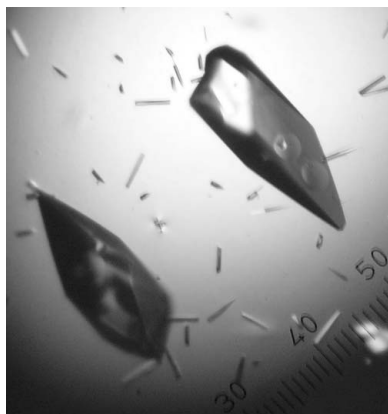# crystallization communications

Crystallization and preliminary X-ray analysis of *Thermoactinomyces vulgaris* R-47 maltooligosaccharide-metabolizing enzyme homologous to glucoamylase

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A maltooligosaccharide-metabolizing enzyme from *Thermoactinomyces vulgaris* R-47 (TGA) homologous to glucoamylase degrades maltooligosaccharides more efficiently than starch, unlike fungal glucoamylases. TGA was crystallized and the state of the protein in solution was analyzed by gel-filtration chromatography. Diffraction data were collected to 3.31 Å resolution. The TGA crystal belongs to the orthorhombic space group $P2_12_12_1$ or $P2_12_12$, with unit-cell parameters $a = 110.2$, $b = 317.6$, $c = 144.9$ Å, and is expected to contain five to eight TGA molecules per asymmetric unit. Gel-filtration and native PAGE analyses indicated that TGA exists as a dimer in solution. This is the first report of the crystallization of an oligomeric glucoamylase.

1. Introduction

Glucoamylase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3) is an exohydrolase that releases β-D-glucose from the non-reducing ends of starch and related oligosaccharides and polysaccharides. Glucoamylase has been extensively used in starch-processing industries and because of its commercial importance (Reilly, 1999) numerous fungal glucoamylases have been studied. Most fungal glucoamylases have a starch-binding domain in addition to a catalytic domain and prefer starch to maltooligosaccharides (Coutinho & Reilly, 1997; Sauer *et al.*, 2000). Recently, glucoamylases have been found from bacteria and archaea and their substrate specificities reportedly differ from those of fungal glucoamylases. *Thermoanaerobacterium thermosaccharolyticum* glucoamylase shows almost equal levels of activity for maltotetraose and maltoheptaose, as well as starch (Ganghofner *et al.*, 1998). Our previous reports indicated that two glucoamylases from the thermophilic actinomycete *Thermoactinomyces vulgaris* R-47 (TGA) and the methanogenic archaeon *Methanococcus jannaschii* degraded maltooligosaccharides more efficiently than starch (Uotsu-Tomita *et al.*, 2001; Ichikawa *et al.*, 2004).

In the CAZy classification (http://afmb.cnrs-mrs.fr/CAZY/) based on amino-acid sequences, glucoamylases are classified into glycoside hydrolase (GH) family 15, whose members have $(\alpha/\alpha)_6$-barrel catalytic domains (Aleshin *et al.*, 1992, 2003; Ševčík *et al.*, 1998; Mizuno *et al.*, 2004). Of the bacterial and archaeal glucoamylases, only the crystal structure of *T. thermosaccharolyticum* glucoamylase has been reported (Aleshin *et al.*, 2003). The enzyme consists of not only an $(\alpha/\alpha)_6$-barrel catalytic domain but also an N-terminal domain (β-domain) and a helical linker which bacterial, but not fungal, glucoamylases possess. Based on the sequence alignment of TGA and other glucoamylases, TGA is expected to have an N-terminal domain in addition to a catalytic domain like *T. thermosaccharolyticum* glucoamylase, whereas the N-terminal domain of TGA may not be similar to that of *T. thermosaccharolyticum* glucoamylase because of the low identity (12%). The primary structure of TGA is closer to that of three archaeal proteins, *M. jannaschii* glucoamylase (27% identity), *Methanosarcina acetivorans* C2A putative glucoamylase (MA4050, 30%) and *Methanosarcina mazei* Goe1 putative glucoamylase (MM0864, 29%), rather than those of other bacterial glucoamylases such as *T. thermosaccharolyticum* glucoamylase (17%). Thus, the three-dimensional structure of TGA provides insight into the evolution of and the difference in substrate specifi-

cities between glucoamylases. Here, we report the crystallization and gel-filtration analysis of TGA.

