Crystallization and preliminary X-ray analysis of an alkaline serine protease from *Nesterenkonia* sp.

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A novel calcium-independent serine protease from an alkaliphilic bacterium, *Nesterenkonia* sp. AL20, has been purified and crystallized at 296 K using sodium formate as the main precipitant. This enzyme is optimally active at pH 10, exhibits high stability towards autolytic digestion and its stability is not affected by the presence of EDTA or detergents. The triangular prism-shaped crystals diffracted X-rays to beyond 1.5 Å at a synchrotron beamline, with space group $R3$ and unit-cell parameters $a = b = 92.26$, $c = 137.88$ Å. A complete data set has been collected to 1.39 Å resolution. The asymmetric unit is estimated and confirmed by self-rotation function calculation to contain two molecules, giving a crystal volume per protein mass (V_M) of 2.68 Å^3 Da^{-1} and a solvent content of 54%.

1. Introduction

Serine proteases constitute one of the most important groups of industrial enzymes; they have widespread occurrence and exist in many distinct families. Alkaline proteases of the subtilisin family produced by *Bacillus* species form the largest subgroup of serine proteases (Siezen *et al.*, 1991). These enzymes have become commercially important as the protein-degrading component of washing detergents, the most suitable being the proteases from alkaliphilic bacilli (Banerjee *et al.*, 1999; Kalisz, 1988; Outtrup & Boyce, 1990). Subtilisin-like proteases are also produced by other bacteria, archaea, fungi, yeasts and even higher eukaryotes (Anwar & Saleemuddin, 1998).

A large body of information has been assembled on the crystal structure and biological function relationship of several members of the subtilisin family. They all possess a similar arrangement of the catalytic triad, consisting of Ser, His and Asp residues, in an α/β protein scaffold (Siezen & Leunissen, 1997). Another common feature is the presence of one or more calcium-binding sites. The bound calcium plays an important role in providing stabilization against thermal denaturation and autolytic digestion; enhanced calcium binding is thus observed for the proteases with higher thermostability (Bryan *et al.*, 1986; Frömmel & Höhne, 1981; Strausberg *et al.*, 1995). The subtilisin family of serine proteases has been subjected to extensive enzymology, structure and engineering studies in order to understand their catalytic mechanism, specificity and stability and also to improve their function in industrial applications (Cunningham & Wells, 1987; Heringa *et al.*, 1995; Siezen *et al.*, 1991; Wells & Estell, 1988).

Recently, an alkaline protease secreted by an alkaliphilic and moderately halophilic microorganism, *Nesterenkonia* sp. AL20, isolated from an alkaline soda lake in the East African Rift Valley, has been purified and characterized (Gessesse *et al.*, 2003; Bakhtiar, Andersson *et al.*, 2003; Bakhtiar, Estiveira *et al.*, 2003). Although its activity profiles suggested the enzyme to be a subtilisin-like protease, its activity and stability were calcium-independent. The enzyme, with a molecular weight of 22 876 ± 5 Da, is optimally active at pH 10 and 343 K. It shows good stability at 323 K in the presence of EDTA and even detergents. These features of the AL20 protease imply that it may be more stable than subtilisin under normal laundry conditions. In order to understand these unusual features and to prepare for protein engineering, we have initiated structural studies of the AL20 protease. Here, we report its crystallization and preliminary structural analysis.

2. Experimental

### 2.1. Protein purification and crystallization

The alkaline protease was purified from the culture supernatant of *Nesterenkonia* sp. AL20 according to the procedure described elsewhere (Gessesse *et al.*, 2003), comprising ammonium sulfate precipitation, ion-exchange chromatography and gel filtration. The purified AL20 protease was obtained with a recovery of 57% and an overall purification factor of 48-fold, having a specific activity of about 2200 U mg^{-1}. Fractions containing the enzyme in 10 mM Tris–HCl buffer pH 8.0 were pooled

and concentrated to 2.8 mg ml^{-1} for crystallization.

The initial crystallization conditions were screened using the sparse-matrix method (Jancarik & Kim, 1991). All crystallization experiments were performed by the hanging-drop vapour-diffusion method using Hampton Research crystallization kits I and II at 294 K in 24-well VDX plates (Hampton Research, USA). In each trial, a hanging drop of 1 µl of protein solution was mixed with 1 µl of reservoir solution and equilibrated against 500 µl of reservoir solution. Additive screens (Hampton Research, USA) were also tried to optimize the crystallization conditions.

2.2. Data collection and processing

An AL20 protease crystal with dimensions of about $0.5 \times 0.3 \times 0.3$ mm was briefly soaked in cryoprotectant (reservoir solution with 110% precipitant concentration plus 5% glycerol; in this case, the cryoprotectant contains about 3.2 M sodium formate in 0.1 M sodium citrate pH 6.9 with 5% glycerol), picked up with a nylon CryoLoop (Hampton Research, USA) and plunged directly into liquid nitrogen. The frozen crystal was transferred to a cold nitrogen stream (Oxford Cryostream Cooler) at 100 K. Diffraction data were collected by the oscillation method using a MARCCD detector (X-ray Research, GmbH) at the crystallographic beamline BL711 at the MAX-II synchrotron laboratory, Lund (Sweden) with a wavelength of 1.076 Å. The crystal-to-detector distance was set to 80 mm with an oscillation range of 0.5° per
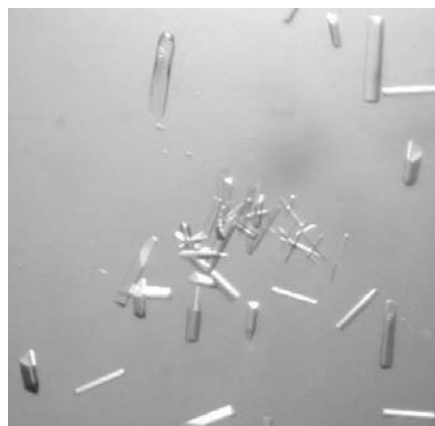
image. The exposure time was typically 60 s per frame. The diffraction data were processed using the program *XDS* and merged with *XSCALE* (Kabsch, 1988). The data-collection statistics are listed in Table 1.

