Crystallization and Preliminary X-ray Diffraction Studies of an Alkaline Protease from *Bacillus lentus*

Various crystal forms of the subtilisin-type protease Savinase (EC 3.4.21.14) from the alkalophilic bacterium *Bacillus lentus* have been obtained. The first were orthorhombic needles, space group *P*2₂₁2₁, with unit cell dimensions *a* = 75.3 Å, *b* = 53.4 Å, *c* = 61.5 Å. The crystals diffract to at least 1.8 Å resolution, and the data to 2.0 Å have been recorded on film using synchrotron radiation. The second crystal form grows as similar orthorhombic needles, also in *P*2₁2₁2₁, with cell dimensions *a* = 75.5 Å, *b* = 47.4 Å, *c* = 62.5 Å, mainly differing from the first in the shorter b-axis. Data have been recorded to 2.8 Å. The third form is monoclinic, space group *P*2₁, with dimensions *a* = 40.7 Å, *b* = 64.4 Å, *c* = 43.0 Å, \( \beta = 119^\circ \). Data to a spacing of 2.4 Å have been recorded for this form.

Savinase is a serine protease from *Bacillus lentus* that has been extensively studied due to its importance in biological systems and its potential for industrial use. The enzyme was purified using ion-exchange chromatography on CM-Sepharose CL-6B, followed by gel filtration on Sephacryl S-200 in 0.01 M citric acid buffer (pH 6.0) and 0.005 M calcium chloride. In a separate purification step, 10% PMSF, 2 mM CaCl₂, and 50 mM citrate buffer were added to a concentration of 3 mm. The protein concentration was approximately 23 mg/ml.

Three crystal forms of native or inhibited Savinase have been obtained using different precipitants and crystallization conditions. All crystals were grown at a controlled temperature of 18°C. The orthorhombic needles of the inhibited enzyme were grown by the hanging-drop vapour diffusion method (Davies & Segal, 1971) within two to three days. Reservoirs containing 12 to 15% (w/v) polyethylene glycol (PEG) 4000 were equilibrated against 15-mM droplets containing 6 mg protein/ml, 8 to 10% precipitating agent, 2 mM CaCl₂, 1 mM PMSF inhibitor, and 50 mM citrate buffer adjusted to pH 6.0. Needle-shaped crystals grow to a size of about 0.4 mm x 0.4 mm x 3.0 mm. Crystals could also be obtained using the same method from 22% saturated ammonium sulphate.

From precession photographs, the space group was determined to be *P*2₁2₁2₁. The unit cell dimensions are *a* = 75.3 Å, *b* = 53.4 Å, *c* = 61.5 Å (1 Å = 0.1 nm). These yield a unit cell volume of 247,500 Å³, one molecule in the asymmetric unit and a packing density parameter, \( V_m \), of 2.2 Å³/dalton (Matthews, 1968). Synchrotron radiation from the storage ring DORIS at DESY, Hamburg.

† Abbreviations used: PMSF, phenylmethylsulphonylfluoride; PEG, polyethylene glycol.
was used to collect a complete data set to 2.0 Å resolution from one crystal. The films were evaluated with the MOSCO program system (Machin et al., 1983). The merging R(I) factor for this data set is 7.5%. R is defined as:

$$\sum_{\mathbf{hkl}} \sum_I I - I_0 \over \sum_{\mathbf{hkl}} I$$

where I is the average of i equivalent intensities, I_0.

A second crystal form has more recently been obtained, under essentially identical growth conditions. The crystals are again long needles, of dimensions similar to those of the first form, space group P2_12_12_1, and cell dimensions a = 75.5 Å, b = 47.4 Å, c = 62.5 Å. These differ from the previous crystals mainly in the b-axis, which is about 6 Å shorter. One molecule per asymmetric unit gives a V_m of 2.70 Å³/dalton. Crystals of this form can be obtained from both native and PMSF-inhibited protein, and from both PEG 4000 and saturated ammonium sulphate solutions as described above. Indeed, it has recently proved impossible to reproduce crystals of the first form. Photographic data for the second form have been recorded to a resolution of 2.8 Å (unpublished results) using an Elliot GX18 rotating anode X-ray source, with a merging R of 7.8%.

The third crystal form of the native protein is grown in 15-ml hanging drops containing 20 mg protein/ml, 4% PEG 4000, 0.33 M NaCl, 1.5 mM CaCl_2, 18 mM-citrate buffer (pH 6.0), equilibrated against reservoirs containing 10% PEG 4000, 1 M NaCl, 5 mM-CaCl_2, 50 mM-citrate buffer (pH 6.0). The crystals grow up to a size of 0.5 mm × 0.5 mm × 1.5 mm. They are monoclinic, space group P2_1, with cell dimensions a = 40.7 Å, b = 64.4 Å, c = 43.0 Å and β = 119°. There is one molecule per asymmetric unit, with a V_m of 1.8 Å³/dalton. The cell is similar to, but not identical with, that reported for Subtilisin NOVO (Drenth & Hol, 1967). The crystals give excellent X-ray diffraction patterns. Photographic data have been recorded to a nominal resolution of 2.4 Å using the rotating anode. The merging R factor for these data is 5.1%. The high quality of the diffraction pattern is clearly related to the tight molecular packing in these crystals.

For all three crystal forms, the position of the molecule in the cell has been located from rotation and translation functions with Subtilisin Carlsberg as a model (McPhalen et al., 1985). The co-ordinates were kindly provided by Professor James. A three-dimensional structure analysis of the monoclinic and at least one of the orthorhombic forms of the enzyme will be carried out to high resolution. The comparison of the resulting structural information with other well-defined structures of this enzyme family will aid in the elucidation of those features that are responsible for the specific properties of this enzyme.

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