Note

Purification and Characterization of a Subtilisin Inhibitor from Seeds of Foxtail Millet, Setaria italica

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We have reported that the whole grain of foxtail millet, Setaria italica, contains at least three trypsin inhibitors (FMTI-I, -II, and -III) and a cysteine proteinase inhibitor (FMCPI).1 During purification of these inhibitors, we also found that the FMTI-II fraction isolated by DEAE-Sepharose CL-6B chromatography has a substance inhibiting subtilisin BPN'. This paper describes purification and characterization of a subtilisin inhibitor (FMSI) from foxtail millet grain.

Protein was measured by the method of Lowry et al.2) using bovine serum albumin as a standard. Protein concentrations of enzyme solutions were measured spectrophotometrically with extinction coefficients as described previously.3) The concentration of Streptomyces subtilisin inhibitor (SSI)4) solution was also measured with the extinction coefficient of $E_{\text{cm}}^{1%=0.829}$ (at 276 nm).5) The purity of subtilisin BPN' (Nagase Biochemicals, Osaka) was measured by titration with SSI at a $10^{-7}$ M enzyme concentration. Subtilisin activity was routinely measured by the following method: the reaction mixture consisted of 2.2 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM CaCl$_2$, 0.1 ml of enzyme solution (about 0.1 mg/ml in 50 mM Na-acetate buffer (pH 5.5) containing 5 mM CaCl$_2$), and 0.1 ml of succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (Peptide Institute, Inc., Osaka) solution (20 mg/ml in dimethylsulfoxide). After incubation for 10 min at 37°C, the reaction was stopped by adding

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Table 1. **Purification of Foxtail Millet Subtilisin Inhibitor**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (IU)</th>
<th>Specific activity (IU/mg protein)</th>
<th>Yield (%)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>21,900</td>
<td>1,870</td>
<td>0.0854</td>
<td>100</td>
<td>1.00</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>12,300</td>
<td>287</td>
<td>0.0233</td>
<td>15.3</td>
<td>0.273</td>
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<tr>
<td>Salting-out</td>
<td>4,550</td>
<td>215</td>
<td>0.0473</td>
<td>11.5</td>
<td>0.554</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>300</td>
<td>71.0</td>
<td>0.237</td>
<td>3.80</td>
<td>2.78</td>
</tr>
<tr>
<td>CM-Sephadex C-25</td>
<td>66.5</td>
<td>52.1</td>
<td>0.783</td>
<td>2.79</td>
<td>9.17</td>
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<tr>
<td>SP-Sephadex C-25</td>
<td>3.88</td>
<td>29.1</td>
<td>7.50</td>
<td>1.56</td>
<td>87.8</td>
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<tr>
<td>Sephadex G-50</td>
<td>0.920</td>
<td>23.1</td>
<td>25.1</td>
<td>1.24</td>
<td>294</td>
</tr>
</tbody>
</table>

* Starting material was 2 kg of defatted flour of foxtail millet grain.
1.0 ml of 10% acetic acid and the absorbance of the mixture was measured at 410 nm. Inhibitory activities of FMSI toward proteinases were estimated from the residual enzyme activities in the presence of the inhibitor. In the routine assay system, one subtilisin unit was the amount of enzyme that caused the release of p-nitroaniline corresponding to 1.0 absorbance change at 410 nm per min and one inhibitor unit (1 IU) was defined as the amount of inhibitor required for complete inhibition of one subtilisin unit. The inhibition constant (K_i) of the FMSI complex with subtilisin BPN' was measured at 25°C and pH 8 using a 10^{-10} M enzyme concentration as described previously except for the use of a different substrate, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Sigma Chemical Co., St. Louis, U.S.A.) solution (20 mg/ml in dimethylsulfoxide) instead of a

![Graph](image)

**Fig. 2.** Titration Curve and Calculated K_a Value Generated by Computer Treatment Using a Nonlinear Least-Squares Analysis Program.

The data were for subtilisin BPN' interacting with FMSI at 25°C in 0.1 M Tris-HCl buffer (pH 8.0) containing 10 mM CaCl_2 and 0.005% (w/v) Triton X-100.
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tryptic substrate.

The purification of FMSI is summarized in Table I. The initial five purification methods were exactly the same as those used in the purification of FMTI-II. However, in the CM-Sephadex chromatography, FMTI-II was adsorbed to a column, while FMSI was obtained in an unadsorbed fraction. As a result, the inhibitors were completely separated. After adjustment to pH 3 with 50 mM HCl, this fraction was put on a column (1.5 x 31 cm) of SP-Sephadex C-25 equilibrated with 50 mM Na-acetate–HCl buffer (pH 3.0) and FMSI was eluted with a linear gradient of 0–0.5 mM NaCl in the buffer. The inhibitor was then chromatographed on a Sephadex G-50 column (2.5 x 97 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.1 mM NaCl. The symmetrical protein peak associated with subtilisin inhibitory activity was collected, desalted, and lyophilized. This final preparation of FMSI had a single protein band in disc-PAGE and SDS-PAGE as shown in Fig. 1.

The molecular weight of FMSI was estimated to be 6600 by SDS-PAGE. The amino acid composition of FMSI (residues/mol) based on a molecular weight of 6600 was as follows: Asx 7, Thr 4, Ser 6, Glx 4, Pro 4, Gly 7, Ala 5, Val 10, Met 1, Ile 2, Leu 3, Tyr 1, Phe 3, Lys 4, Arg 3, and Trp 1. FMSI is composed of 65 amino acid residues and is lacking in Cys and His residues. The titration of a 10^{-7}M active subtilisin BPN' with FMSI indicated that the inhibition was linear up to almost 100% inhibition and the stoichiometry was a 1:1 molar ratio. Figure 2 represents the titration curve and the calculated $K_i$ value generated by a computer treatment after the titration of subtilisin BPN' with FMSI at a 10^{-10}M enzyme concentration. The data fitted the theoretical equation of Bieth quite well and $K_i=5.2 \times 10^{-12} M$ was obtained at pH 8.0 and 25°C. On the other hand, FMSI had no inhibitory effect on bovine chymotrypsin, bovine trypsin, papain, or porcine pepsin. The inhibitor was most stable from pH 3 to 10, where virtually optimal inhibitor activity was maintained after 24 hr of incubation at 37°C. FMSI was also heat-stable at acidic and neutral pHs: at pHs 2 and 7, the inhibitor still had about 70% of its original activity after 30 min of incubation at 100°C. However, it was completely inactivated by 30 min of incubation at 100°C and pH 10.

Purified FMSI is heat-stable. However, heat treatment (80°C, 10 min) of the grain extract in the purification process caused a considerable loss of the inhibitory activity (Table I), suggesting that foxtail millet grain may contain another subtilisin inhibitor that is thermolabile. The amino acid composition of FMSI is characterized by a high content of Val and a lack of Cys. Similar inhibitors have been found in legumes and are classified in the potato inhibitor I family. We, therefore, assume that FMSI also belongs to the potato inhibitor I family.

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References


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