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Preliminary X-ray crystallographic analysis of thermolysin in the presence of 4 *M* NaCl

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The activity of thermolysin (EC 3.4.24.27) is greatly enhanced by high concentrations of neutral salts. For instance, 4 *M* NaCl enhances the activity 13–15-fold [Holmquist & Vallée (1976), *Biochemistry*, **15**, 101–107; Inouye (1992), *J. Biochem. (Tokyo)*, **112**, 335–340]. To clarify the structural basis of the activation of thermolysin by high concentrations of NaCl, we have developed a new method to introduce 4 *M* NaCl into the $P6_122$ crystal of thermolysin originally grown without NaCl. The crystal obtained by this method diffracted X-rays to 2.43 Å. No unit-cell parameter change was observed except the length of the *c* axis, which was elongated by 9.6% by the introduction of 4 *M* NaCl.

1. Introduction

Thermolysin (EC 3.4.24.27) is a thermostable neutral metalloproteinase secreted by *Bacillus thermoproteolyticus* and catalyzes the hydrolysis of peptide bonds involving hydrophobic amino-acid residues (Endo, 1962; Matsubara & Feder, 1971). A thermolysin molecule requires one zinc ion for enzymatic activity and four calcium ions for structural stability (Latt *et al.*, 1969; Feder *et al.*, 1971; Tajima *et al.*, 1976). The amino-acid sequence (Titani *et al.*, 1972; O'Donohue *et al.*, 1994) and three-dimensional structure (Holmes & Matthews, 1982) have been reported and the kinetic mechanism of the reaction has been proposed (Hangauer *et al.*, 1984; Mock & Aksamawati, 1994).

Thermolysin is remarkably activated by high concentrations of neutral salts such as NaCl and NaBr (Inouye *et al.*, 1996). The enzymatic activity increases in an exponential fashion with increasing salt concentration; the activity is enhanced 13–15-fold by adding 4 *M* NaCl at pH 7.0 and 298 K (Inouye, 1992). The activation is caused by an increase in the molecular activity, k_{cat}, but not by a decrease in the Michaelis constant K_m (Inouye, 1992; Inouye *et al.*, 1996).

The order of cations in the efficiency of thermolysin activation is $Na^+ > K^+ > Li^+$ (Inouye, 1992; Inouye *et al.*, 1994, 1996) and is different from the order of the Hofmeister series that corresponds to the degree of strong hydration: $Li^+ > Na^+ > K^+$ (Pundak *et al.*, 1981; Inouye, 1991). The difference suggests that the activation efficiency of a cation depends on its interactions with charged groups on the enzyme rather than its size or hydration potential (Inouye *et al.*, 1997). The concentration of NaCl does not cause any detectable change in the circular-dichroism (CD) spectrum of thermolysin (Inouye *et al.*, 1998*a*,*b*). To clarify the molecular mechanism of thermolysin activation by a high concentration of NaCl, we have performed preliminary crystallographic analysis of the enzyme in the presence of 4 *M* NaCl. In this paper, we report a new method to introduce a high concentration of NaCl into thermolysin crystals originally grown without NaCl and some preliminary results of the X-ray crystallographic analysis.

2. Materials and methods

2.1. Materials

A three-times crystallized and lyophilized preparation of thermolysin was purchased from Daiwa Kasei (Osaka, Japan). This preparation was used without further purification. All other reagents were of analytical grade.

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

NaCl concentration (*M*)	0	4.0
X ray source	SPring 8 BL38B1	SPring 8 BL41XU
Temperature (K)	100	100
Wavelength (Å)	1.000	1.000
Space group	$P6_122$	$P6_122$
Unit-cell parameters (Å)	$a = b = 92.62$, $c = 129.22$	$a = b = 93.82$, $c = 141.63$
Resolution range (Å)	18.41–1.79 (1.85–1.79)	19.65–2.43 (2.52–2.43)
No. of unique reflections	29546	13662
Completeness (%)†	99.0 (99.7)	100.0 (99.9)
$R_{\rm merge}$† for all data (%)	5.0 (10.1)	11.6 (21.9)
$\langle I\rangle/\langle\sigma(I)\rangle$	99.3 (60.6)	32.3 (11.4)
Redundancy	20.1 (19.8)	40.1 (41.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

2.2. Crystallization of thermolysin

Thermolysin was crystallized according to the procedure described previously (Holmes & Matthews, 1982) with some minor modifications. Thermolysin was dissolved in solution *A* [10 m*M* Tris–HCl pH 7.5, 2.5 *M* CsCl, 45%(*v*/*v*) DMSO (dimethyl sulfoxide)]. Insoluble material was removed by centrifugation and the clarified supernatant was used for crystallization trials. The enzyme concentration was estimated using an absorbance value, *A* (1 mg ml^{-1}) at 277 nm of 1.83 (Inouye, 1992). 2 µl drops (∼ 40 mg ml^{-1}) were equilibrated against 500 µl distilled water by the hanging-drop vapor-diffusion method. In this case, the vapor diffused from the reservoir to the drop and its volume was increased. The supersaturation of thermolysin was achieved because of the low solubility of thermolysin in the low concentrations of DMSO or salts. Hexagonal crystals appeared in a few days at 293 K.

