band was noted in each case, indicating that these inhibitor preparations were essentially homogeneous (Fig. 4).
Effects of pH on the formation of inhibitor-trypsin complexes

As given in Fig. 5, both BTI IIIa and IIIb2 seemed to form complexes with trypsin even at pH's below 3 at which dissociation of complexes into enzyme and inhibitor take place ordinarily. BTI IIIa formed complexes with trypsin constantly at pH's above 5, whereas the formation of complexes of BTI IIIb2 with the enzyme decreased gradually with the rising of pH and rapidly at pH's above 8.

Effects of incubation time on the trypsin-inhibition at various pH's

To confirm the results described above, BTI IIIa and IIIb2 were preincubated at 30°C with trypsin at various pH's and intervals, and then the inhibitor activity was determined at pH 7.6. The activity of BTI IIIa against trypsin remained constant except near pH 3.5 (Fig. 6). Similar results were observed in the cases of BTI I, IIIa and IIb.

On the other hand, only the incubation at pH 1.3 resulted in no change with time in the activity of BTI IIIb2 against trypsin (Fig. 6). A considerable decrease in inhibitory activity of the inhibitor was noticed at pH 4.5. Moreover, activity of the inhibitor decreased gradually at pH 7.0 and very rapidly at pH 9.0 with time. Similar results were obtained with BTI IIc and IIIb1.

Thermal and pH stabilities

Upon treatment of BTI IIIa and IIIb2 at different temperatures and pH's for 10 min, both inhibitors were highly thermostable in acidic and neutral pH's. At alkaline pH's, however, the treatment at higher temperatures led to rapid inactivation of the inhibitors, and finally to complete inactivation at pH 11 (Fig. 7).
Proteinase-inhibition profiles of the inhibitors

Figure 8 shows the inhibitions of trypsin and chymotrypsin by the BTI's. BTI I, IIb, and IIa strongly inhibited trypsin and also weakly inhibited chymotrypsin. BTI IIa had a stoi-

![Graph showing inhibition profiles of trypsin and chymotrypsin by BTI's](image)

**Fig. 6.** Effects of Incubation Time on Trypsin-Inhibitions by BTI IIIa and IIIb2 at Various pH's.

A quarter ml of each inhibitor solution of the indicated pH's was incubated with 0.25 ml of trypsin at 30°C and various intervals. To the reaction mixture, 0.5 ml of 0.4 M phosphate buffer was added to make the final volume 1 ml and to adjust the pH to 7.6. This mixture was subjected to the routine determination of the activity of inhibitor described in EXPERIMENTAL. The trypsin solutions treated in the same manner except for the replacement of the inhibitors by the buffer solutions of various pH's were used as controls. The inherent activity of the inhibitor solution used in this experiment corresponded to 64% inhibition of trypsin.

![Graph showing thermal and pH stabilities of BTI IIIa and IIIb2](image)

**Fig. 7.** Thermal and pH Stabilities of BTI IIIa and IIIb2.

Inhibitor solutions of various pH's were allowed to stand for 10 min at different temperatures. After preincubation of 0.5 ml of the inhibitor solutions with 0.5 ml of trypsin at 30°C and pH 7.6 for 5 min, the residual activity of the enzyme was determined using the casein digestion method described in EXPERIMENTAL. The inherent activity of the inhibitor solutions used in this experiment corresponded to 64% inhibition of trypsin.

![Graph showing enzyme-inhibition profiles by purified BTI's](image)

**Fig. 8.** Enzyme-Inhibition Profiles by the Purified BTI's.

— , trypsin (29 µg/ml); — , chymotrypsin (27 µg/ml).