2. Material and methods

2.1. Purification, crystallization and data collection

TGA was produced as described previously (Ichikawa *et al.*, 2004). The previous purification method of TGA was changed to the following as the enzyme is stable at 328 K for 30 min. The crude TGA solution was incubated at 328 K for 30 min to denature other proteins and after Q-Sepharose HP chromatography the Sephacryl S-200 HR chromatography step was omitted. Purified TGA was concentrated using Centricon Plus-20 (Millipore) for crystallization and the protein concentration was determined by the measurement of absorbance at 280 nm using the formula of Gill & von Hippel (1989).

TGA crystals were grown by the hanging-drop vapour-diffusion method at 293 K. 1.5 µl TGA solution (20 mg ml^{-1}) was mixed with 1.5 µl crystallization reservoir solution containing 10%(w/w) poly-ethylene glycol 5000 monomethyl ether, 186 mM glucose, 10 mM dithiothreitol and 1.0 M lithium chloride in 100 mM MES pH 6.1. To perform data collection at cryogenic temperature, the crystal was transferred to a cryoprotectant solution containing 20%(w/v) poly-ethylene glycol 6000, 20%(v/v) 2-methyl-2,4-pentanediol and 2.5 mM CaCl$_2$ in 40 mM MES pH 6.1. Diffraction data were collected at the PF-AR NW-12 beamline (Tsukuba, Japan) and the data set was processed with the program *HKL*2000 (Otwinowski & Minor, 1997).

2.2. Gel-filtration chromatography

Gel-filtration chromatography was carried out using a TSKgel G3000SW (Tosoh). The buffer solution of concentrated TGA solution was substituted with 0.2 M sodium phosphate buffer pH 7.0 using a Centricon Plus-20 (Millipore). Standard proteins (thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; albumin, 67.0 kDa; ovalbumin, 43.0 kDa; ribonuclease A, 13.7 kDa; Amersham Bioscience) were used. 20 µl TGA (0.5 mg ml^{-1}) and 20 µl standard proteins (0.5–1.0 mg ml^{-1}) were applied onto a TSKgel G3000SW column buffered with a 0.2 M sodium phosphate buffer pH 7.0 and eluted with the same buffer at a flow rate of 1.0 ml min^{-1}.

2.3. Native and SDS–PAGE

Native PAGE analysis was performed according to the method of Ornstein & Davis (1964). Thyroglobulin (669 kDa), ferritin (443 kDa), lactate dehydrogenase (140 kDa), albumin (66.3 kDa) and trypsin inhibitor (20.1 kDa; Daiichi Pure Chemicals Co. Ltd) were used as molecular-weight standards. Native TGA and the molecular-weight standards were loaded onto a gradient gel (5–20%, Wako Pure Chemical Industries Ltd).

SDS–PAGE was carried out using 10%(w/v) gel. Phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa) and carbonic anhydrase (30.0 kDa) were used as molecular-weight standards for SDS–PAGE. These protein bands were visualized with Coomassie Brilliant Blue R-250.

3. Results and discussion

TGA crystals reaching dimensions of 0.5 × 0.2 × 0.2 mm grew within 4 d (Fig. 1). Data-collection statistics are summarized in Table 1. The TGA crystal belongs to the orthorhombic space group $P2_12_12_1$ or $P2_12_12$, with unit-cell parameters a = 110.2, b = 317.6, c = 144.9 Å and unit-cell volume 5.0×10^6 Å^3. Assuming the crystal to contain five to

Table 1
Data-collection statistics.

Values for the highest resolution shell (3.43–3.31 Å) are given in parentheses.

Temperature (K)	100
Resolution (Å)	3.31
No. of measured reflections	520921
No. of unique reflections	76469
Completeness (%)	99.7 (98.6)
R_{merge}†	0.099 (0.287)
$I_0/\sigma(I_0)$	25.7 (5.1)
Space group	$P2_12_12_1$ or $P2_12_12$
Unit-cell parameters	
a (Å)	110.2
b (Å)	317.6
c (Å)	144.9
$\alpha = \beta = \gamma$ (°)	90

† $R_{\mathrm{merge}} = \sum \sum |I_i - \langle I \rangle| / \sum \langle I \rangle$.

eight TGA molecules per asymmetric unit, the solvent content is calculated to be 42–64% ($V_{\mathrm{M}} = 2.1$–3.4 Å^3 Da^{-1}) using the program *MATTHEWS_COEFF* from the *CCP*4 software suite (Collaborative Computational Project, Number 4, 1994).

