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Crystallization and preliminary X-ray diffraction studies of a novel alkaline serine protease (KP-43) from alkaliphilic *Bacillus* sp. strain KSM-KP43

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A novel alkaline serine protease (KP-43) which belongs to a new class of the subtilisin superfamily was crystallized by the sitting-drop vapour-diffusion method with ammonium sulfate as a precipitant. The crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 43.50 (2), b = 110.4 (1), c = 168.9 (1) Å. The crystals diffract X-rays beyond 1.9 Å resolution using Cu $K\alpha$ radiation from a rotating-anode generator and are suitable for high-resolution crystal structure analysis.

1. Introduction

Subtilisins and related 'high-alkaline' proteases are important materials for improving the efficiency of laundry detergents. These proteases, which facilitate the release of proteinaceous materials such as grime, blood and milk, have been added to compact the volume of the detergents. World use of detergent proteases has increased in recent years (Houston, 1997). These enzymes are classified as 'high alkaline', a subclass of the subtilisin family (Siezen & Leunissen, 1997). In order to resist the highly alkaline conditions of typical laundry detergents, various 'high-alkaline' proteases, such as M-protease (Kobayashi *et al.*, 1995), PB92 (Zuidweg *et al.*, 1972) and Savinase (Betzel *et al.*, 1992), have been isolated from several strains of alkaliphilic *Bacillus* species. However, these 'high-alkaline' proteases are sensitive to oxidizing agents (Stauffer & Eston, 1969).

KP-43 protease (DNA Data Bank of Japan, accession No. AB051423) was found in an alkaliphilic *Bacillus* sp. strain KSM-KP43 (FERM BP-6532). This protease resists oxidizing agents in addition to highly alkaline conditions and high temperature (343 K). Multiple amino-acid sequence comparisons among KP-43 protease and related enzymes in the subtilisin family indicate that KP-43 protease is subtilisin-like but does not belong to any subfamilies of the subtilisin family (Siezen & Leunissen, 1997; Saeki *et al.*, 2000). All subtilisin-family proteases have three completely conserved catalytic residues, Asp, His and Ser. These three residues (the catalytic triad) are located far from each other in the primary structure but are close in the three-dimensional structure and form the active centre of this enzyme (Wright *et al.*, 1969). Amino-acid sequences around these three catalytic residues are well conserved among all members of the family, including KP-43 protease. However, the total amino-acid sequence homology of KP-43 with known subtilisins and related enzymes is only about 25%. In addition, the molecular weights of all proteases belonging to these subfamilies are around 28 kDa, whereas that of KP-43 protease is 45 kDa. Compared with typical proteases of these subfamilies, KP-43 protease has an additional domain in the C-terminal region. These features suggest that the KP-43 protease should be classified into a new category (Saeki *et al.*, 2000).

The three-dimensional structures of several subtilisin family proteases, subtilisin BPN′ (Wright *et al.*, 1969), subtilisin Carlsberg (Bode *et al.*, 1987), subtilisin Novo (Drenth *et al.*, 1972), M-protease (Yamane *et al.*, 1995) and Savinase (Betzel *et al.*, 1992), have been determined. The structures of these proteases show the common structurally conserved core and a similar topology of secondary-structure elements (Siezen & Leunissen, 1997). The common core, which consists of about 190 residues including the catalytic triad, superimposes well among these structures (r.m.s.d.

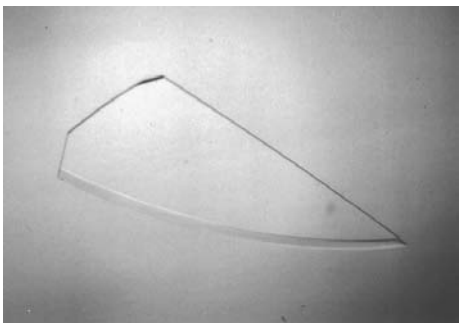

Figure 1
A crystal of KP-43 protease from *Bacillus* sp. strain KSM-KP43.

