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Crystallization and preliminary X-ray crystallographic studies of glutamic acid specific proteinase from *Bacillus licheniformis* complex with Z-Leu-Glu-CH_2Cl.

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Abstract

A glutamic acid specific proteinase from *Bacillus licheniformis* has been crystallized as a complex with the inhibitor Z-Leu-Glu-CH_2Cl. Crystals were grown by the vapor-diffusion method using sodium formate as a precipitant. The crystals diffracted to about 2.0 Å resolution and belonged to the trigonal space group $P3_121$ ($P3_221$) with unit-cell parameters $a = b = 134.3$, $c = 109.7$ Å. A total of 26 964 independent reflections were obtained up to 2.2 Å resolution, the merging R factor being 0.05 for 42 614 measurements.

Introduction

Glutamic acid specific proteinases from various sources have been reported (Drapeau, Boily & Houmard, 1972; Kakudo, *et al.*, 1992; Yoshida, *et al.*, 1988; Niidome, Yoshida, Ogata, Ito & Noda, 1990; Kitadokoro, *et al.*, 1993). They recognize negatively charged residues of glutamic acid and aspartic acid.

Recently, Nienaber, Breddam & Birktoft (1993) reported the three-dimensional structure of the acidic amino-acid specific proteinase from *Streptomyces griseus*. We also investigated by X-ray analysis the three-dimensional structure of a proteinase with broad substrate specificity (SFase-2), and compared it with the related proteinase structures of *S. griseus* proteinase A and *S. griseus* proteinase B. Based on the SFase-2 structural parameters, the tertiary structure of the acidic amino-acid specific proteinase (SFase-1), which possesses 57% amino-acid sequence identity, was studied to elucidate its substrate-recognition mechanism (Kitadokoro, Tsuzuki, Okamoto & Sato, 1994).

The important key residues for substrate recognition of the acidic amino-acid specific proteinases produced from *Streptomyces* may be the three histidine residues, His158, His168 and His185 (Kitadokoro *et al.*, 1994). The substrate-recognition pocket may be charged positively by this histidine cluster and, therefore, will preferentially recognize the negatively charged Glu and Asp residues on substrate molecules.

Glutamic acid specific proteinase from a pathogenic *Staphylococcus aureus* strain V8 (V8 proteinase) has been widely used as a tool for analysis of the protein sequence (Drapeau *et al.*, 1972). The V8 proteinase has been subjected to nucleotide sequencing (Carmona & Gray, 1987), and also a crystallographic study (Gehrig, Delbaere & Drapeau, 1985). However, its tertiary structure has not been determined. We screened various amino-acid specific proteinases from non-pathogenic microorganisms and found a glutamic acid specific protease from *Bacillus licheniformis* (BLase). Its enzymatic characterization and gene cloning were reported (Kakudo *et al.*, 1992). A glutamic acid specific proteinase from Alcalase™, which has the same protein sequence and character as BLase, was reported by Svendsen & Breddam (1992).

It would be very interesting to study how BLase hydrolyzes the C-terminal bonds of the acidic amino acids Glu and Asp. As the sequence similarities between SFase-1 and BLase are low (Kitadokoro, *et al.*, 1993), and BLase does not contain the histidine cluster, it may recognize its respective substrates in a different manner from the acidic amino-acid specific proteinases from Streptomycetes. This paper will discuss the crystallization and preliminary crystallographic data of the BLase complex with its inhibitor of Z-Leu-Glu-CH_2Cl.

Experimental

The glutamic acid specific proteinase (BLase) was purified from culture filtrates of *B. licheniformis*, strain ATCC 14580, as described (Kakudo *et al.*, 1992). Z-Leu-Glu-CH_2Cl was prepared in our laboratory. Purified BLase was inhibited by a tenfold molar excess of Z-Leu-Glu-CH_2Cl in a solution consisting of 10 mM Tris buffer, pH 8.0, 2 mM $CaCl_2$, and 5% dimethyl sulfoxide, for 1 h at 298 K, concentrated to 10 mg protein ml^{-1} by use of Centricon 10 tubes (Amicon). The crystallization conditions were screened by the hanging-drop vapor-diffusion method, using the macromolecular crystallization reagent kit, Crystal Screen (Hampton Research). This kit consists of 50 precipitating reagents which were developed by Jancarik & Kim (1991). Each hanging droplet on a siliconized coverslip consisted of 3 µl of the protein solution (10 mg ml^{-1}) plus 3 µl of one of the precipitating reagents. The reservoir contained 0.7 ml of the same reagent.

