A well characterized soybean protease inhibitor, the Bowman-Birk Inhibitor, has been crystallized at room temperature in the presence of polyethylene glycol 4000 by vapor diffusion against an ammonium sulfate solution containing 2-methyl-2,4-pentanediol. An x-ray diffraction study reveals that the inhibitor crystallizes in a hexagonal unit cell of symmetry P6₁,2̅ with dimensions a = b = 91.36(2) Å and c = 63.93(2) Å. Each of the 12 asymmetric units contains 2 molecules of molecular weight 8000. The crystal, which diffracts barely to 3-Å spacings, is fairly stable to x-irradiation and has a solvent content of approximately 52% by volume.

A group of proteins which have relatively small molecular weights (ranging from 8,000 to 24,000) and are capable of inhibiting the activities of trypsin and chymotrypsin are widely spread in various parts of plants throughout the plant kingdom (1). Their roles in nature, however, are not yet fully understood (2, 3).

Two such protease inhibitors from soybean seeds have been extensively investigated. Kunitz (4) first isolated and crystallized a soybean trypsin inhibitor with a molecular weight of 21,500 (5), and the conformation of its complex with porcine trypsin has been elucidated at 2.6-Å resolution (6). The second soybean inhibitor, known as the Bowman-Dik inhibitor (7-10), has also been called inhibitor AA (8) or inhibitor 1.9 S (9). These different preparations of the second inhibitor were found by Frattali (10) to be electrochemically identical and biologically similar to, but different in molecular weight from, his preparation (25,000 for inhibitor AA; 16,400 for inhibitor 1.9 S; 7,975 for Frattali's preparation), a vexing discrepancy which was explained by Millar et al. (11) as a consequence of the concentration-dependent association of the protein.

Being unusually rich in cystine (as high as 20%) (9, 10), the Bowman-Dik inhibitor has dual and independent activities against enzymes of animal or microbial origins, having trypsin-like and chymotrypsin-like specificities (8, 10, 12). The complete amino acid sequences have already been established by Odani and Ikenaka (13), with a total of 71 amino acid residues, thus confirming the monomer molecular weight of roughly 8,000. The same authors also reported the antiprotease sites (13), the preparation and characterization of active fragments (14), and the locations of the seven disulfide bridges in the molecule (15).

Recently, by use of a different purification procedure, Hwang et al. (16) obtained five distinct but closely related protease inhibitors (molecular weight range, 7,000 to 8,000) from Tracy soybean seeds. One of these (PI I) has been crystallized in our laboratory to a size suitable for x-ray structural studies (17). Of the other four, one species (PI V) has been identified as the Bowman-Birk inhibitor, on the basis of amino acid composition, isoelectric point, partial sequence study, and inhibitory activities (16).

Although the biological and physicochemical properties of this classical inhibitor have been well documented, the understanding of its specificities at the molecular level depends on x-ray crystallographic studies. Here we report the first crystallization of the Bowman-Birk inhibitor and its crystallographic data.

**MATERIALS AND METHODS**

The Bowman-Birk inhibitor was isolated and purified from soybean seeds of the 'Tracy' variety, harvested in Oak Ridge, Tenn., in 1974. According to the procedures described by Hwang et al. (16), after electrophoresis of the crude product on DEAE-cellulose (Whatman DE52), the major fractions were pooled, dialyzed, and lyophilized in the usual manner. Thus, from 100 g of defatted soybean meal, 53 mg of homogeneous Bowman-Birk inhibitor having an electrophoretic mobility identical with that of PI V (16) were obtained.

The e absorption value at 280 nm was estimated spectrophotometrically as 4.2, a value in close agreement with those reported by other investigators (8-10). Molecular weight determination by gel filtration on a Sephadex G-50 column (2.5 x 35 cm), with 0.05 M sodium cacodylate/HCl buffer (pH 5.6) as an eluent, yielded a value of 9,000 ± 1,000.

Crystals of the purified Bowman-Birk inhibitor were grown by the vapor diffusion technique (18, 19). Approximately 4 to 5 mg of the lyophilized inhibitor were dissolved in 200 μl of 0.05 M sodium cacodylate/HCl buffer (pH 5.6), followed by centrifugation. To 30-μl droplets of the protein solution, 5 μl of 20% (w/v) polyethylene glycol 4000 containing 0.02% of sodium azide were added. The transparent droplets were then placed in a glass reservoir (15 cm in diameter, 6.5 cm high) which contained 120 ml of a mixture consisting of 28 ml of saturated ammonium sulfate, 4 ml of 2-methyl-2,4-pentanediol, and water. Kept at room temperature (24°C), the droplets gradually lost water and absorbed the organic solvent. After 3 to 5 days, chunky, hexagonal prism crystals of usable size (some longer than 1 mm) could be obtained (Fig. 1), although they tended to aggregate. It should be pointed out that the presence of 2-methyl-2,4-pentanediol is essential. The choice of buffer and its concentration and pH are probably less important controlling factors in this case; this is supported by the fact that identical crystals could also be obtained by

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1 The abbreviation used is: PI, protease inhibitor.