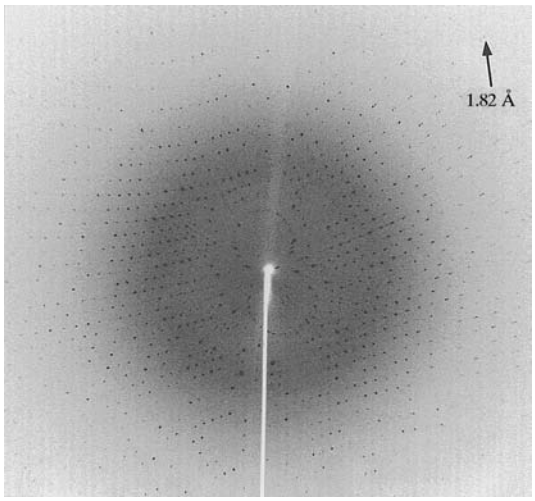

Figure 2
X-ray diffraction patterns from a crystal of KP-43 taken with Cu $K\alpha$ radiation. The crystal-to-detector distance and the oscillation range are 150 mm and 1.0°, respectively.

of C^{α} atoms is less than 2.0 Å) (Siezen & Leunissen, 1997). However, KP-43 protease has several insertions and a long C-terminal extension which are assumed to play a dominant role in its resistance to oxidizing agents, highly alkaline conditions and high temperature (Saeki *et al.*, 2000). Therefore, the three-dimensional structure of KP-43 protease is indispensible for the investigation of the resistance mechanism towards these severe conditions and to design more efficient enzymes. Here, we report the crystallization and preliminary X-ray diffraction studies of KP-43 protease from an alkaliphilic *Bacillus* sp. strain KSM-KP43.

2. Materials and methods

KP-43 protease from alkaliphilic *Bacillus* sp. strain KSM-KP43 was prepared as described by Saeki *et al.* (2000). It is composed of 434 amino-acid residues and its molecular mass is 45 331 Da. The purity of the sample used for crystallization was more than 90% judged by SDS–PAGE.

Crystallization of KP-43 was carried out by the sitting-drop vapour-diffusion method at 293 K. The protein solutions contained 35 mg ml^{-1} KP-43 protease, 10 m*M* Tris–HCl pH 7.5 and 2.0 m*M* calcium chloride, whereas the reservoir solutions contained 0.1 *M* HEPES–NaOH pH 7.3, 1.7 *M* ammonium sulfate, 10 m*M* calcium chloride and 10%(*v*/*v*) 1,4-dioxane. Mixtures of protein solution and the same volume of the reservoir solution were equilibrated against the reservoir solution. Plate-shaped crystals grew in about 10 d to approximate dimensions of 1.8 × 0.4 × 0.05 mm (Fig. 1).

The crystals were mounted and sealed in a glass capillary with a trace amount of crystal-stabilizing buffer composed of 0.1 *M* HEPES–NaOH pH 7.3, 1.2 *M* ammonium sulfate, 10 m*M* calcium chloride and 10%(*v*/*v*) 1,4-dioxane. Diffraction data were collected at room temperature using Cu $K\alpha$ radiation generated by a Rigaku UltraX18 rotating-anode generator with a working voltage of 40 kV and a current of 100 mA. The crystal-to-detector distance was set to 150 mm and diffraction images were recorded on an R-AXIS IV image plate with 1.0° oscillation and 6 min exposure per frame (Fig. 2). All data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Molecular replacement was carried out using the program *AMoRe* (Navaza, 1994) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Data collection and processing

The crystals of KP-43 diffract X-rays beyond 1.9 Å resolution (Fig. 2). They belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 43.50 (2), b = 110.4 (1), c = 168.9 (1) Å. The statistics of the intensity data are shown in Table 1. Assuming one KP-43 molecule per asymmetric unit, the Matthews coefficient V_M is calculated to be 2.2 Å^3 Da^{-1} and the solvent content of the crystal is calculated to be 45% (Matthews, 1968). No reasonable solution was obtained from the molecular-replacement method using several crystal structures of subtilisin family serine proteases (PDB codes 1cnm, 1st3, 1mme and 1be6). A search for heavy-atom derivatives is now in progress.

Table 1
Data-collection statistics for KP-43 protease.

Space group	$C222_1$
Unit-cell parameters† (Å)	
a	43.49 (2)
b	110.4 (1)
c	168.9 (1)
Resolution‡ (Å)	100–1.9 (1.93–1.90)
Total No. of reflections	132 663
No. of unique reflections	29752
$I/\sigma(I)$	19.2
Completeness‡ (%) [$I \geq \sigma(I)$]	90.9 (69.1)
$R_{\rm merge}$‡§ (%)	6.6 (28.9)

† Estimated standard deviations in parentheses. ‡ Values for the highest resolution shell in parentheses. § $R_{\rm merge} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$.

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