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Crystallization and Preliminary Studies of Lima Bean Trypsin Inhibitor

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ABSTRACT **Crystals of lima bean trypsin inhibitor (LBTI) were obtained by using the vapor phase equilibration technique with sodium/potassium tartrate as the precipitating agent. The space group was determined to be cubic, $I2_13$ with a = 110.2 Å. These crystals diffract to about 1.9 Å resolution. Preliminary analysis of self-rotation maps (calculated from native x-ray intensity data) suggests the presence of two monomers in the asymmetric unit. LBTI is very thermostable and retains activity even after boiling for 10 minutes. This property is exploited as part of its purification procedure. Proteins 27:311–314**

Key words: thermostability; cubic; trypsin-agarose/sepharose chromatography vapor diffusion method; self-rotation function

INTRODUCTION

Inhibition of proteolytic activity involves the interaction of a proteinase inhibitor with its respective proteinase. In animal systems this process serves an important role in regulation of self-defense mechanisms, blood coagulation, intracellular protein catabolism, and processing of precursor proteins, and in plants it is proposed to function in a defensive mode.[1–4] There are two classes of proteinase inhibitors: those that are synthetic and those that are naturally occurring. Our interest lies in the naturally occurring endogenous protein inhibitors. These types of inhibitors almost always occur as proteins.

One of the most widely studied class of enzymes is serine proteinases and their proteinaceous inhibitors; kinetic, biochemical, and structural studies have resulted in consistent descriptions of their mechanism of action.[1,3] In general, the trypsin inhibitors can be classified into three categories based on molecular weight and the number a disulfide bonds.[3] The first category consists of the small BPTI-Kunitz-type trypsin inhibitors, which are characterized by three conserved disulfide bonds and molecular weights of approximately 6,000 Da. Bovine pancreatic trypsin inhibitor (BPTI) is representative of this type of inhibitor molecule. The second class consists of the Bowman-Birk trypsin inhibitors, which contain a large number of disulfide bonds, are very thermostable, and range in molecular weights from 8,000 to 10,000 Da. Peanut inhibitor A-II is an example of this class of inhibitors. The third class of trypsin inhibitors is the STI-Kunitz-type inhibitors, which contain two conserved disulfide bonds and have molecular weights of 18,000–22,000 Da. Soybean trypsin inhibitor (STI) typifies this class. A complete review of these inhibitors can be found elsewhere in the literature.[1,3,4]

We report here the characterization (i.e., purification, crystallization, and preliminary x-ray studies) of a relatively new type of trypsin inhibitor from lima beans. It has an estimated molecular weight of 15,500 Da and contains approximately 16 cysteines (unpublished results). It appears to be most closely related to the Bowman-Birk class of trypsin inhibitor except for the large difference in molecular weight. This protein is very thermostable and can be boiled for 10 minutes without affecting its trypsin inhibitor activity.

MATERIALS AND METHODS

Purification

Lima bean trypsin inhibitor was obtained from Sigma and further purified by affinity chromatography on a trypsin-agarose/sepharose column. The purification strategy for LBTI is a modified version of the technique developed by Sheih.[5] The primary modification is the addition of a heat treatment before affinity chromatography. Crude lima bean trypsin inhibitor is dissolved in 1% NaCl. It is then subjected to a boiling water bath for 10 minutes, after which it is quenched on ice for 10 minutes. The resulting soluble LBTI is separated from the denatured contaminating protein by centrifugation at 16,000*g* for 10 minutes and 20°C (room temperature).

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Further purification of lima bean trypsin inhibitor is carried out based on its ability to bind trypsin. An affinity column prepared from 2.0 ml of trypsin/agarose and 0.75 ml of Sephadex G-10 was set up and rinsed with 0.5 M NaCl, 1% Triton X-100. The supernatant fraction from the heat treatment was loaded onto the column and washed first with 0.4 M NaCl, 1% Triton X-100 in phosphate buffered saline (PBS) and then with only PBS. The trypsin inhibitor was eluted from the affinity column by the addition of 0.2 M glycine pH 2.2, and later dialyzed into a buffer containing 0.5% NaCl. SDS-PAGE analysis of the purified LBTI and the crude LBTI indicated that the contaminating proteins were removed.