Next, gel-filtration chromatography was performed using a TSKgel G3000SW column to analyze the oligomerization state of TGA in solution. The molecular weight of native TGA was estimated to be 155 kDa, suggesting that this enzyme exists as a dimer (Fig. 2a). Native PAGE analysis was also carried out. Generally, the mobility of protein in native PAGE depends on the pI value of protein. The pI values of thyroglobulin, ferritin, lactate dehydrogenase, albumin and trypsin inhibitor used in this experiment were 5.1, 4.2, 4.5, 5.1 and 4.5, respectively. The pI value of 5.7 for TGA calculated from the amino-acid sequence was close to those of the molecular-weight standards, which allowed us to determine the molecular weight of native TGA using these standards with native PAGE analysis. The result also supported the idea that TGA exists as a dimer because the molecular weight of native TGA was calculated as 150 kDa (Figs. 2b, 2c and 2d). These findings indicated that native TGA exists as a dimer in solution.

A glucoamylase from *T. thermosaccharolyticum* has been reported to be present as a monomer by gel-filtration and sucrose-gradient centrifugation analyses (Specka *et al.*, 1991). In contrast, a glucoamylase from the hyperthermophilic archaeon *Sulfolobus solfataricus*

Figure 1
TGA crystals.

0.25 mm
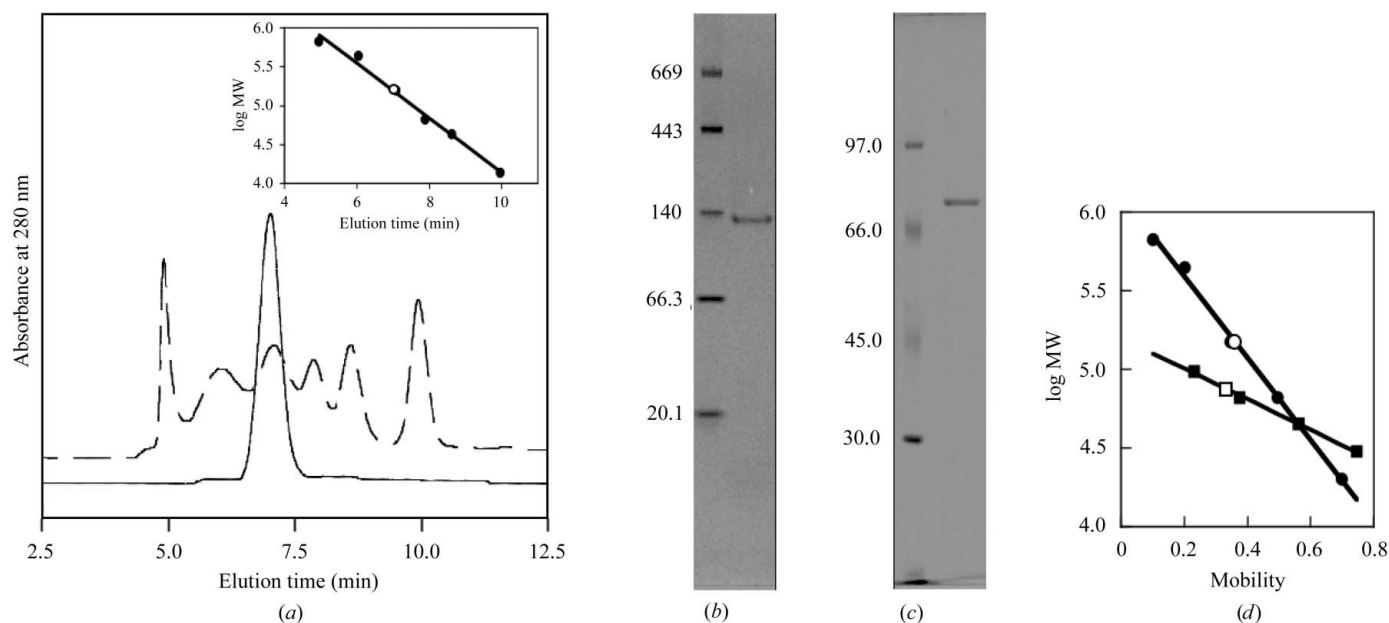
Figure 2
Gel filtration, native PAGE and SDS–PAGE analyses of TGA. (*a*) Gel-filtration chromatography. Molecular-weight standards (dotted line) and TGA (unbroken line) were eluted with 0.2 *M* sodium phosphate buffer pH 7.0 through a TSKgel G3000SW (Tosoh). The inset shows a plot of elution time *versus* logarithm of molecular weight; (*b*) native-PAGE; (*c*) SDS–PAGE; (*d*) plots of mobility *versus* logarithm of molecular weight obtained from native PAGE (circles) and SDS–PAGE (squares).

