Acta Cryst. (1997). D**54**, 441–443

Preliminary X-ray crystallographic analysis of Bowman–Birk trypsin inhibitor from barley seeds

HYUN KYU SONG AND SE WON SUH* *at Department of Chemistry and Center for Molecular Catalysis, College of Natural Sciences, Seoul National University, Seoul 151–742, Korea. E-mail: sewonsuh@plaza.snu.ac.kr*

(*Received 6 May 1997; accepted 7 August 1997*)

Abstract

Bowman–Birk trypsin inhibitor from barley seeds has been crystallized at room temperature using polyethylene glycol as precipitant. The crystal is tetragonal, belonging to the space group $P4_12_12$ (or $P4_32_12$), with unit-cell parameters of $a = b = 62.48$ and $c = 94.63$ Å. The asymmetric unit contains one molecule of Bowman–Birk trypsin inhibitor with corresponding crystal volume per protein mass (V_m) of 2.89 Å^3 Da^{-1} and the solvent content of 57% by volume. The crystals diffract to at least 1.9 Å Bragg spacing upon exposure to synchrotron X-rays. X-ray data to 1.9 Å have been collected from a native crystal.

1. Introduction

Plant seeds contain a large number of different types of serine protease inhibitors which block trypsins from the animal, fungal and bacterial origins. Among many types of trypsin inhibitors from plants, Kunitz- and Bowman–Birk type inhibitors have been most extensively studied. Members of the Kunitz-type inhibitor family have a molecular mass of about 20 kDa and two disulfide bridges, whereas those of the Bowman–Birk family are smaller and rich in cysteine residues (Onesti *et al.*, 1991). The Bowman–Birk type protease inhibitor was first isolated from leguminous seeds (Bowman, 1946) and its properties were characterized (Birk *et al.*, 1963). The Bowman–Birk inhibitors (BBI) from dicotyledonous seeds such as soybean are 8 kDa double-headed proteins. That is, both trypsin and chymotrypsin are inhibited by the two reactive sites in a single inhibitor molecule. In contrast, the 8 kDa inhibitors from monocotyledonous seeds are single headed. Monocots also have a 16 kDa double-headed inhibitor (Prakash *et al.*, 1996). Recent results from laboratory animal tests and other experiments have shown that several plant BBI's are anticarcinogenic (Kennedy, 1993). They are stable at cooking temperature and also toward the acidic pH found in the digestive system of humans and animals (Birk, 1987). Human populations consuming a large amount of BBI in their diet have been shown to exhibit lower rates of colon, breast, prostate and skin cancers (Birk, 1993).

Several trypsin inhibitors are found in various tissues of barley (Mikola & Kirsi, 1972; Ogiso *et al.*, 1975; Boisen & Djurtoft, 1982; Odani *et al.*, 1983; Nagasue *et al.*, 1988). Among them a trypsin inhibitor purified from barley rootlet was characterized as a BBI. It contains two reactive sites and inhibits trypsin in a molar ratio of 1:2. The amino-acid sequence of this barley rootlet trypsin inhibitor (BRTI) was determined (Nagasue *et al.*, 1988) and it was found to consist of 124 amino-acid residues. BRTI shows an intramolecular sequence identity of 55% between the amino-terminal half (positions 1–62) and the carboxy-terminal half (positions 63–124). A comparison with soybean BBI indicated the reactive sites of BRTI to be Arg17 and Arg75.

Three-dimensional structures of several BBI's have been reported. They include structures of the tracy bean inhibitor PI-II (Chen *et al.*, 1992), the peanut inhibitor A-II (Suzuki, Yamane *et al.*, 1993), and soybean inhibitor (Werner & Wemmer, 1992; Voss *et al.*, 1996), which have been analyzed in the free form, and those of the inhibitors from adzuki bean (Tsunogae *et al.*, 1986) and mung bean (Lin *et al.*, 1993), which have been determined in complex with porcine trypsin. Crystallization of BBI from wheat germ complexed with bovine pancreatic β-trypsin has been reported (Suzuki, Kurasawa *et al.*, 1993). However, no crystal structure of monocotyledonous BBI has been reported. It is important to compare the tertiary structure of BBI in cereal grains, a monocot, with those in leguminous plants, a dicot, in order to understand not only the difference in structure and function but also the evolution of BBI. Therefore, we have initiated the structure determination of BBI from barley seeds. It has been successfully purified and crystallized. In this paper, crystallization condition and preliminary X-ray data are reported. Crystals of BBI from barley seeds diffract to at least 1.9 Å resolution and thus they are suitable for structure determination at high resolution.