Crystallization and Preliminary X-ray Diffraction

Purified LBTI samples were crystallized by the vapor diffusion hanging drop method. Diffraction quality crystals were obtained using the following strategy: the hanging droplet (5 µl) contained the protein (≈10 mg/ml) in the sodium/potassium tartrate buffer, and the reservoir buffer (600 µl) contained 1.0 M sodium/potassium tartrate and 0.1 M HEPES buffer at pH 7.5. For indexing purposes, a small dataset was collected as follows: 20 data frames were collected at 0.3° oscillation per frame. These frames of data yielded 400 or more strong intensities with high signal-to-noise ratio (i.e., high σ values), and that were subsequently used for autoindexing, thereby yielding initial cell parameters. The Molecular Simulations software, XGEN and the Marsys program were used for autoindexing. Precession photographs were also taken on a Charles Supper camera mounted on a Rigaku generator with a sealed tube. These photographs were used to confirm the space group. Native diffraction datasets have been collected (to 2.0 Å resolution) from two different crystals and processed using the Molecular Simulation software XGEN. The search for suitable heavy atom derivatives for phasing is currently under way.

Self-Rotation Maps

To explore the packing of LBTI protomers in the unit cell, native x-ray intensity data was used to compute a number of self-on-self rotation maps. These calculations were carried out using the program XPLOR.[6] Several maps were calculated using 4–8 Å, 5–10 Å and 6–10 Å resolution data with 6σ cutoff. The principal kappa (κ) sections of 180°, 120°, 90°, 72°, and 60° were analyzed for peaks that would indicate presence of noncrystallographic axes in the asymmetric unit.

RESULTS AND DISCUSSION

We have characterized a relatively new type of trypsin inhibitor from lima beans. This protein, which is denoted as lima bean trypsin inhibitor (LBTI), has an estimated monomeric molecular weight of 15,500 Da (see Fig. 1) and contains approximately 16 cysteines (unpublished results). Figure 1 also shows that LBTI was purified to adequate homogencity for crystallization experiments. The protein appears to be most closely related to the Bowman-Birk class of trypsin inhibitor except for the large difference in molecular weight. The molecule is very thermostable and can be boiled for 10 minutes without affecting its trypsin inhibitor activity.

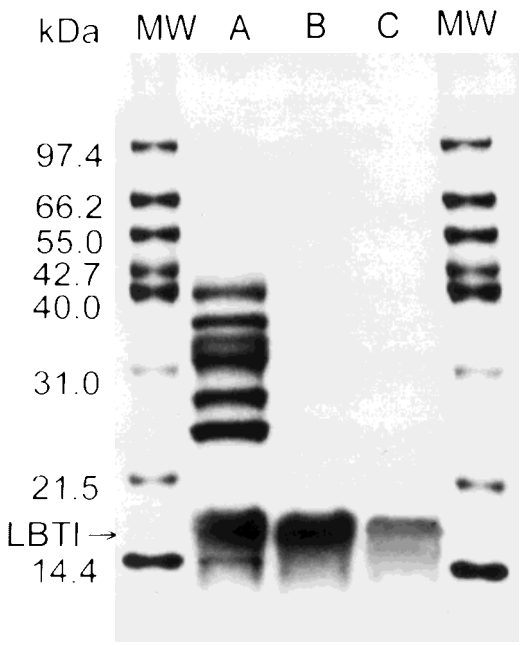

Fig. 1. SDS-PAGE gel showing results from purification of LBTI. Lane A is crude material purchased from Sigma Chemical Company. Lane B shows LBTI after 10 minutes of boiling and refolding (activity is fully recovered). Lane C shows final purity after the heat-treated material is passed through trypsin-agarose affinity chromatography. The two lanes marked MW contain molecular weight markers.

The resulting crystals appeared cubic morphologically (see Fig. 2), reaching dimensions of 0.8 mm along the edges, but were not birefringent. The initial autoindexing yielded a primitive rhombohedral cell with $a_r = 95.91$ Å and $\alpha = 109.47°$. This was converted to a cubic body centered cell with $a_c = 2a_r(\sqrt{3})^{-1}$ (International Tables for Crystallography, Volume A, pp 77). Thus, the space group is $I2_13$, and with cell dimensions of $a = 110.75$ Å. This yields a unit cell volume of 1,358,533 Å^3. There are 24 asymmetric units in this space group. Thus, using the formula[7]:

$$\varphi_p = (nv_pM) \div (NV)$$

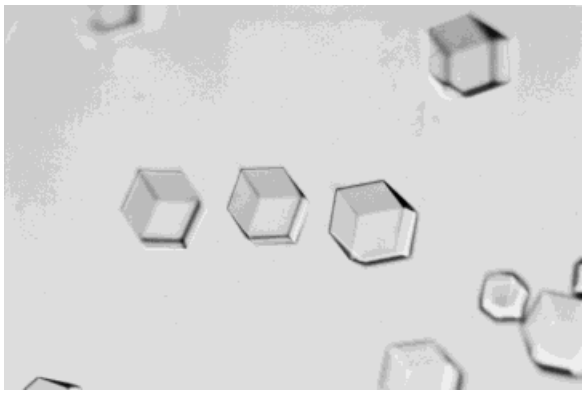

Fig. 2. Cubic crystals of LBTI grown from solutions of sodium/potassium tartrate and HEPES through vapor phase equilibration in hanging drops. These crystals grew to dimensions of 0.8 mm along the edges in about 10–15 days at 20°C.

