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# Crystallization and preliminary X-ray studies on the trypsin inhibitor I-2 from wheat germ and its complex with trypsin.

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## Abstract

A Bowman-Birk type trypsin inhibitor I-2, $M_r$ = 14 000, 123 amino-acid residues, isolated from wheat germ, and its complex with trypsin have been crystallized. For I-2 two morphologically different crystal forms were obtained. Crystal form 1 is tetragonal, $P4_122$ or $P4_322$, with $a$ = 55.45 (2), $c$ = 129.1 (2) Å and $V = 3.97\,(2) \times 10^5$ Å$^3$. The crystals diffract X-rays very anisotropically, to less than 6 Å resolution normal to the $c^*$ direction, but up to 3 Å resolution in the other directions. Crystal form 2 is monoclinic, space group $C2$. The cell parameters show significant variation even for crystals in the same batch. The median parameters are: $a$ = 83.9, $b$ = 41.5, $c$ = 45.7 Å, $\beta$ = 95.9° and $V = 1.58 \times 10^5$ Å$^3$. The diffraction pattern is isotropic and reflections up to 2.2 Å resolution were observed. The crystals of the complex between bovine trypsin and I-2 (2:1) belong to the orthorhombic space group $P2_12_12_1$ with $a$ = 73.49 (2), $b$ = 120.56 (3), $c$ = 70.04 (2) Å and $V = 6.206\,(5) \times 10^5$ Å$^3$. The crystals diffract up to 2.3 Å resolution, and contain one complex of 60 100 Da in an asymmetric unit.

## Introduction

Many proteinase inhibitors including Bowman-Birk type inhibitors (Laskowski & Kato, 1980) have been found in leguminous plants. The double-headed Bowman-Birk type proteinase inhibitors (BBI) of leguminous plants are rather small proteins made of 60-80 amino-acid residues, containing as many as seven disulfide bridges. In order to clarify the structure and inhibiting mechanism of BBI we have studied the crystal structures of A-II from peanut (Suzuki *et al.*, 1987, 1993), and the complex of AB-I from adzuki bean with bovine trypsin (Tsunogae *et al.*, 1986). It was found that the structure of the domain of AB-I which interacted with trypsin was essentially the same as that of free A-II, and that the anterior portion of AB-I, containing the reactive site, interacted with trypsin in a manner similar to other families of inhibitors. This interaction scheme was maintained stably and rigidly by the supportive interactions of the posterior segment following the reactive site (Tsunogae *et al.*, 1986). The structure and binding interaction observed in the complex of mung bean trypsin inhibitor with porcine trypsin (Tang *et al.*, 1990) are similar to those of the complex of AB-I and bovine trypsin.

Cereal grains also contain many proteinase inhibitors. Two groups of the BBI type trypsin inhibitor, I ($M_r$ = 14 000, 123 amino-acid residues) and II ($M_r$ = 7000, 61 amino-acid residues), based on their molecular sizes, were isolated from wheat germ (Odani, Koide & Ono, 1986). Among these inhibitors, the amino-acid sequence of I-2*b*, a major component of group I, and that of II-4, one of the components of group II, were studied. The 56 N-terminal residues for I-2*b* and the 53 N-terminal residues foı II-4 show a high degree of sequence homology; group I essentially has a duplicated structure of group II, leading to a tandem structure such as ovomucoid. Group II is a single-headed trypsin inhibitor, though it is highly homologous to the double-headed BBI of leguminous plants, such as A-II or AB-I. It is therefore of importance to compare the tertiary structures of BBI's in cereal grains with those in leguminous plants, in order to understand not only the function but also the evolution of BBI. We have thus initiated the structural study of I-2, the major component of group I. Here the crystallization and preliminary X-ray studies on I-2 and its complex with bovine $\beta$-trypsin are presented.

## Experimental

### *Purification*

Wheat germ was kindly donated by Nippon Flour Mills Co. The trypsin inhibitors were isolated and purified according to the Odani procedure (Odani, Koide & Ono, 1986). The inhibitor I-2 could be separated further into three components, I-2*a*, I-2*b* and I-2*c* by reverse-phase chromatography. In the present study, however, this purification step was omitted because both I-2*a* and I-2*c* are highly homologous to I-2*b* and their levels are fairly low compared with that of I-2*b*.

$\beta$-Trypsin was purified from bovine pancreatic trypsin (Sigma Chemicals) by ion-exchange chromatography using SP-Sephadex C-50. Purified $\beta$-trypsin and I-2 were mixed in a molar ratio of 1:1 and the 2:1 complex between $\beta$-trypsin and I-2 (formula weight = 60 100) was separated from the 1:1 complex by Sephadex G-75 gel-filtration chromatography.