2 To increase the yield, a second ammonium sulfate precipitation of the crude seed extract (16) was also carried out after the first, at 40% saturation. The subsequent elution patterns of the 40 to 60% cut thus obtained remained similar to those of the original report.
 Preliminary Crystallographic Data for Bowman-Birk Inhibitor

FIG. 1. Crystals of the Bowman-Birk inhibitor grown at room temperature (24°C) by the vapor diffusion technique. Although they tend to aggregate, chunky crystals with hexagonal cross-section (inset) can be separated for x-ray examination.

The substitution of 0.01 M Na₂HPO₄/citric acid buffer (pH 4.5) for the sodium cacodylate/HCl buffer.

Precession photographs were recorded at room temperature with nickel-filtered copper Kα radiation from an Elliott rotating anode generator operated at 40 kV and 40 mA. An Enraf-Nonius camera with a crystal-to-film distance of 75 mm was used.

RESULTS AND DISCUSSION

A photograph with the x-ray beam parallel to the hexagonal prism axis showed that the hk0 zone of the reciprocal lattice had 6 mm symmetry (Fig. 2a), which was also present on the parallel upper levels. The h0l zone showed mm symmetry (Fig. 2b), as did the hhl zone (not shown). The only systematic absences observed were l ≠ 6n for 00l reflections. These observations are consistent only with the hexagonal space group of P6₃22 or P6₅22.

On the films, measurable intensities appeared to extend barely to 3 Å spacings. Nevertheless, the crystals were fairly stable to x-irradiation; even after 40 h of continuous exposure to the filtered x-ray beam (40 kV, 40 mA), no significant deterioration was observed.

Twelve strong reflections in the 2θ range of 19 to 21° were centered with an Oak Ridge computer-controlled diffractometer (20) by use of Cu Kα, (λ = 1.5418 Å) radiation, and the lattice parameters were refined by the least squares method. The resulting parameters are a = b = 91.36(2) Å and c = 63.93(2) Å. The mosaic spread of the crystal was estimated to be roughly 0.4° about each of three perpendicular directions.

There are 12 asymmetric units in the unit cell volume (V) of 4.61 × 10⁵ Å³. The crystal density (D) was measured as 1.32(1) g/cm³ at room temperature by immersing crystals in a bromobenzene/xylene density gradient column calibrated with potassium bromide solutions (21). The mass of the asymmetric unit (M'), protein plus solvent, is calculated to be 30,500 daltons by the formula M' = 0.6023DV/12. If the molecular weight is taken as 8,000 (10, 13, 16), the amount of protein in the crystal would then be 52% by volume, which lies outside the normal range. Only at n = 2 does the value of V₉ (2.40) fall within the normal range. Since the calculated partial specific volume is 0.69 ml/g for this protein (13, 16), the solvent in the crystal would correspond to a solution dimer.

The 2 protein molecules of the asymmetric unit may correspond to a solution dimer. The inhibitor has been found to undergo reversible self-association to form dimers and trimers

Various protease inhibitors of other plant origins have also been observed to exist in solution as dimers, trimers, and even tetramers (24-27).
The solutions from which crystallization took place had initial protein concentrations larger than 10 mg/ml, at which the protein is presumably aggregated, with a weight average molecular weight of about 14,500 (Ref. 11, Fig. 1). As the diffusion process went on, the protein concentration increased, so that further dimerization could take place.

A set of preliminary x-ray data for a native crystal (0.5 mm in length and 0.25 mm in average width) have been collected by the ω-scan technique to a 2θ maximum value of 22° (equivalent to 4.04 Å resolution) by use of the Oak Ridge computer-controlled diffractometer. To investigate the possible existence of a noncrystallographic 2-fold rotation axis, relating the two monomers of the dimer in the asymmetric unit, rotation functions were calculated with Crowther's program (28), which was modified for the present space group. The results showed that the only prominent nonorigin peak had Euler angle parameters θ1 = 30°, θ2 = 62.5°, and θ3 = 30°. The peak is consistent with the existence of a local 2-fold axis, which (because of the high symmetry) has 12 possible alternative orientations, lying in the {101} planes at angles of 17 and 73° to the c axis.

A search for isomorphous heavy atom derivatives is under way.

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REFERENCES


A right-handed Cartesian axial system was employed with X along a and Z along c. The Euler angle rotations were defined as θ1 about Z, followed by θ2 about the new Y, and θ3 about the new Z. All observed data with spacings smaller than 12 Å were used in three calculations, in which the values 15, 20, and 25 Å were alternatively employed for the radius of the Patterson sphere. The prominent nonorigin peak was highest (23% of the origin peak height) when a Patterson radius of 15 Å was employed. The rotation function peak for a local 2-fold axis, with one of the orientations stated in the text, would be expected to be roughly one-sixth as high as the origin peak.
Preliminary crystallographic data for Bowman-Birk inhibitor from soybean seeds.

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