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Crystallization and preliminary X-ray analyses of the active and the inactive forms of family GH-8 chitosanase with subclass II specificity from *Bacillus* sp. strain K17

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Chitosanase from *Bacillus* sp. strain K17 (ChoK) belongs to glycoside hydrolase family 8 and exhibits subclass II specificity. The purified protein is structurally stable over a wide pH range (3–10), but is active in a much narrower pH range (4.5–7.5), with optimal activity around pH 6.0. The protein has been successfully crystallized at two different pH values corresponding to the active and inactive states. The crystals diffract to 1.5 and 2.0 Å resolution, respectively.

1. Introduction

Chitosan is a β-1,4-poly-D-glucosamine that can be obtained by N-deacetylation of chitin, an abundant natural polysaccharide. Chitosan and its partially degraded oligosaccharides are useful materials for a wide range of medical, agricultural, chemical, textile and food applications. Chitosanase (EC 3.2.1.132) hydrolyzes the β-1,4-linkage of chitosan. This protein is used in the chitosan industry to facilitate the production of chitosans of certain lengths. Chitosanases from different sources are divided into three subclasses (I, II and III) according to their substrate specificities (Fukamizo *et al.*, 1994). Chitosanases of every subclass hydrolyze the β-1,4-linkage when both the -1 and +1 sites are occupied by glucosamine residues. However, the chitosanases in subclasses I and III are able to accept N-acetylated glucosamine at the -1 site and at the +1 site, respectively. The X-ray structures reported for the latter two subclasses are similar to each other (Marcotte *et al.*, 1996; Saito *et al.*, 1999). Recently, we purified a new type of chitosanase (ChoK) from *Bacillus* sp. strain K17 (Fukazawa *et al.*, 2001). This protein exhibits stricter specificity, belonging to subclass II, suggesting that it adopts a structure different from the other chitosanases.

According to the Henrissat's classification of glycoside hydrolases (CAZY; http://afmb.cnrs-mrs.fr/CAZY/index.html; Henrissat, 1991; Henrissat & Bairoch, 1993; Davies & Henrissat, 1995; Henrissat & Bairoch, 1996), ChoK belongs to family GH-8 (Yatsunami *et al.*, 2002). This strongly suggests that the tertiary structure of ChoK is different from those of the other two types of chitosanases, which belong to family GH-46 (Marcotte *et al.*, 1996; Saito *et al.*, 1999). The GH-8 family includes cellulases, xylanases and lichenases. Despite having a different substrate specificity to that of chitosanase, these proteins still catalyze hydrolysis of the β-1,4-linkage; for example, chitosanase hydrolyzes between glucosamine residues, cellulase between glucose residues, xylanase between xylose residues and lichenase hydrolyzes only the β-1,4-linkage of a linear β-glucan having mixed β-1,4- and β-1,3-linkages. Thus, the differing substrate specificities of these proteins from the GH-8 family suggest that diversification from a common tertiary structure may have occurred. Comparison of their detailed structures will provide a structural basis for understanding their specificities and reaction mechanisms, which will be useful for protein engineering.

In addition to the above two families, GH-8 and GH-46, chitosanases are also found in families GH-5 (Tanabe *et al.*, 2003), GH-75 (Shimosaka *et al.*, 1996; Cheng & Li, 2000; Zhang *et al.*, 2001) and GH-80 (Park *et al.*, 1999). Owing to this diversity in classification, it is difficult to understand the correlation between structure and function. Therefore, X-ray analysis of ChoK is necessary in order to reveal the tertiary structure of this example of a protein with a high specificity and in order to elucidate its reaction mechanism. In addition, structural comparisons of this protein with those of other glycoside hydrolases from family GH-8 will provide insights into the structural evolution and functional diversification of the family.

2. Materials and methods

After *Bacillus* sp. strain K17 had been cultivated aerobically at 303 K overnight, cells were removed by centrifugation at 7000*g* for 10 min to obtain the culture supernatant. 10 m*M* Tris–HCl buffer pH 7.5 solutions were used in the following steps of protein isolation. Proteins precipitated with 80% saturated ammonium sulfate were dissolved and the solution was dialyzed at 277 K and then applied onto a DEAE-Toyopeal 650M (Tosoh) column equi-

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librated with the same buffer. Bound proteins were eluted with a 0–1 *M* linear gradient of NaCl. In subsequent purification steps, 20 m*M* Tris–HCl buffer pH 7.5 was used. Pooled fractions exhibiting chitosanase activity were dialyzed against 20 m*M* Tris–HCl buffer pH 7.5 and applied onto a Mono Q (Amersham Biosciences) column. Proteins were eluted with the same NaCl linear gradient. Finally, the active fraction, after dialysis, was applied onto a Superose 12 (Amersham Biosciences) gel-filtration column. Protein concentration was measured by the Lowry method (Lowry *et al.*, 1951).