A half ml of trypsin or chymotrypsin solution was incubated at pH 7.6 and 30°C for 5 min with 0.5 ml of increasing amounts of BTI's. The residual activities of the enzymes was determined using the casein digestion method as described in EXPERIMENTAL.
TABLE II. DISSOCIATION CONSTANTS (Ki) OF THE
COMPLEXES OF BTI I, IIa, IIb, AND IIIa
WITH ENZYMES

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>I</th>
<th>IIa</th>
<th>IIb</th>
<th>IIIa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>$10^{-11}$</td>
<td>$10^{-11}$</td>
<td>$10^{-11}$</td>
<td>$10^{-11}$</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>$10^{-7}$</td>
<td>*</td>
<td>$10^{-7}$</td>
<td>$10^{-7}$</td>
</tr>
</tbody>
</table>

*, no inhibition.

chirometric inhibition against trypsin like BTI I, IIb, and IIIa, but no inhibition against chymotrypsin. BTI IIc and IIIb1 significantly inhibited trypsin but had negligible or no inhibition against chymotrypsin. The inhibition profile of BTI IIIb2 for these enzymes was almost the same as that of BTI IIIb1. The dissociation constants for the complexes of BTI I, IIa, IIb, and IIIa with trypsin and chymotrypsin are listed in Table II. The dissociation constants for the complexes of BTI IIc, IIIb1, and IIIb2 with trypsin could not be estimated since these inhibitors were temporary ones.

These seven inhibitors had no effect on subtilisin, Aspergillus sydowii proteinase, neutral subtilopeptidase, papain, or pepsin.

DISCUSSION

At least twelve different types of proteins having inhibitory activity against trypsin were found in buckwheat seeds. Among them, the seven main inhibitors were isolated by a purification procedure of extraction, heat treatment, (NH₄)₂SO₄ fractionation, acrinol treatment, gel filtration, ion-exchange chromatography, and crystallization. They were referred to as buckwheat trypsin inhibitors (BTI) I, IIa, IIb, IIc, IIIa, IIIb1, and IIIb2. The homogeneities of the purified inhibitors were established by polyacrylamide gel electrophoresis: The yield of each inhibitor varied from 10 to 50 mg per kg of grains. For BTI I, IIa, IIb, and IIIa, an overall purification of 130-fold was achieved and the average recovery of each inhibitor was 8%. Those of BTI IIc, IIIb1, and IIIb2 were 70-fold and 7%, respectively.

As reported earlier,⁴,⁵ the formation of complexes of proteinases with potato proteinase inhibitors I, IIa, and IIb rapidly decreased at pH’s below 6. This suggests that the dissociation of complexes into inhibitor and enzyme takes place at acidic pH’s. However, the present data (Fig. 5) show that BTI’s seem to form the complexes with trypsin even at pH’s below 3 and the reason for this remains obscure. However, it could be mentioned that BTI’s have a strong affinity for the enzyme even in the presence of an excess of casein substrate (pH 7.6). The activities of BTI, I, IIa, IIb, and IIIa remained constant at pH’s above 5, whereas the activities of BTI IIc, IIIb1, and IIIb2 decreased gradually with the rising of pH and rapidly at pH’s above 8. The decrease in trypsin inhibitory activities of BTI IIc, IIIb1, and IIIb2 on the incubation at alkaline pH’s may be attributed to the digestion of the inhibitors with the enzyme, suggesting that BTI IIc, IIIb1, and IIIb2 are typical temporary inhibitors of trypsin whereas BPI I, IIa, IIb, and IIIa are permanent ones.

Of the proteinases tested, only trypsin was powerfully inhibited by BTI’s. In consideration of the molecular weights of BTI I, IIa, IIb, and IIIa, which will be described in the following paper,²² it is very likely that these inhibitors inhibit trypsin at the molar ratio of one to one, and that these inhibitors are single-headed. It is not certain whether or not BTI IIc, IIIb1, and IIIb2 are single-headed, since these inhibitors act as temporary inhibitors on trypsin.

BTI IIIa and IIIb2 somewhat resemble trypsin inhibitor III of Ikeda et al.¹⁹ in terms of their thermal stabilities. The comparison with the three trypsin inhibitors of Ikeda et al.¹⁹ and correlation among BTI’s will be discussed in the following paper.²²

REFERENCES