Note

Purification of Bowman-Birk Type Inhibitor of High Molecular Weight from Wisteria Seeds

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Trypsin and chymotrypsin inhibitory proteins have been isolated from a number of sources and well characterized.1) The existence of Bowman–Birk type inhibitors in wisteria seeds was reported by Norioka et al.2) using gel filtration of a crude extract. In the course of isolation of plant cystatin from wisteria seeds,3) we found a Bowman–Birk type inhibitor of high molecular weight. In this communication, we described the purification of the inhibitor to homogeneity from wisteria seeds and some properties of the inhibitor.

Trypsin inhibitory activity was measured as described by Kassell.4) A sample of extract was incubated in a total volume of 4.7 ml of 95.7 mM Tris–HCl buffer, pH 8.3, containing 4.79 mM CaCl2, 42.6 nM trypsin, and 1.06 mM $\beta$-benzoyl-L-arginine-$\beta$-nitroanilide for 20 min at 37 °C. After addition of 1 ml of 30% acetic acid, absorbance at 410 nm was measured. Assay for chymotrypsin inhibitory activity was done as above, except for the use of succinyl-L-phenylalanyl-L-prolyl-L-phenylalanine-$\beta$-nitroanilide instead of $\beta$-benzoyl-L-arginine-$\beta$-nitroanilide as the substrate. One inhibitory unit (IU) was defined as the amount of inhibitor that suppressed the liberation of one jimol of $\beta$-nitroaniline per min by the proteases. Chymotrypsin activity was measured spectrophotometrically at 410 nm using the same reaction mixture as described above with or without WTI-I. Amino acid composition was analyzed with Yanagimoto LC-8A apparatus. Before hydrolysis of the protein, cysteine residues were S-carboxymethylated under reducing conditions. The active trypsin and chymotrypsin was measured by titration with $\beta$-nitrophenyl-$\beta$-guanidinobenzoate 5) and $\beta$-nitrophenyl-$\alpha$-$\beta$-acetyl-$\alpha$-$\beta$-benzylcarbazate, 6) respectively.

Seeds of Wisteria floribunda (500 g) were homogenized in 1 l of 50 mM Tris–HCl buffer, pH 9.0. After centrifugation, the resultant supernatant was fractionated with ammonium sulfate (70% saturation). The precipitate was dissolved in 50 mM Tris–HCl buffer, pH 8.6 (Buffer A), and put on Bio-gel P6 column (4.8 x 50 cm). The flow-through fraction was put on DEAE-cellulofine AH column (4.8 x 45 cm) equilibrated with Buffer A, and elution was done in a linear gradient of NaCl from 0 to 0.4 M. Fractions rich in the inhibitory activity were concentrated and put on a Sephadex G-75 column (4.8 x 55 cm) equilibrated with Buffer A. The inhibitor fraction was then put on a DEAE-Toyopearl 650M column (1.6 x 27 cm) equilibrated with Buffer A, and elution was done in a linear gradient of NaCl from 0 to 0.2 M. The inhibitory activity was separated into four peaks, and the first and largest peak was further purified. The fraction was put on a CM-Toyopearl 650M column (1.6 x 27 cm) equilibrated with 10 mM sodium acetate buffer, pH 4.7, and elution was done in a linear gradient of NaCl from 0 to 0.2 M. The inhibitor activity was separated into one large and two small peaks. The largest fraction was put through a second CM-Toyopearl column, and elution was done in a gradient of NaCl from 0 to 0.15 M. Three inhibitor peaks were obtained and the largest fraction of the three, which was designated as WTI-I, was put on a DEAE-Toyopearl 650S column (1.6 x 27 cm) and elution was done in a gradient of NaCl from 0 to 0.15 M. A single inhibitor peak was obtained and the elution pattern of the inhibitory activity coincided with that of absorbance at 280 nm.

WTI-I was purified about ten-fold and the recovery was about 1.7% of the total inhibitory activity in the wisteria seeds. The low purification ratio and recovery seem to be due to the presence of several kinds of trypsin inhibitory proteins. As shown in Fig. 1, WTI-I gave a single protein...
WTI-I and trypsin and/or chymotrypsin were incubated in 0.1M Tris-acetate buffer, pH 7.5, containing 0.1M Na₂SO₄ at 37°C for 10 min. The mixture was put on a TSK gel G2000SWₓL column (7.8 x 30 mm) and elution was done with the same buffer. A, WTI-I (49 nM) and trypsin (23 nM); B, WTI-I (49 nM) and chymotrypsin (23 nM); C, WTI-I (24 nM); trypsin (23 nM); chymotrypsin (23 nM).

The titration experiment showed that WTI-I formed an equimolar complex with trypsin. The dissociation constant of the complex was calculated to be 3 nM by the method of Green and Work⁷) using 80 nM of trypsin. The dissociation constant of the complex of chymotrypsin and WTI-I could not be measured by Green and Work's method, suggesting the low affinity of WTI-I for chymotrypsin.

Lineweaver-Burk analysis showed that WTI-I inhibited chymotrypsin activity competitively with an inhibition constant of 210 nM. Pepsin, elastase, and papain were not inhibited by WTI-I.

The formation of WTI-I-protease complex was investigated by gel-filtration HPLC using a TSK gel G2000SWₓL column (Fig. 2). When WTI-I and trypsin was mixed in the molar ratio of 2 to 1 and the mixture was put on the column, a peak corresponding to the complex was observed at 11.16 min with a small peak of free WTI-I (12.56 min) (Fig. 2A). When chymotrypsin was used instead of trypsin, a large peak corresponding to the complex of WTI-I and chymotrypsin were observed at 10.54 min, in addition to a small peak corresponding to free WTI-I (Fig. 2B). Trypsin and chymotrypsin were eluted at 12.05 and 11.37 min, respectively, from the same column under the same conditions. The equimolar mixture of WTI-I, trypsin, and chymotrypsin gave a peak eluted earlier (10.36 min) than the complex of WTI-I and either protease (Fig. 2C), indicating the formation of a triple complex.

This study showed that WTI-I was rich in half-cystine and had two distinct reactive sites, trypsin- and chymotrypsin-binding sites. The results clearly indicate that WTI-I is a Bowman-Birk type inhibitor, although its molecule is definitely larger than the typical inhibitors of this type.

References