A Soybean Trypsin Inhibitor

CRystallization and X-RAY Crystallographic Study

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Five trypsin and α-chymotrypsin inhibitors which have low molecular weights (ranging from 6800 to 8600) and are present in soybean seeds of the Tracy variety have been isolated and purified, and single crystals which give x-ray diffraction data beyond 3-Å spacings have been obtained from one of them. The trypsin inhibitor crystallizes in a monoclinic unit cell of symmetry P2₁ and dimensions \( a = 25.919(7) \text{ Å}, b = 43.23(1) \text{ Å}, c = 19.905(5) \text{ Å}, \) and \( \beta = 103.63(2)^\circ \). The asymmetric unit contains 1 molecule of molecular weight 6800. The crystal, which has been found to be unusually stable to x-radiation, has a solvent content of approximately 26% by volume.

Soybean trypsin inhibitors are a group of small proteins which account for a minor portion of the proteins present in the seeds. Although the role of the inhibitors in the seeds is not fully understood, they are thought to be the cause of pancreatic hypertrophy in rats and chicks ingesting raw soybeans (1, 2). To date, several soybean trypsin inhibitors have been isolated (with molecular weights ranging from 8,000 to 24,000), and their biological and physicochemical properties have been studied (2-11). Kunitz (3) first crystallized a soybean trypsin inhibitor with a molecular weight of 21,500 (4), and its molecular conformation in a complex form with porcine trypsin has been partially elucidated at 9.6-Å resolution (19).

During the course of attempts to isolate soybean proteins with high amounts of sulfur-containing amino acids, we developed a simple method of extraction and purification which has resulted in the isolation of five different, but closely related, inhibitors of trypsin and α-chymotrypsin (a complete description is being prepared for publication). The inhibitors are all rich in sulfur-containing amino acids (15 to 22%) and have molecular weights ranging from 6800 to 8600. One of these is probably the Bowman-Birk inhibitor (10), which has also been called inhibitor AA (2, 6). Of the other four, we have succeeded in obtaining crystals of one species, the first case known to us of the crystallization of a soybean trypsin inhibitor (by itself) to a size and quality appropriate for x-ray diffraction studies.

MATERIALS AND METHODS

Isolation and Some Properties of Soybean Trypsin Inhibitor—The soybean trypsin inhibitor was obtained from seeds of the Tracy variety, harvested in Oak Ridge, Tenn. in 1974. Since the purification procedures used in this research and the properties of the resulting trypsin inhibitor were not exactly the same as those previously reported (2-11), some brief information is given. Defatted soybean meal was extracted with 76 mEq Tris/HCl buffer (pH 8.6) containing 5 mM 2-mercaptoethanol. After centrifugation followed by dialysis overnight against running water at 4°C, the supernatant was adjusted to pH 4.8 with the use of 6 N HCl. The precipitate was centrifuged off, and the resulting supernatant was adjusted to pH 7.6 with 5 N NaOH before solid ammonium sulfate was added to 40% saturation. The residue, collected by centrifugation, was dialyzed against water and lyophilized. Further purification, details of which will be described elsewhere, involves reiterative procedures of gel filtration with the use of Sephadex G-100 and Sephadex G-75, and DEAE-cellulose chromatography. The fraction which appeared as the first peak (Peak I) on gradient elution of a DEAE-cellulose column was dialyzed against water and lyophilized. From 100 g of defatted meal, usually about 40 mg of the pure Peak I inhibitor can be obtained.

Disc gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified Peak I preparation showed a single band in each case. The electrophoretic mobility value of 280 nm was determined as 3.86. The NH₂-terminal residue was found to be aspartic acid by dansylation (14, 15). Determination of the anitryptic activity (10, 16) showed that 1 μg of Peak I inhibitor was capable of inhibiting 0.72 μg of active trypsin to the 50% activity level. The amino acid composition was different from those previously reported for soybean inhibitors (2, 4, 8-10). The minimal molecular weight of the protein calculated on the basis of the amino acid composition was 6800 ± 500 (the smallest known among this family of proteins), the value used in the subsequent crystallographic calculations.

2 The best estimates, in integers, of the number of residues per inhibitor (also per 6800 g) were lysine, 3; histidine, 1; asparagine, 4; aspartic acid, 8; threonine (extrapolated to zero hours of hydrolysis), 4; serine (extrapolated to zero hours of hydrolysis), 7; glutamic acid, 4; proline, 4; glycine, 4; alanine, 2; half-cystine (as cysteic acid (17)), 10; valine, 1; methionine (as methionine sulfone (17)), 1; isoleucine, 3; leucine, 7; tyrosine (extrapolated to zero hours of hydrolysis), 1; phenylalanine, 1; and tryptophan (determined spectrophotometrically (18)), 0. Thus the total number of residues is 64. The results are the average of 22- and 72-hour hydrolysates.