2. Experimental

2.1. *Purification*

Unmilled barley (*Hordeum vulgare* L.) seeds were obtained from the Wheat and Barley Research Institute, Rural Development Administration, Korea. Barley seeds were ground to flour. The flour was extracted with buffer *A* (50 m*M* sodium phosphate at pH 6.0). The suspension was centrifuged for 20 min at 8540*g* (8000 rev min^{-1}, Sorvall GSA rotor) and the supernatant was brought to 60% saturation with ammonium sulfate. Most of BBI from barley seeds precipitated in 60% saturation with ammonium sulfate. The precipitated protein was re-dissolved in buffer *A* and was subjected to dialysis against the same buffer. An ion-exchange chromatographic step was performed on a carboxymethyl-cellulose column (2.5 × 38 cm), which was previously equilibrated with buffer *A*. The protein was eluted with a linear gradient of 0 to 0.3 *M* sodium chloride in buffer *A* at a flow rate of 50 ml h^{-1}. Further purification of trypsin inhibitor was obtained by gel filtration on a Sephacryl S-200 HR column (2.5 × 76 cm), which was previously equilibrated with buffer *A* containing 50 m*M* sodium chloride. Elution was performed with the above buffer at a flow rate of 8 ml h^{-1}. The purified trypsin inhibitor was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of 0.1%(*w*/*v*) sodium dodecyl sulfate (Laemmli, 1970) and its activity was checked by a method using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, a

chromogenic trypsin substrate (Chung *et al.*, 1983). This procedure yielded approximately 10 mg of homogeneous BBI from 1 kg of barley seeds. Purified BBI was subject to N-teminal sequencing on a pulsed-liquid phase sequencer (Applied Biosystems model 473A) at the Inter-University Center for Natural Sciences Research Facilities, Seoul National University. The determined amino-terminal sequence of the purified BBI from barley seeds, Ala-Gly-Lys-Lys-Arg-Pro-Trp-Lys-, was identical to the published sequence for barley rootlet Bowman–Birk type trypsin inhibitor (Nagasue *et al.*, 1988).

2.2. *Crystallization*

The purified protein solution was concentrated to about 15 mg ml^{-1} by using a YM 10 membrane (Amicon) and then dialyzed against 50 m*M* sodium citrate buffer (pH 5.98) for crystallization. The protein concentration was estimated by measuring the absorbance at 280 nm, assuming a correspondence of 1.0 mg ml^{-1} concentration to the unit absorbance at 280 nm for the 1.0 cm path length. Crystallization was performed by the hanging-drop vapor-diffusion method at room temperature (about 295–297 K) using 24-well tissue-culture plates (Flow Laboratories). The optimized crystallization condition is as follows. The reservoir solution [23%(*w*/*v*) PEG 3000, 100 m*M* sodium citrate, final pH 6.23] was prepared by mixing appropriate volumes of 1.0 *M* sodium citrate (pH 5.65) and 50%(*w*/*v*) PEG 3000 (Merck) and adjusting the final volume with water. The hanging drop on a siliconized cover slip was prepared by mixing equal volumes of the above protein solution and the reservoir solution.

2.3. *Data collection*

A crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax after filling both ends with the mother liquor. X-ray experiments were carried out using graphite-monochromatized Cu *Kα* X-rays from a rotating-anode generator (Rigaku RU-200BH), running at 40 kV and 70 mA with a 0.3 mm focus cup. The first set of native X-ray diffraction data was collected on a FAST area detector system (Enraf–Nonius) using the *MADNES* software (Messerschmidt & Pflugrath, 1987). The unit-cell parameters were determined by the autoindexing and parameter refinement procedure of *MADNES* software. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982).

The second set of native X-ray data was collected at 283 K using a Weissenberg camera for macromolecular crystallography at the BL-6A2 experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of synchrotron X-rays was 1.000 Å and a 0.1 mm collimator was used. A Fuji image plate (20 × 40 cm) was placed at a distance of 429.7 mm from the crystal. The oscillation range per image plate was 10.5°, with a speed of 2.0° s^{-1} and a coupling constant of 2.0° mm^{-1}. An overlap of 0.5° was allowed between two contiguous image plates. The diffraction patterns recorded on the image plates were digitized by a Fuji BA100 scanner. The raw data were processed using the program *WEIS* (Higashi, 1989).

3. Results

Tetragonal crystals of BBI from barley seeds were obtained when the reservoir solution contained 23%(*w*/*v*) PEG 3000, 100 m*M* sodium citrate, final pH 6.23. They grew to typical dimensions of 0.7 × 0.4 × 0.4 mm within a week (Fig. 1). The crystal diffracts to at least 2.3 Å with Cu Kα X-rays from a rotating-anode source and is very stable in the X-ray beam. X-ray data have been collected to approximately 2.5 Å Bragg spacing from a native crystal. The final merged native data set consisted of 28 815 measurements of 6464 unique reflections with an $R_{\rm merge}$ (on intensity) of 5.9% (rejecting 3.6% outliers). The space group was determined by examining the intensity distribution of the X-ray data. The crystal belongs to the tetragonal space group $P4_12_12$ (or $P4_32_12$), with unit-cell dimensions of $a = b = 62.58$, and $c = 94.85$ Å.

