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Crystallization and preliminary X-ray analysis of a complex between the Bowman–Birk trypsin inhibitor from barley and porcine pancreatic trypsin

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A 1:2 complex between the Bowman–Birk trypsin inhibitor from barley seeds and porcine pancreatic trypsin has been crystallized at 291 K using polyethylene glycol as precipitant. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 67.10$, $b = 88.38$ and $c = 203.65$ Å. The asymmetric unit contains two monomers of the complex, with a corresponding V_m of 2.41 Å^3 Da^{-1} and a solvent content of 49%. Native data to 2.2 Å resolution have been collected at 100 K using synchrotron X-rays.

1. Introduction

Among the many kinds of trypsin inhibitors from plants, Bowman–Birk and Kunitz-type inhibitors have been the most extensively characterized. Members of the Bowman–Birk family are small proteins rich in cysteine residues, whereas those of the Kunitz-type inhibitor family have a molecular mass of about 20 kDa and two disulfide bridges (Birk, 1987). The Bowman–Birk inhibitors (BBIs) from dicotyledonous seeds such as soybean are 8 kDa double-headed proteins; that is, a single inhibitor molecule contains two reactive sites and inhibits both trypsin and chymotrypsin simultaneously. In contrast, the 8 kDa inhibitors from monocotyledonous seeds are single-headed. Interestingly, monocots also possess double-headed inhibitors with approximately twice the above molecular mass (Prakash *et al.*, 1996). Recent results from laboratory animal tests and other experiments have shown that several plant BBIs are anticarcinogenic (Kennedy, 1993). They are stable at cooking temperature and also towards the acidic pH found in the digestive systems 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 different trypsin inhibitors are present in various tissues of barley, a monocot (Mikola & Kirsi, 1972; Ogiso *et al.*, 1975; Boisen & Djurtoft, 1982; Odani *et al.*, 1983; Nagasue *et al.*, 1988). Among the barley inhibitors, a trypsin inhibitor isolated from the rootlet was characterized as belonging to the BBI family. The amino-acid sequence of this barley rootlet trypsin inhibitor was determined (Nagasue *et al.*, 1988) and it was found to consist of 124 amino-acid residues. Its amino-terminal half (residues 1–62) and carboxy-terminal half (residues 63–124) showed an intramolecular sequence identity of 55%. The two reactive sites were found to be Arg17 and Arg75 through a comparison with the sequence of soybean BBI. Thus, this barley BBI is a double-headed inhibitor capable of inhibiting trypsin in a 1:2 molar ratio. Three-dimensional structures of several BBIs from dicots have been reported, including those from tracy bean, peanut, soybean, adzuki bean and mung bean (Tsunogae *et al.*, 1986; Chen *et al.*, 1992; Werner & Wemmer, 1992; Lin *et al.*, 1993; Suzuki *et al.*, 1993; Voss *et al.*, 1996). However, relatively little is known about the structures of double-headed BBIs from monocots or their complexes with trypsin. We have previously reported the crystallization of the double-headed BBI from barley seeds in its uncomplexed state (Song & Suh, 1998). In our attempts to solve the structure of the inhibitor by multiple isomorphous replacement, some difficulties were experienced in preparing heavy-atom derivatives, probably owing to a lack of reactive side chains. Therefore, it was hoped that the crystallization of the inhibitor in complex with trypsin would be useful in solving the phase problem and in understanding the interaction between the inhibitor and trypsin. Here, we have crystallized the 1:2 complex between the double-headed BBI from barley seeds and porcine pancreatic trypsin (PPT) as the first step towards its structure determination. The crystallization conditions and preliminary X-ray data are reported.

2. Experimental

2.1. Purification

BBI from barley seeds was purified as described previously (Song & Suh, 1998) and porcine pancreatic trypsin purchased from Sigma (T-7418) was used without further purification. For the complex formation, BBI and PPT were mixed in a 1:2 molar ratio and were left for 2 h at 277 K. This solution was loaded onto a Superdex-75 gel-filtration

Table 1
Statistics on synchrotron data collection.

Number of crystals used	1
Temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 67.10, b = 88.38, c = 203.65
Number of measured reflections	366835
Number of unique reflections	60321
$R_{\rm merge}$† (%)	6.8
Data completeness (%)	
50–2.2 Å shell	96.1
2.24–2.2 Å shell	92.6

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over the i measurements of reflection h.

column (HiLoad 16/60, Pharmacia) which had previously been equilibrated with buffer *B* (50 m*M* Tris–HCl pH 8.0) containing 100 m*M* NaCl. Elution was performed with the above solution at a flow rate of 1 ml min^{-1}. The fractions containing the complex were then applied to a Mono-S column (HR5/5, Pharmacia). The complex was eluted with a linear gradient of 0–0.5 *M* NaCl in buffer *B*. During the purification stages, the protein was monitored by 12% sodium dodecyl sulfate polyacrylamide-gel electrophoresis. The purified complex was concentrated to about 14 mg ml^{-1} using a YM 10 membrane (Amicon) and then dialyzed against 50 m*M* Tris–HCl (pH 8.0), 100 m*M* NaCl before mixing with the reservoir solution 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 unit absorbance for the 1.0 cm path length.