### *Crystallization*

Crystallization was carried out by the hanging-drop vapor-diffusion method. Each droplet was composed of 5 µl of an aqueous solution containing 20 mg ml$^{-1}$ protein and an equal amount of a reservoir solution. Two morphologically different crystal forms, 1 and 2, were obtained for I-2. Square bipyramidal crystals of form 1 with maximum dimensions of 1.0 × 0.3 × 0.3 mm were obtained within a month, when a 50 m*M* sodium acetate-HCl buffer solution (pH 3.6-3.8) containing 0.9 *M* ammonium sulfate was used as the reservoir solution. Alternatively pillar-shaped crystals of form 2 with maximum dimensions of 2.0 × 0.2 × 0.2 mm were obtained within a month when 0.1 *M* sodium phosphate buffer at pH 7.0-7.5 containing 10% (*w*/*v*) polyethylene glycol 6000 (PEG 6000) was used as the reservoir solution. Pillar-shaped crystals of trypsin-I-2 (2:1) complex up to 1 mm in length were grown within two weeks, when the precipitant was 8-10% (*w*/*v*) PEG 4000 with 1-3% (*w*/*v*) 1,4-dioxane and the reservoir solution was 0.05 *M* sodium phosphate buffer at pH 7-8. Typical crystals obtained are shown in Fig. 1. Precession photographs of forms 1 and 2, and the complex are shown in Fig. 2. The crystallographic data of the three crystals are given in Table 1.




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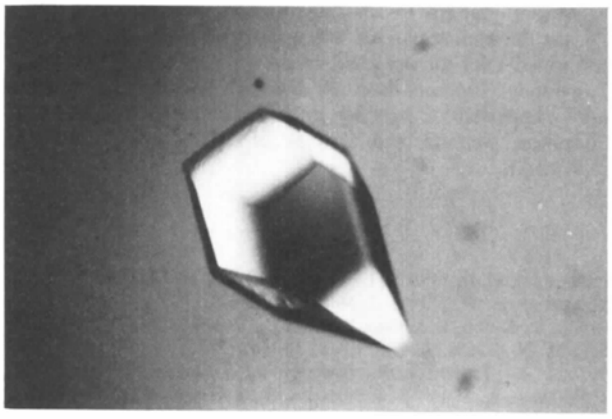
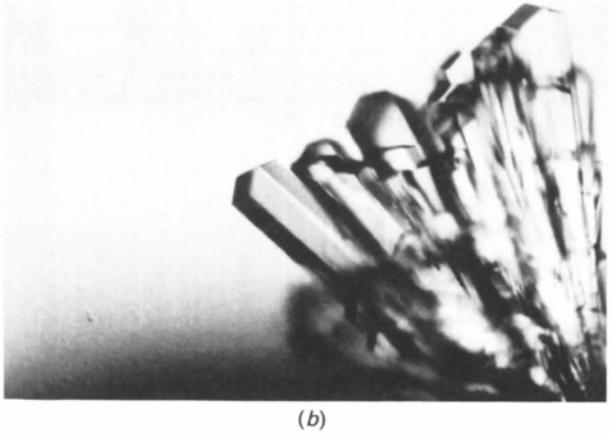
### Results and discussion

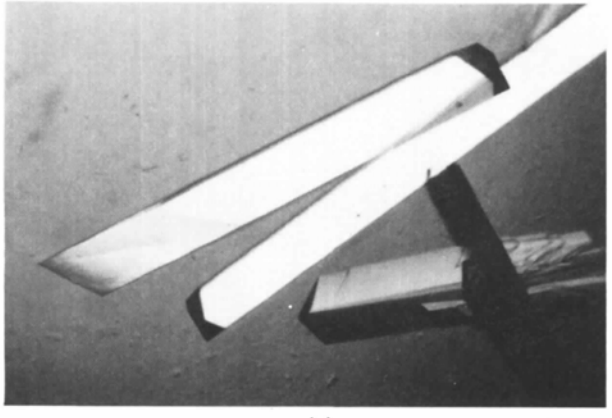
The diffraction power of crystal form 1 is, as a whole, weak. This may be attributed to the high solvent content (65%) in the crystals. Furthermore the crystals diffract X-rays very anisotropically to less than 6 Å resolution normal to the $c^*$ direction and to 3 Å parallel to $c^*$. This suggests that disorder occurs as a result of loose packing on a (001) plane in the



(*a*)

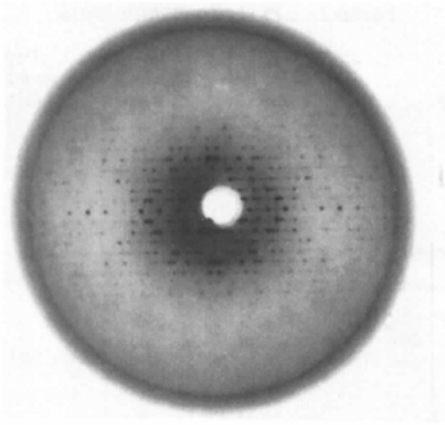


(*b*)