3. Results and discussion

Initial screening of the crystallization conditions showed thin needle-like crystals in condition No. 33 of Crystal Screen I (4.0 M sodium formate only). This condition was further optimized by fine screening of the sodium formate and protein concentrations, buffer content and pH values. The addition of $3\%(v/w)$ ethanol, 0.01 M L-cysteine or 0.01 M NAD$^+$ seemed to improve the crystal quality; however, good-quality crystals were also obtained without additives.

As shown in Fig. 1, triangular prism-shaped crystals could be obtained with typical dimensions of $0.2 \times 0.1 \times 0.1$ mm in 2.9 M sodium formate with 0.1 M sodium citrate pH 6.9 in 4–5 d at room temperature. The macroseeding procedure using a nylon CryoLoop could produce larger and better-looking single crystals with the same crystal morphology as those shown in Fig. 1. Crystals grown under such conditions are very reproducible, particularly with macroseeding. Some crystals of AL20 diffracted to beyond 1.5 Å resolution on beamline BL711 of the MAX-II synchrotron laboratory, Lund.

A complete data set to 1.39 Å resolution was collected and processed as summarized in Table 1. According to the statistics, it was obvious that the crystal could diffract to higher resolution, but the resolution was physically limited by the detector setting. The crystal belongs to space group $R3$, with unit-cell parameters $a = b = 92.26$, $c = 137.88$ Å, $\gamma = 120°$. There are two molecules per asymmetric unit as estimated by the calculated V_M (Matthews, 1968) value of 2.68 Å^3 Da^{-1}. The solvent content for such a crystal is then approximately 54%. A self-rotation function was calculated on the scaled data (the resolution range 10–5 Å was used for calculation) from the native crystal using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994), as shown in Fig. 2. In
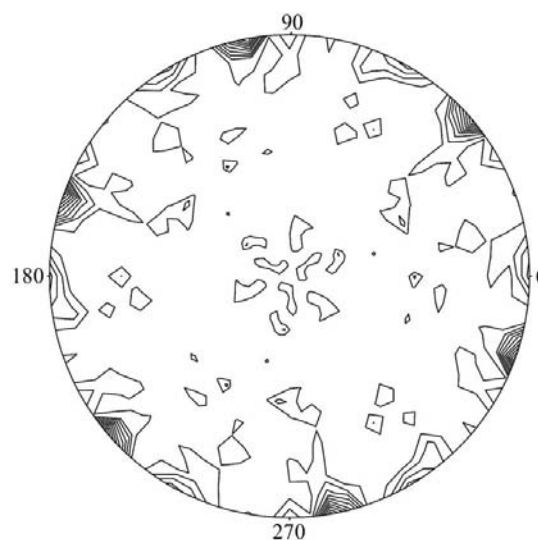
the $\kappa = 180°$ section, the self-rotation map clearly shows there to be three NCS (non-crystallographic symmetry) twofold axes related by the crystallographic threefold symmetry (Fig. 2). These twofold NCS fit well with the asymmetric unit content estimated above.

Determination of the primary sequence of the AL20 alkaline protease is under way and the structure solution is also progressing by MIR (multiple isomorphous replacement) methods. Derivatives have been prepared by soaking native protein crystals in different heavy-atom compounds; some of the soaked crystals seem to diffract well and to contain heavy-metal sites.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Wavelength (Å)	1.076
Resolution (Å)	30.0–1.39 (1.47–1.39)
Completeness (%)	97.4 (90.0)
R_{merge}† (%)	4.9 (19.3)
$I/\sigma(I)$	13.7 (4.3)
Space group	$R3$
Unit-cell parameters (Å, °)	$a = 92.26$, $b = 92.26$, $c = 137.88$, $\gamma = 120.0$
Possible No. of unique reflections	88606
Total No. of reflections collected	283174
V_M (Å^3 Da^{-1})	2.68
Molecules per asymmetric unit	2

† $R_{merge} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$, where the summation is over all reflections.

Figure 1
Triangular prism-shaped rod crystals of AL20 alkaline protease grown in 2.9 M sodium formate, 0.1 M sodium citrate pH 6.9 at room temperature; these crystals can be used as seeds to obtain even larger crystals. In this figure, the typical width of the crystals is about 0.05–0.1 mm.

Figure 2
The self-rotation function map of an AL20 protease crystal calculated in the resolution range 10–5 Å and plotted at the $\kappa = 180°$ section to show possible twofold NCS (non-crystallographic symmetry). Contour levels start from 2σ (σ is the root-mean-square deviation from mean density), with an increment of 1σ. The map is viewed down the threefold axis. The obvious twofold NCS peaks are about 11σ high and are all located at $\omega \simeq 90°$, with $\varphi \simeq 40$, 100, 160, 220, 280 and 340°.

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