SDS–PAGE (Laemmli, 1970) was used to evaluate the purity of the protein. The isolated protein (ChoK) was incubated at 373 K for 3 min in the presence of 2%(*w*/*v*) SDS and 5%(*v*/*v*) 2-mercaptoethanol and then applied to a 12.5%(*w*/*v*) polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB).

A chitosanase assay was performed following the method of Sakihama *et al.* (2004). Chitosan 10B (Funakoshi) was used at 0.25%(*w*/*v*) concentration as a substrate. During reaction at 310 K for 10 min in 100 m*M* MOPS–Na_2CO_3 buffer pH 6.5, the amount of liberated reducing sugars was measured calorimetrically by the Rondle–Morgan method (Rondle & Morgan, 1955). To estimate the pH-dependence, the enzymatic activity was measured at different pH values at 310 K for 30 min. In order to evaluate the effect of pH on stability, the protein solutions were first incubated at high concentration at various pH values at 310 K for 30 min, after which the residual activities were measured at low concentration at pH 6.5 at 310 K for 10 min.

Crystals of ChoK were grown at pH 6.4 and 3.7 by the hanging-drop vapour-diffusion method at 277 K. Purified protein at 7 mg ml^{-1} concentration was used as the protein solution. Needle-shaped crystals (ChoK-*a*) with dimensions of 1.0 × 0.10 × 0.10 mm were obtained within one week in a droplet prepared by mixing 2 µl protein solution and 2 µl reservoir solution at pH 6.4 along with 0.1 *M* 1,4-piperazine-bis(2-ethanosulfonic acid), 13%(*w*/*v*) polyethylene glycol (PEG 4000) and 0.1 *M* ammonium sulfate.

Crystals (ChoK-*i*) obtained within one week from a droplet prepared by mixing 2 µl protein solution and 2 µl reservoir solution at pH 3.7 containing 0.1 *M* sodium citrate, 20%(*w*/*v*) PEG 4000 and 0.4 *M* ammonium sulfate are of needle shape and 1.2 × 0.12 × 0.12 mm in size. Several crystals of ChoK-*a* and ChoK-*i* suitable for X-ray experiments were mounted in nylon cryoloops (Hampton Research) with the reservoir solution containing 25%(*v*/*v*) glycerol and 15%(*v*/*v*) glycerol as a cryoprotectant and stored in liquid nitrogen.

X-ray data from ChoK-*a* and ChoK-*i* were measured at 100 K using synchrotron radiation (λ = 0.900 Å) at SPring-8 (BL44XU). Diffraction patterns were recorded on a 3 × 3 array CCD detector (PX210; Oxford Instruments Co.) positioned 153 mm away from the ChoK-*a* crystal and 193 mm away from the ChoK-*i* crystal. Diffraction data for both ChoK-*a* and ChoK-*i* were integrated from 1° oscillation images. Each frame was exposed for 10 s for a total oscillation range of 180°. The data for ChoK-*a* and ChoK-*i* were processed with *d*TREK* (Pflugrath, 1999) to yield final resolutions of 1.5 and 2.0 Å, respectively. The intensities were converted to structure-factor amplitudes using the program *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project Number 4, 1994).