has been reported to exist as a tetramer by gel-filtration and sedimentation-equilibrium analytical ultracentrifugation analyses (Kim *et al.*, 2004). Comparing the substrate-specificities and the states in solution of these glucoamylases, *S. solfataricus* glucoamylase and TGA most effectively hydrolyze maltotriose but hardly affect starch and exist in an oligomeric state in solution, while *T. thermosaccharolyticum* glucoamylase showed almost equal levels of activity for starch, maltotetraose and maltoheptaose and was present as a monomer in solution. *Lactobacillus brevis* maltose phosphorylase, classified into GH family 65, is structurally very close to *T. thermosaccharolyticum* glucoamylase (Egloff *et al.*, 2001) and forms dimer in the crystal. This maltose phosphorylase is an inverting-type enzyme that exists as a dimer in solution, like TGA (Hüwel *et al.*, 1997). These findings raise the possibility that the oligomerization state of glucoamylases correlates with the substrate specificities.

In conclusion, TGA has been crystallized and shown to exist as a dimer in solution; this is the first report of the crystallization of an oligomeric glucoamylase.

References

Aleshin, A. E., Feng, P. H., Honzatko, R. B. & Reilly, P. J. (2003). *J. Mol. Biol.* **327**, 61–73.

Aleshin, A., Golubev, A., Firsov, L. M. & Honzatko, R. B. (1992). *J. Biol. Chem.* **267**, 19291–19298.

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.

Coutinho, P. M. & Reilly, P. J. (1997). *Proteins*, **29**, 334–347.

Egloff, M. P., Uppenberg, J., Haalck, L. & van Tilbeurgh, H. (2001). *Structure*, **9**, 689–697.

Ganghofner, D., Kellermann, J., Staudenbauer, W. L. & Bronnenmeier, K. (1998). *Biosci. Biotechnol. Biochem.* **62**, 302–308.

Gill, S. C. & von Hippel, P. H. (1989). *Anal. Biochem.* **182**, 319–326.

Hüwel, S., Haalck, L., Conrath, N. & Spener, F. (1997). *Enzyme Microb. Technol.* **21**, 413–420.

Ichikawa, K., Tonozuka, T., Uotsu-Tomita, R., Akeboshi, H., Nishikawa, A. & Sakano, Y. (2004). *Biosci. Biotechnol. Biochem.* **68**, 413–420.

Kim, M. S., Park, J. T., Kim, Y. W., Lee, H. S., Nyawira, R., Shin, H. S., Park, C. S., Yoo, S. H., Kim, Y. R., Moon, T. W. & Park, K. H. (2004). *Appl. Environ. Microbiol.* **70**, 3933–3940.

Mizuno, M., Tonozuka, T., Suzuki, S., Uotsu-Tomita, R., Kamitori, S., Nishikawa, A. & Sakano, Y. (2004). *J. Biol. Chem.* **279**, 10575–10583.

Ornstein, L. & Davis, B. J. (1964). *Ann. N. Y. Acad. Sci.* **121**, 321.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Reilly, P. J. (1999). *Starch/Stärke*, **51**, 269–274.

Sauer, J., Sigurskjold, B. W., Christensen, U., Frandsen, T. P., Mirgorodskaya, E., Harrison, M., Roepstorff, P. & Svensson, B. (2000). *Biochim. Biophys. Acta*, **1543**, 275–293.

Ševčík, J., Solovicová, A., Hostinová, E., Gasperík, J., Wilson, K. S. & Dauter, Z. (1998). *Acta Cryst.* D**54**, 854–866.

Specka, U., Mayer, F. & Antranikian, G. (1991). *Appl. Environ. Microbiol.* **57**, 2317–2323.

Uotsu-Tomita, R., Tonozuka, T., Sakai, H. & Sakano, Y. (2001). *Appl. Microbiol. Biotechnol.* **56**, 465–473.