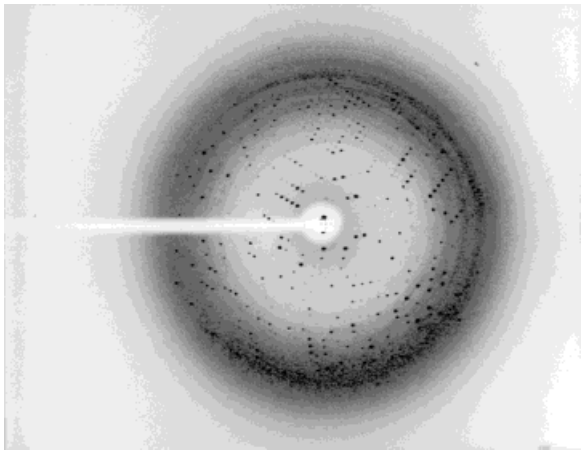

Fig. 3. A 1° oscillation diffraction photograph from an LBTI crystal. Measurable diffraction can be observed to 1.9 Å resolution. These crystals are quite stable in the x-ray beam.

where φ_p is the volume fraction of the crystal occupied by protein; n is the number of protomers per unit cell; V_p is the mean partial specific volume of unsolvated protein ($\approx$0.74 m3/g); M is the molecular weight of one protomer; N is Avogadro's number (6.023×10^{23} mole^{-1}); V is the volume of the unit cell, and two possibilities for (n) the number of protomers in the unit cell arise: 24 and 48. For $n = 24$ (i.e., one protomer in the asymmetric unit), the fractional solvent content is 67% and Matthew's coefficient parameter (V_m) is 3.66 Å^3/Da. On the other hand, if $n = 48$ (i.e., two protomers in the asymmetric unit), the fractional solvent content is 33% and Matthew's coefficient parameter is 1.83 Å^3/Da. The normal ranges of values of V_m and solvent content were compiled by Matthews.[8] Both V_m values (3.66 and 1.83 Å^3/Da) are at the extreme ends of the normal ranges, but are likely. There are two reasons at this point to argue for two protomers in the asymmetric unit ($n = 48$): First, these crystals diffract well to 1.9 Å (90% complete data to 2.0 Å have been collected and processed). Figure 3 shows a 1° oscillation diffraction data frame from crystals of LBTI. The general observation is that crystals with very high solvent content (>60%) tend not to diffract well beyond 2.2 Å. Second, the self-rotation map shows a very strong nonorigin peak at kappa (κ) = 180° ($\phi = 0°$ and $\psi = 45°$), indicating the presence of a noncrystallographic twofold symmetry (see Fig. 4). The remainder of the strong peaks can be generated from this peak by symmetry considerations.

We have collected and processed 90% complete native x-ray intensity data to 2.0 Å. Efforts are currently underway to search for heavy atom derivatives to solve the phase problem. There is also concurrent effort to determine the sequence of LBTI. Sequence data will be useful in the determination of the three-dimensional structure from x-ray data.

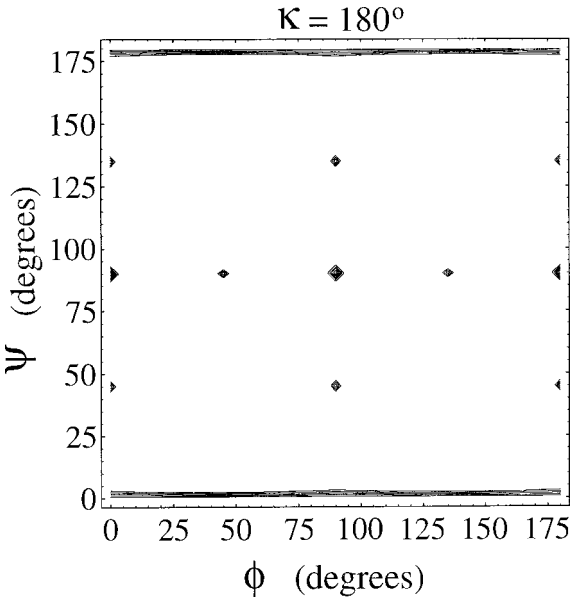

Fig. 4. A $\kappa = 180°$ self-on-self rotation map computed from native x-ray intensity data. A unique peak occurs at $\phi = 0°$ and $\psi = 45°$. The remainder of the peaks can be generated from this peak by symmetry considerations.

The structural analysis of this protein will yield unique insights into a new class of trypsin inhibitors and, perhaps, shed some light on the structural basis for the thermostability of this LBTI.

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