3. Results and discussion

SDS–PAGE showed a single band, suggesting that the protein with chitosanase

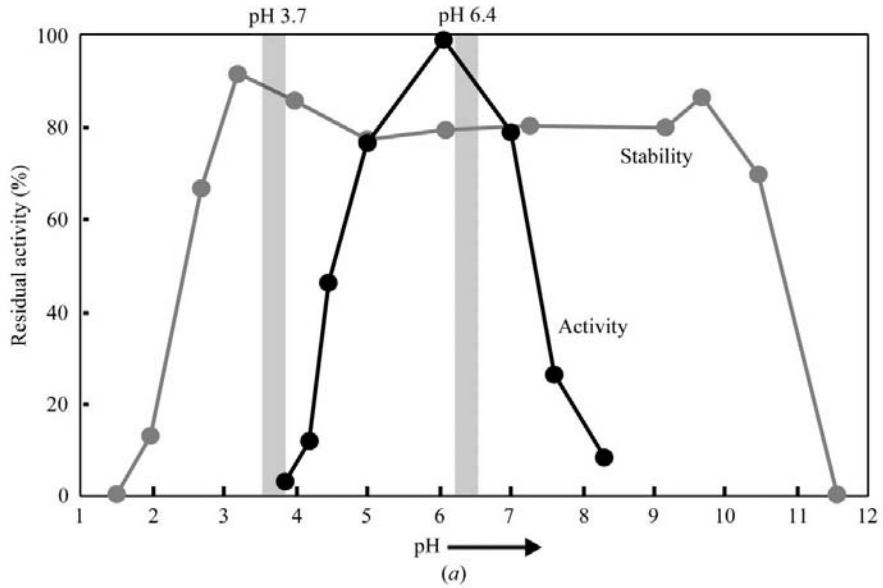

Figure 1
(*a*) Effects of pH on stability and activity of ChoK and (*b*) crystals obtained at pH 3.7 and at pH 6.4. Chitosan 10B (Funakoshi) was used at 0.25%(*w*/*v*) concentration as a substrate. Using the Rondle–Morgan method (Rondle & Morgan, 1955), the enzyme activities were evaluated at various pH values by measuring the amounts of liberated reducing sugars calorimetrically at 310 K for 10 min. The buffer solutions used were glycine–HCl (pH 2.0–3.0), acetate–sodium acetate (pH 3.0–6.0), MOPS–Na_2CO_3 (pH 6.0–8.0), $NaHCO_3$–Na_2CO_3 (pH 8.0–10.0) and Na_2HPO_4–NaOH (pH 10.0–12.0). To evaluate the stability at different pH values, the protein solutions were incubated in advance at the various pH values at 310 K for 30 min and the activities were then measured at pH 6.5.

Table 1
Crystal data and statistics of data collection.

Values in parentheses are for the outer shell.

Crystal	ChoK-*i*	ChoK-*a*
Crystal data		
Space group	*I*222	$P2_12_12_1$
Unit-cell parameters (Å)		
a	85.1	70.8
b	91.1	98.1
c	131.5	115.8
Data collection		
Resolution range (Å)	39.5–2.00 (2.07–2.00)	40.3–1.50 (1.55–1.50)
Observed reflections	252468	930840
Unique reflections	34863	128931
Average multiplicity	7.2	7.2
Completeness (%)	99.8 (100)	99.6 (99.9)
$R_{\rm merge}$ (%)	7.2 (27.5)	7.6 (27.8)
$I/\sigma(I)$	9.4 (3.0)	8.3 (2.4)
Z†	1	2

† Number of protein molecules in the asymmetric unit.

activity was highly purified. Fig. 1(*a*) shows the dependence of activity on pH and the effect of pH on stability. The two types of crystals obtained at different pH values are shown in Fig. 1(*b*). Crystal data and statistics of data collection of the two crystal forms are given in Table 1.

The two different crystal forms, ChoK-*a* and ChoK-*i*, were obtained at pH 6.4 and pH 3.7, respectively. ChoK-*a* crystals belong to space group $P2_12_12_1$. From the unit-cell parameters, a = 70.8, b = 98.1, c = 115.8 Å, the number of protein molecules in the asymmetric unit is estimated to be two, according to the Matthews criteria (Matthews, 1968). The crystal diffracts well to 1.5 Å resolution. The ChoK-*i* crystal belongs to space group *I*222, with unit-cell parameters a = 85.1, b = 91.1, c = 131.5 Å. In this case, there is only one molecule is in the asymmetric unit and the resolution is restricted to 2.0 Å.

As shown in Fig. 1(*a*), the purified protein is structurally stable over a wide range of pH values from 3 to 10, but is active in a much narrower pH range from 4.5 to 7.5, with optimal activity around pH 6.0. Crystals grown at pH 6.4 are close to the point of maximum activity, while at pH 3.7 activity is completely abolished. Therefore, the two crystal forms, ChoK-*a* and ChoK-*i*, obtained at the two pH values might represent structures in the active and inactive states, respectively. According to the proposed reaction mechanism of cellulase from *Clostridium thermocellum* (Alzari *et al.*, 1996; Guérin *et al.*, 2002), which also belongs to the GH-8 family, the catalytic residues are two acidic amino acids sensitive to pH. Therefore, the two structures of ChoK-*a* and ChoK-*i* probably differ in the conformations of some parts of the structure related to the activity. As the two crystal forms diffract to reasonably high resolution, it is expected that their structures will not only suggest the reaction mechanism, but will also provide some structural reasons for the difference in substrate specificity on comparison with structures reported for the subclass I and III chitosanases (Marcotte *et al.*, 1996; Saito *et al.*, 1999).

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