3 An estimated value of 6800 ± 400, obtained by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (19), agreed with that by the gel filtration method using Sephadex G-75. However, because the molecular weight is small and the protein has an unusual amino acid composition (see Footnote 2), this value cannot be regarded as having the usual accuracy (20).

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The complete amino acid sequence of this protein has been reported (13).
Crystallization - The purified Peak I trypsin inhibitor was crystallized by the vapor diffusion technique in a manner similar to that described for abrus lectin (21). Fifteen milligrams of lyophilized protein were dissolved in 0.5 ml of 0.02 M sodium acetate buffer (pH 4.5) containing 0.1 M NaCl. After the pH value was readjusted to 4.5 with a small amount of 0.1 N HCl, the sample was centrifuged. Clear droplets (0.05 to 0.05 µl) of the protein solution were then kept in a glass reservoir which contained 100 ml of 15% saturated ammonium sulfate. The crystals usually start to appear after a few hours at room temperature (24°C), and grow to at least 0.5 mm in length within 2 days. Some typical sizes and shapes of the crystals are shown in Fig. 1. The crystals withstand mechanical manipulation at 4°C, and can be stored in mother liquor for long periods without change in the x-ray pattern. They deteriorate, however, almost instantly when approximately 50 µl of 15% 2-methyl-2,4-pentanediol solution is added to the droplet containing the crystals.

X-ray Diffraction - X-ray investigations were carried out on crystals mounted by conventional means in capillaries at 4°C. Diffraction patterns were recorded at room temperature with nickel-filtered Cu Kα radiation from an Elliott rotating anode generator operated at 40 kV and 40 mA. A Nonius precession camera with a crystal-to-film distance of 75 mm and a 0.25-mm collimator were used.

RESULTS AND DISCUSSION

The x-ray diffraction patterns showed that the crystals belong to the monoclinic system. The only observed systematic absences were 0k0 for k odd, indicating the probable space group to be P2₁. The hk0 reciprocal lattice zone is shown in Fig. 2.

Twelve strong reflections in the 2θ range of 22-29° were centered with an Oak Ridge computer-controlled diffractometer (22) using Cu Kα (λ = 1.5418 Å) radiation, and cell parameters were refined by the least squares method. The cell parameters thus obtained are a = 25.919(7) Å, b = 43.23(1) Å, c = 19.906(5) Å, and β = 103.63(2)°. The unit cell volume is 21,673 Å³.

Intensity data were collected from a thin tabular crystal (Fig. 1) of dimensions 0.03 x 0.10 x 0.49 mm; the longest dimension, corresponding to the b direction, was nearly parallel to the c-axis of the diffractometer. The mosaic spread of the crystal was estimated to be only 0.25° in each of the three reciprocal axial directions. The intensities of 1273 independent reflections with 2θ ≤ 35° (equivalent to a minimum crystal spacing of 2.71 Å) were measured with the θ-2θ technique. The crystal was unusually stable to x-radiation, as evidenced by the fact that the intensity of a reference reflection fluctuated within 3% of the average value during the course of data collection. Measurable intensities extend beyond 3-Å spacings. Structure determination thus appears to be feasible.

The density of the crystals, determined by flotation in mixtures of xylene and bromobenzene, was found to be 1.23 g/cm³. The molecular weight, M', of the asymmetric unit, protein plus solvent, may then be calculated by use of the formula M' = DVN/N, where D = crystal density (g/cm³), V = unit cell volume (cm³), N = Avogadro's number, and Z = number of asymmetric units per unit cell. For space group P2₁, Z = 2 and M' = 8028. The amount of protein in the crystal is therefore (6800n/8028) x 100, or 85n% by weight, where n = number of molecules per asymmetric unit. This result alone requires the presence of only 1 molecule in the asymmetric unit. The value for Vₖ, the ratio of protein volume to protein weight, is 1.59 Å³/dalton. For n = 2, the values for Vₖ are far smaller than the commonly observed range of 1.68 to 3.53 (23) for crystals of globular proteins,

while for n = 1, the value of 1.59 lies on the lower limit for the observed range; therefore, n = 1 is clearly established. Since the calculated partial specific volume of this protein is 0.705 cm³/g, the solvent content in the crystal would be 28% by volume. This atypical low solvent content may be due to the intrinsic shape of the molecule itself, or may be attributed to an overestimation of the molecular weight. For example, if the molecular weight (6800) has been overestimated by 500 (1 standard deviation), the value of Vₖ would be 1.72 Å³/dalton,
and the crystal solvent content would be 32% by volume. Regardless of possible errors in the molecular weight determination, the more reliable crystallographic and density data indicate that these crystals have an unusually low solvent content.

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