2.3. Introduction of 4 *M* NaCl into thermolysin crystals

To introduce 4 *M* NaCl into the crystals, the crystals were picked up in nylon loops (Hampton Research, Aliso Viejo, USA) and soaked in solution *B* [35 m*M* Tris–HCl pH 7.5, 4 *M* NaCl, 4.9%(*v*/*v*) DMSO with thermolysin saturated] for 3 h at 293 K. These crystals did not diffract X-rays. Another method was attempted. A siliconized glass cover slide with a hanging droplet (∼20 µl) containing thermolysin crystals was sealed onto a 24-well sitting-drop plate (Hampton Research), where 100 µl of solution *C* [35 m*M* Tris–HCl pH 7.5, 4.8 *M* NaCl, 4.9%(*v*/*v*) DMSO with thermolysin saturated] was on the platform and 500 µl of solution *C* was in the deep-well as the reservoir. After incubation for 2 d at 293 K, the crystals moved down from a droplet under the cover glass slide to the bottom of the platform on the sitting-drop plate. The concentration of NaCl on the platform should have been decreased from 4.8 to ∼4.0 *M* after the fusion with the droplet not containing NaCl.

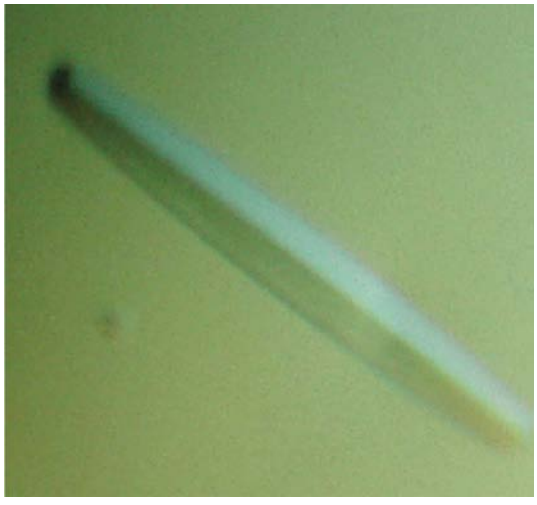

Figure 1
A typical crystal of thermolysin grown by the hanging-drop vapor-diffusion method at 293 K.

2.4. X-ray diffraction data collection

Prior to data collection, the crystals of thermolysin was picked up in a nylon loop (Hampton Research), transferred to a cryoprotectant solution [4.9%(*v*/*v*) DMSO, 30%(*v*/*v*) glycerol and 35 m*M* Tris–HCl pH 7.5 for native crystals and 4.9%(*v*/*v*) DMSO, 30%(*v*/*v*) glycerol, 4 *M* NaCl and 35 m*M* Tris–HCl pH 7.5 for 4 *M* NaCl introduced crystals] and flash-frozen in liquid nitrogen. Diffraction data were collected at BL38B1 for native crystals and at BL41XU for 4 *M* NaCl introduced crystals at SPring-8 (Harima, Japan). The diffraction data were indexed and scaled with *HKL*2000 and *SCALEPACK* (Otwinowski & Minor, 1997). The statistics of data collection are summarized in Table 1.

The structures were solved with *MOLREP* (Vagin & Teplyakov, 1997) using protein atoms from PDB entry 8tln as the search model. The models were refined using *REFMAC*5 (Murshudov *et al.*, 1997) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) and model building was performed with *ARP*/*wARP* (Perrakis *et al.*, 2001) and *XtalView* (McRee, 1999).

3. Results and discussion

The crystals grew to dimensions of 0.05 × 0.05 × 0.5 mm in a few days in the absence of NaCl (Fig. 1). The crystals diffracted X-rays to