Crystals were mounted and sealed in 1.0 mm glass capillaries with a small portion of mother liquor. X-ray diffraction

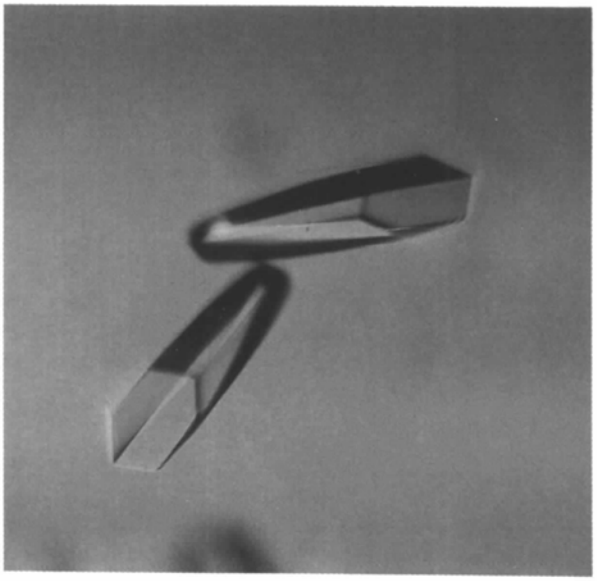

Fig. 1. Crystals of the complex of glutamic acid specific proteinase of *Bacillus licheniformis* with Z-Leu-Glu-CH_2Cl.

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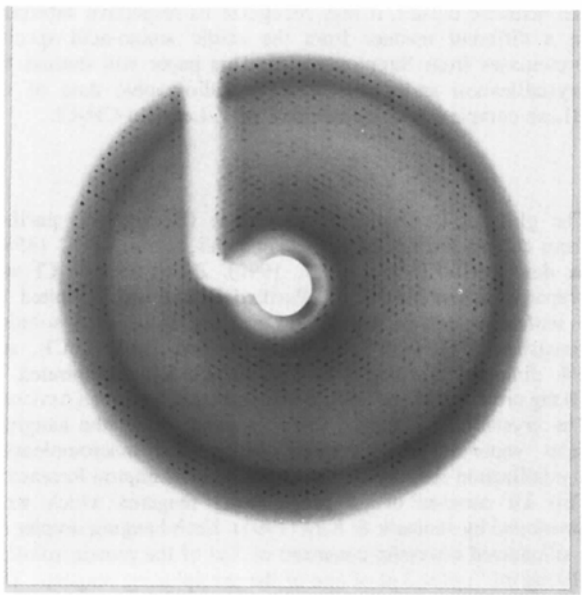
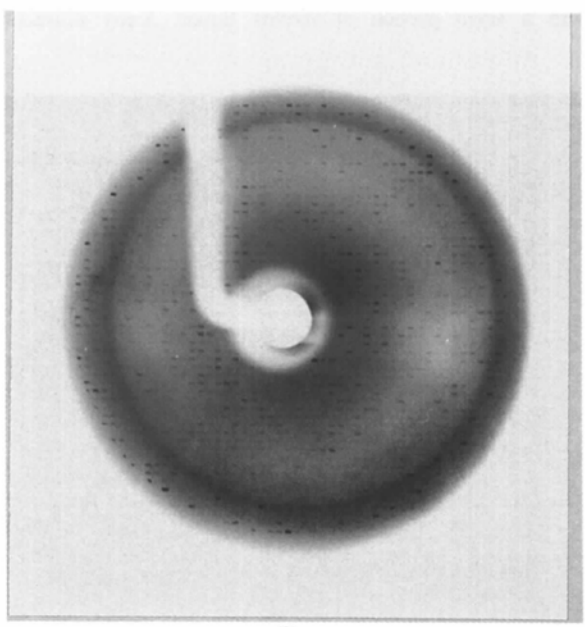
photographs were taken at room temperature with a precession camera using Ni-filtered Cu $K\alpha$ radiation from a sealed X-ray tube operated at 40 kV, 35 mA.