The diffraction data extended to at least 1.9 Å with synchrotron X-rays. The synchrotron data consisted of 133 929 measurements of 13 611 unique reflections with an $R_{\rm merge}$ (on intensity) of 5.8% (rejecting 1.1% outliers). The merged data set is 88.0% complete to 1.9 Å, with the shell completeness between 2.0 and 1.9 Å being 74.1%. The presence of one molecule of trypsin inhibitor in the asymmetric unit gives a crystal volume per protein mass (V_m) of 2.89 Å^3 Da^{-1} with a corresponding solvent content of 57% by volume. These values are within the frequently observed ranges for protein crystals (Matthew, 1968). A search for suitable heavy-atom derivatives is in progress.

We thank the Inter-University Center for Natural Science Research Facilities for providing the X-ray equipment, which was supported in part by the Korea Science and Engineering Foundation Specialization Support Fund. This work was supported by grants from Korea Science and Engineering Foundation through the Center for Molecular Catalysis at Seoul National University and Korea Ministry of Education (BSRI-96–3418). We also thank Professor N. Sakabe, Dr A. Nakagawa, and Dr Watanabe for assistance during data collection at BL-6A2 of Photon Factory, Japan.

Fig. 1. Photograph of tetragonal crystals of Bowman–Birk trypsin inhibitor from barley seeds. The crystal has approximate dimensions of 0.7 × 0.4 × 0.4 mm. See the text for detailed crystallization conditions.

References

Birk, Y. (1987). *Proteinase Inhibitors.* In *Hydrolytic Enzymes*, edited by A. Neuroberger & K. Brocklehurst, pp. 257–300. Amsterdam: Elsevier.

Birk, Y. (1993). *Protease inhibitors of plant origin and role of protease inhibitors in human nutrition.* In *Protease Inhibitors as Cancer Chemopreventive Agents*, edited by W. Troll & A. R. Kennedy, pp. 97–106. New York: Plenum Press.

Birk, Y., Gertler, A. & Khalef, S. (1963). *Biochem. J.* **87**, 281–284.

Boisen, S. & Djurtoft, R. (1982). *J. Sci. Food Agric.* **33**, 431–440.

Bowman, D. E. (1946). *Proc. Soc. Exp. Biol. Med.* **63**, 547–550.

Chen, P., Rose, J., Love, R., Wei, C. H. & Wang, B.-C. (1992). *J. Biol. Chem.* **267**, 1990–1994.

Chung, C. H., Ives, H. E., Almeda, S. & Goldberg, A. L. (1983). *J. Biol. Chem.* **258**, 11032–11038.

Higashi, T. (1989). *J. Appl. Cryst.* **22**, 9–18.

Kabsch, W. (1988). *J. Appl. Cryst.* **21**, 916–924.

Kennedy, A. R. (1993). *Anticarcinogenic activity of protease inhibitors.* In *Protease Inhibitors as Cancer Chemopreventive Agents*, edited by W. Troll & A. R. Kennedy, pp. 9–64. New York: Plenum Press.

Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.

Lin, G., Bode, W., Huber, R., Chi, C. & Engh, R. A. (1993). *Eur. J. Biochem.* **212**, 549–555.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Messerschmidt, A. & Pflugrath, J. W. (1987). *J. Appl. Cryst.* **20**, 306–315.

Mikola, J. & Kirsi, M. (1972). *Acta Chem. Scand.* **26**, 787–795.

Nagasue, A., Fukamachi, H., Ikenaga, H. & Funatsu, G. (1988). *Agric. Biol. Chem.* **52**, 1505–1514.

Odani, S., Koide, T. & Ono, T. (1983). *J. Biol. Chem.* **258**, 7998–8003.

Ogiso, T., Noda, T., Sako, Y., Kato, Y. & Aoyama, M. (1975). *J. Biochem.* **78**, 9–17.

Onesti, S., Brick, P. & Blow, D. W. (1991). *J. Mol. Biol.* **217**, 153–176.

Prakash, B., Selvaraj, S., Murthy, M. R. N., Sreerama, Y. N., Rao, D. R. & Gowda, L. R. (1996). *J. Mol. Evol.* **42**, 560–569.

Sakabe, N. (1991). *Nucl. Instrum. Methods A*, **303**, 448–463.

Suzuki, A., Kurasawa, T., Tashiro, C., Hasegawa, K., Yamane, T. & Ashida, T. (1993). *Acta Cryst.* D**49**, 594–596.

Suzuki, A., Yamane, T., Ashida, T., Norioka, S., Haram, S. & Ikenazka, T. (1993). *J. Mol. Biol.* **234**, 722–734.

Tsunogae, Y., Tanaka, I., Yamane, T., Kikkawa, J., Ashida, T., Ishikawa, C., Watanabe, K., Nakamura, S. & Takahashi, K. (1986). *J. Biochem.* **100**, 1637–1646.

Voss, R.-H., Ermler, U., Essen, L.-O., Wenzl, G., Kim, Y.-M. & Flecker, P. (1996). *Eur. J. Biochem.* **242**, 122–131.

Weissman, L. (1982). *Strategies for extracting isomorphous and anomalous signals.* In *Computational Crystallography*, edited by D. Sayre, pp. 56–63. Oxford University Press.

Werner, M. H. & Wemmer, D. E. (1992). *Biochemistry*, **31**, 999–1010.