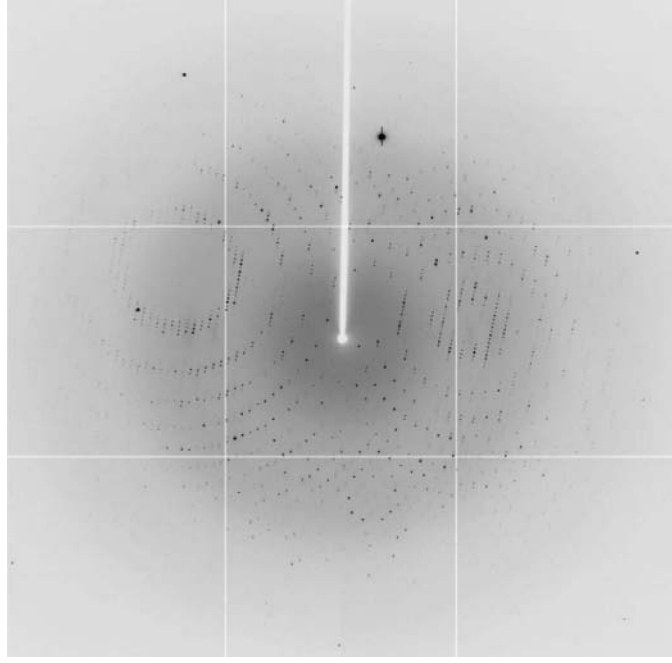

Figure 2
X-ray diffraction image from a thermolysin crystal in the presence of NaCl at high concentrations. The edge of the detector corresponds to a resolution of 1.81 Å.

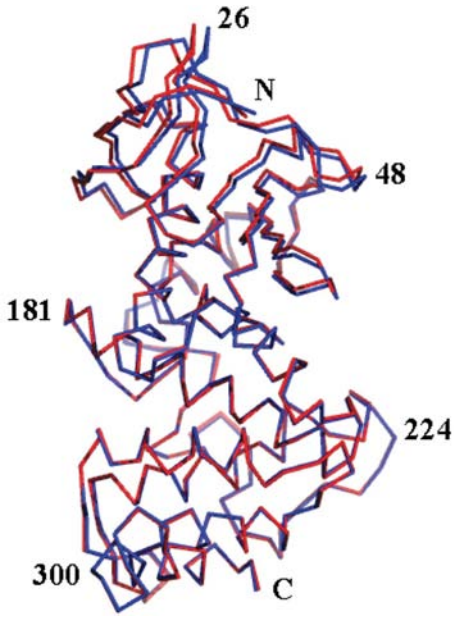

Figure 3
Superposition of the backbone structures of thermolysin in the absence (dark blue) and the presence of 4 *M* NaCl. The structures are superimposed based on the C-terminal domain (residues 78–90, 135–194 and 200–316; Holland *et al.*, 1992). The figure was prepared using *PyMOL* (DeLano, 2002). A colour version of this figure is available in the online edition of the journal.

1.79 Å. They belonged to space group $P6_122$ and the unit-cell parameters were $a = b = 92.62$, $c = 129.22$ Å. The crystal contained one molecule in an asymmetric unit, giving a crystal volume per protein weight (V_M) of 2.4 Å^3 Da^{-1} and a solvent content of 47.3% (Matthews, 1968).

Our initial attempt to introduce 4 *M* NaCl into the crystals obtained in the absence of NaCl by a direct soaking of 4 *M* NaCl failed due to changes in osmotic pressure as well as thermolysin solubility (Inouye *et al.*, 1998*a*,*b*). The crystals obtained by this method did not diffract X-rays. The solubility of thermolysin is maximal at 2.0–2.5 *M* NaCl, which is about ten times higher than that in the absence of NaCl. Thus, the crystal lattice has been disordered during the course of solubility increase and decrease by changing the NaCl concentration from 0 to 4 *M*.

We then tried to introduce 4 *M* NaCl into the crystals by mixing a droplet containing thermolysin crystals with solution *C* containing 4.8 *M* NaCl and saturated thermolysin. The crystals obtained by this method diffracted X-rays to 2.43 Å (Fig. 2). The space group was $P6_122$ and the unit-cell parameters were $a = b = 93.82$, $c = 141.63$ Å. Thus, no change was observed in the space group and the lengths of the *a* and *b* axes, but the *c* axis was elongated by 12.41 Å (9.6%) by introducing 4 *M* NaCl into the crystal (Table 1). This change in the length of *c* axis was reproducible.

The NaCl concentration changes more slowly in the latter method. The fast change in NaCl concentration caused by direct soaking attacked the crystal lattice and may have caused the deterioration of crystals, while the slower change of NaCl concentration affected the crystal lattice only a little and the crystals containing 4 *M* NaCl were able to diffract X-rays to 2.43 Å. We have solved crystal structures of thermolysin in the absence and in the presence of 4.0 *M* NaCl. The overall structures were quite similar with a root-mean-square (r.m.s.) deviation of 0.3 Å for C^α atoms. Fig. 3 shows the backbone trace of the two structures. When their C-terminal domains are superposed, their N-terminal domains are shifted slightly (up to ∼1.5 Å) due to a hinge-bending conformational change. The structure in the presence of 4 *M* NaCl has a more open conformation when compared with the structure in the absence of NaCl. This conformational change would be one of the reasons for the different length of the *c* axis.

In conclusion, we developed a new method to introduce 4 *M* NaCl into the $P6_122$ thermolysin crystal. The introduction of NaCl is supported by the change in the length of *c* axis of the unit cell and in the conformation of thermolysin. The detailed description of structural changes in the active site and the discussion on the molecular mechanism of salt-depended activation of thermolysin will be reported elsewhere.

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