X-ray diffraction data were collected using a Rigaku R-AXIS IIC imaging plate on a Rigaku R-200 X-ray generator with a fine focus operated at 40 kV and 100 mA. Cu $K\alpha$ X-rays were selected with a graphite monochromator. A single crystal with dimensions $0.5 \times 0.3 \times 0.3$ mm was mounted in a glass capillary.



(*a*)



(*b*)

Fig. 2. Precession photographs ($\mu = 10°$) of the (*a*) *hk*0 and (*b*) *h*0*l* zones of the Z-Leu-Glu-BLase crystals. The crystal-to-film distance was 60 mm, the crystals diffracted to about 2.0 Å.

Results and discussion

Among 50 precipitants, hexagonal rod-shaped crystals appeared in tube 33 (4.0 *M* sodium formate, Crystal Screen) at 293 K after 2 weeks. Under the best conditions which were found by surveying around the conditions of tube 33 [2.8 *M* sodium formate, 0.1 *M* 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5], the crystals grew to a size of $1.0 \times 0.3 \times 0.3$ mm (Fig. 1).

The crystallographic data were obtained by precession photographs taken with an Enraf–Nonius camera. The crystals diffract beyond 2.0 Å resolution. Fig. 2 shows the precession photographs ($\mu = 10°$) of the *hk*0 zone and the *h*0*l* zones, which indicate that the crystals belong to the trigonal space group $P3_121$ ($P3_221$), with cell dimensions $a = b = 134.3$, $c = 109.7$ Å. They are apparently unaffected by several days of exposure to the X-ray beam. Assuming that there are five or six molecules per asymmetric unit, respectively (5 or 6×24 kDa), the volume of the molecule is calculated as 2.74 or 2.28 Å^3 Da^{-1} and the solvent content is 55.0 or 46.01%, respectively (Matthews, 1968).

A total of 42 614 reflections [$I > \sigma(I)$] up to 2.2 Å resolution were measured by rotating the crystal from 0 to 30° about the c^* axis with a rotation interval of 1.0° per frame. These data were merged and scaled by the Rigaku software (Sato *et al.*, 1992) to yield 26 964 unique reflections giving an internal merging R factor [$R_{\rm merge} = \Sigma|I(h) - \langle I(h)\rangle|/\ \Sigma I(h)$, where $I(h)$ and $\langle I(h)\rangle$ are the intensity values of individual measurements and of the corresponding mean values] of 5.5%, comprising 81.9% of all data expected to 2.2 Å resolution.

The search for suitable heavy-atom derivatives to solve the structure by multiple isomorphous replacement methods is in progress.

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References

Carmona, C. & Gray, G. L. (1987). *Nucleic Acids Res.* **15**, 6757.
Drapeau, G. R., Boily, Y. & Houmard, J. (1972). *J. Biol. Chem.* **247**, 6720–6726.
Gehrig, L. M. B., Delbaere, L. T. J.& Drapeau, G. R. (1985). *J. Mol. Biol.* **185**, 651.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kakudo, S., Kikuchi, N., Kitadokoro, K., Fujiwara, T., Nakamura, E., Okamoto, H., Shin, M., Tamaki, M., Teraoka, H., Tsuzuki, H. & Yoshida, N. (1992). *J. Biol. Chem.* **267**, 23782–23788.
Kitadokoro, K., Nakamura, E., Tamaki, M., Horii, T., Okamoto, H., Shin, M., Sato, T., Fujiwara, T., Tsuzuki, H., Yoshida, N. & Teraoka, H. (1993). *Biochim. Biophys. Acta*, **1163**, 149–157.
Kitadokoro, K., Tsuzuki, H., Okamoto, H. & Sato, T. (1994). *Eur. J. Biochem.* **224**, 735–742.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nienaber, V. L., Breddam, K. & Birktoft, J. J. (1993). *Biochemistry*, **32**, 11469–11475.
Niidome, T., Yoshida, N., Ogata, F., Ito, A. & Noda, K. (1990). *J. Biochem. (Tokyo)*, **108**, 965–970.
Sato, M., Yamamoto, M., Imada, K., Katsube, Y., Tanaka, N. & Higashi, T. (1992). *J. Appl. Cryst.* **25**, 348–357.
Svendsen, I. & Breddam, K. (1992). *Eur. J. Biochem.* **204**, 165–171.
Yoshida, N., Tsuruyama, S., Nagata, K., Hirayama, K., Noda, K. & Makisumi, S. (1988). *J. Biochem. (Tokyo)*, **104**, 452–456.