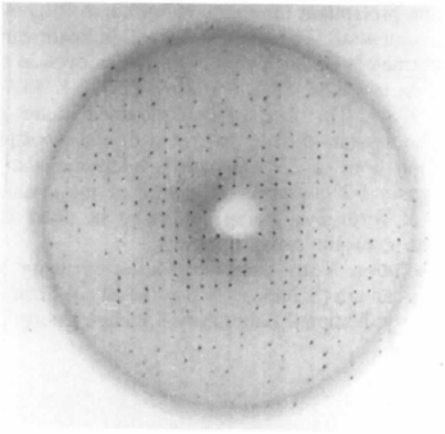


(*c*)

Fig. 1. Crystals of (*a*) form 1 and (*b*) form 2 of trypsin inhibitor I-2, and (*c*) trypsin–I-2 (2:1) complex.



(*a*)



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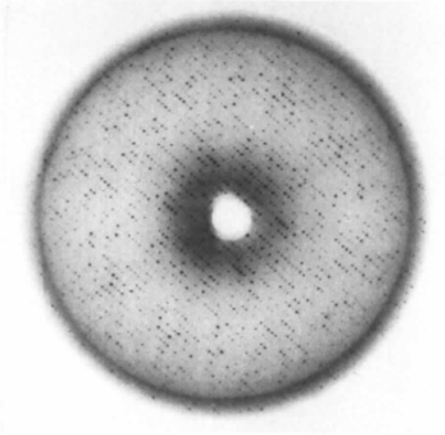

(*c*)

Fig. 2. (*a*) (*h*0*l*) precession photograph of form 1 of trypsin inhibitor I-2, (*b*) (*h*0*l*) precession photograph of form 2 of I-2 and (*c*) (*hk*0) precession photograph of trypsin–I-2 (2:1) complex. The crystal-to-film distance was 60 mm and the precession angle was 15°. The Cu $K\alpha$ radiation was generated by a Rigaku rotating anode at 50 kV and 100 mA.

Table 1. *Crystallographic data*

| | I-2 (form 1) | I-2 (form 2) | Trypsin–I-2 complex (2:1) |
|---|---|---|---|
| Crystal system | Tetragonal | Monoclinic | Orthorhombic |
| Space group | $P4_122$* | $C2$ | $P2_12_12_1$ |
| $a$ (Å) | 55.45 (2) | 83.9† | 73.49 (2) |
| $b$ (Å) | | 41.5† | 120.56 (3) |
| $c$ (Å) | 129.1 (4) | 45.7† | 70.04 (2) |
| $\beta$ ( ) | – | 95.9† | – |
| $V$ ($\times 10^5$ Å$^3$) | 3.97 (2) | 1.58 | 6.21 (1) |
| $V_m$‡ (Å$^3$ Da$^{-1}$) | 3.55 | 2.83 | 2.58 |
| Density (observed) | 1.30 | 1.33 | 1.28 |
| $Z$ | 8 | 4 | 4 |
| Solvent content (%) | 65 | 44 | 49.5 |

* $P4_122$ or $P4_322$.
† Median cell parameters.
‡ Volume of molecular mass per dalton (Matthews, 1968).

crystals. To improve the crystallinity, the effect of metal ions was examined, but improvement on the resolution limit of the crystals could not be detected.

Morphologically different shaped crystals of form 2 grow by changing the precipitant (ammonium sulfate to PEG 6000) and pH (acidic to neutral). The crystals show significant variation of the cell parameters for each crystal specimen even in the same batch, that is, $a$ = 82.6–85.1, $b$ = 40.9–42.0, $c$ = 43.5–47.8 Å, $\beta$ = 94.3–97.4°. The median cell parameters are given in Table 1. The crystals diffract X-rays well and isotropically to the resolution limit of 2.2 Å which is similar to that of A-II and the trypsin–I-2 complex. Screening of the crystallization conditions of form 2 is being continued in order to obtain crystals with constant cell parameters.

High-resolution X-ray data were collected using Sakabe's Weissenberg camera (Sakabe, 1983) at the BL6A2 station at the Photon Factory, National Laboratory for High Energy Physics. Currently we are collecting X-ray data sets for the native and Hg-derivative crystals of form 2 of I-2 at 2.3 Å resolution and are initiating the determination of the heavy-atom sites. The X-ray data to 2.3 Å resolution from one complex crystal have been collected and merged into a single data set of 23 849 reflections with the merging $R$ of 0.082 {$R = \sum[|I(i) - \langle I\rangle|/I(i)]$ where $I(i)$ is the intensity of an individual measurement and $\langle I\rangle$ is the average value}. The structure determination of the complex is in progress using the molecular replacement method.

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