2.2. Dynamic light-scattering studies

The dynamic light-scattering experiment was performed on a Model DynaPro-801 instrument from Protein Solutions (Charlottesville, Virginia). The data were measured at room temperature on the protein complex diluted to 1 mg ml^{-1} concentration in 50 m*M* Tris–HCl pH 8.0 and 200 m*M* NaCl.

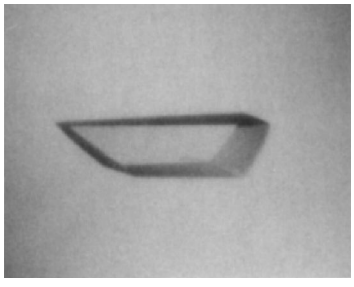

Figure 1
An orthorhombic crystal of the complex between barley Bowman–Birk trypsin inhibitor and porcine pancreatic trypsin. The approximate dimensions of the crystal are 0.6 × 0.3 × 0.1 mm.

2.3. Crystallization

Crystallization was performed using the hanging-drop vapour-diffusion method at 291 K using 24-well tissue-culture plates (Flow Laboratories). The hanging drop was prepared on a siliconized coverglass by mixing equal volumes of the protein solution (14 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl) and the reservoir solution. The coverglass containing the hanging drop was placed over a 1 ml reservoir. The best crystals were obtained when the reservoir solution 15%(*w*/*v*) PEG 8000, 100 m*M* sodium citrate and 1 m*M* dithiothreitol (DTT) at final pH 5.59 was used.

2.4. Data collection

For the first set of native X-ray diffraction data, 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-monochromated Cu $K\alpha$ 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 data were collected at 293 K 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 procedures of the *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).

For the second set of native data, another crystal was equilibrated in the cryoprotectant solution [reservoir solution plus 13.5%(*v*/*v*) glycerol and 15.8%(*v*/*v*) PEG 400] for 1 h after gradually achieving the final cryoprotectant concentration in six solution-exchange steps over a period of 3 h. The crystal was then flash-frozen in a nitrogen-gas stream at 100 K (Oxford Cryosystems Cryostream, Oxford, UK). The data was collected using a Weissenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of the 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 3.5°, 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 data were processed and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993). The space group was determined by examining the systematic absences in the X-ray intensity data.

3. Results

The dynamic light-scattering analysis indicated that the purified barley BBI–PPT complex has an estimated molecular mass of 60 kDa with a polydispersity of 29%. Therefore, the complex contains one molecule of barley BBI (13 823 Da) and two molecules of PPT (23 476 Da). The same complex composition was also suggested by gel filtration on a Superdex-75 column. Well diffracting crystals of the 1:2 complex were obtained at 291 K when the reservoir solution contained 15%(*w*/*v*) PEG 8000, 100 m*M* sodium citrate and 1 m*M* DTT at final pH 5.59. They grew to typical dimensions of 0.6 × 0.3 × 0.1 mm within two weeks (Fig. 1). Large single crystals of excellent diffraction quality grew only when 1 m*M* DTT was present in the reservoir solution. In the absence of DTT, crystals of poor morphology were obtained which only diffracted to 3.2 Å with Cu $K\alpha$ X-rays from a rotating-anode source.

Crystals grown in the presence of DTT diffracted to at least 2.7 Å with Cu $K\alpha$ X-rays from a rotating-anode source and were very stable in the X-ray beam. The first set of X-ray diffraction data was collected at 293 K from a native crystal using Cu $K\alpha$ X-rays. A total of 65 498 measured reflections were merged into 30 705 unique reflections with an $R_{\rm merge}$ (on intensity) of 8.7% and a completeness of 84.6% to 2.7 Å resolution. The systematic absences indicated that the crystals belong to the orthorhombic space group $P2_12_12_1$. The unit-cell parameters were determined to be a = 70.35, b = 91.17 and c = 200.43 Å.

Using synchrotron X-rays, a second set of native X-ray diffraction data extending to 2.2 Å was collected from a crystal flash-frozen at 100 K. A total of 366 835 measured reflections were merged into 60 321 unique reflections with an $R_{\rm merge}$ (on intensity) of 6.8%. The merged data set is 96.1% complete to 2.2 Å resolution, with the shell completeness between 2.24 and 2.20 Å being 92.6%. Table 1 summarizes the statistics for the synchrotron data collection. Upon flash-freezing, the length of the *c* axis increased by

more than 3 Å, while the lengths of the *a* and *b* axes decreased by about 3 Å. The presence of two monomers of the 1:2 complex between barley BBI and porcine trypsin in the asymmetric unit gives a crystal volume per protein mass (V_m) of 2.41 Å^3 Da^{-1} and a solvent content of 49%. These values are within the frequently observed ranges for protein crystals (Matthews, 1968).

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