

Nozomu Okino,^a Yoshimitsu Kakuta,^{a*} Hitomi Kajiwara,^b Masako Ichikawa,^b Yoshimitsu Takakura,^b Makoto Ito^a and Takeshi Yamamoto^{b*}

^aDepartment of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan, and

^bGlycotechnology Business Unit, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan

Correspondence e-mail:
kakuta@agr.kyushu-u.ac.jp,
takeshi.yamamoto@ims.jti.co.jp

Received 25 May 2007
Accepted 27 June 2007

Purification, crystallization and preliminary crystallographic characterization of the α 2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224

Sialyltransferases transfer sialic acid from cytidine-5-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) to the nonreducing termini of the oligosaccharyl structures of various glycoproteins and glycolipids. The newly cloned α 2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224 (from the *Vibrionaceae* family) is composed of two domains: an unknown N-terminal domain and a catalytic C-terminal domain which shares significant homology with the *Pasteurella multocida* multifunctional sialyltransferase. The putative mature form of JT-ISH-224 α 2,6-sialyltransferase was overproduced in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method at 293 K. The crystal belonged to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 90.29$, $c = 204.33$ Å. X-ray diffraction data were collected to 2.5 Å resolution.

1. Introduction

Sialic acids are of great interest for the development of pharmaceuticals and other applications because sialylated molecules play important roles in cell–cell interactions, cell differentiation and various receptor–ligand interactions (Schauer, 2000; Angata & Varki, 2002). In some mucosal pathogens, the sialylation of lipopolysaccharides is a widely conserved structural modification and plays an important role in resistance to the killing effects of normal human serum (Vimr & Lichtensteiger, 2002; Vimr *et al.*, 2004). Furthermore, it has been demonstrated that sialyltransferase (STase) activity is crucial for bacterial pathogenicity. In general, bacterial STases are also important for the enzymatic modification of glycoconjugates and the enzymatic synthesis of glycans because they are more stable and productive than mammalian enzymes. Since α 2,3-STase was first cloned from *Neisseria meningitidis* and *N. gonorrhoeae* (Gilbert *et al.*, 1996), bacterial STases have been cloned from several microorganisms (Gilbert *et al.*, 2000; Yamamoto *et al.*, 1998; Bozue *et al.*, 1999; Shen *et al.*, 1999). To date, the crystal structures of three bacterial STases have been reported: the bifunctional STase CstII from *Campylobacter jejuni* (Chiu *et al.*, 2004), the α 2,3-STase CstI from *C. jejuni* (Chiu *et al.*, 2007) and the multifunctional STase Δ 24PmST1 from *Pasteurella multocida* (Ni *et al.*, 2006, 2007). Very recently, α 2,6-STase from *Photobacterium* sp. JT-ISH-224 (ISH224 α 2,6-STase) has been cloned in our laboratory (Tsukamoto *et al.*, 2007). The deduced amino-acid sequence of this enzyme shows 35.6% identity to the multifunctional STase from *P. multocida*, but no significant homology to the bifunctional STase from *C. jejuni*. Interestingly, the STases from *C. jejuni* and *P. multocida* have bifunctional (α 2,3/ α 2,8-STase) and multifunctional (α 2,3/ α 2,6-STase, α 2,3 sialidase and α 2,3-trans-sialidase) activities, respectively, but ISH224 α 2,6-STase shows only α 2,6-STase activity. Therefore, it is of great interest to elucidate the molecular structure of this enzyme in order to help understand its catalytic mechanism and α 2,6 reaction specificity. In this study, we report the preparation, crystallization and preliminary X-ray analysis of α 2,6-STase from *Photobacterium* sp. JT-ISH-224.



2. Materials and methods

2.1. Preparation of the expression plasmid and recombinant enzyme

DNA encoding α 2,6-STase (Δ 2–17, lacking the N-terminal hydrophobic region) from *Photobacterium* sp. JT-ISH-224 was inserted into pTrc99A (Pharmacia; Tsukamoto *et al.*, 2007). The resulting expression plasmid was transformed into *Escherichia coli* TB1 competent cells. The transformant was cultured in 6 ml LB medium containing 100 μ g ml⁻¹ ampicillin at 303 K for 8 h. The culture was transferred into 300 ml LB medium containing 100 μ g ml⁻¹ ampicillin and 1 mM IPTG and cultured at 303 K for 16 h. The cells were harvested by centrifugation and suspended in buffer A (20 mM bis-Tris buffer pH 6.0 containing 0.336% Triton X-100) and disrupted by sonication. The cell debris was removed by centrifugation (100 000g for 1 h) and the supernatant was filtered through a 0.45 μ m cellulose acetate membrane. The obtained crude enzyme solution was loaded onto a HiLoad 26/10 Q-Sepharose HP column (GE Healthcare). The adsorbed protein was washed with buffer A and eluted with a linear gradient of buffer A with an increasing concentration of NaCl up to 1 M. The pooled fractions showing α 2,6-STase activity were diluted with four volumes of buffer B (20 mM potassium phosphate buffer pH 6.0 containing 0.336% Triton X-100) and applied onto a CHT20-I column (Bio-Rad). The adsorbed protein was washed with the same buffer and eluted with a linear gradient of buffer B with an increasing concentration of potassium phosphate up to 0.5 M. The active fractions were pooled and applied onto a Mono Q 10/100 GL column (GE Healthcare) equilibrated with buffer A. The enzyme was eluted with an increasing NaCl gradient from 0 to 1 M in buffer A. Finally, the active fractions were pooled and applied onto a Superdex 200 HR column (GE Healthcare) equilibrated with 25 mM MES buffer pH 6.0 containing 100 mM NaCl. The eluted α 2,6-STase protein was concentrated to 10 mg ml⁻¹ for crystallization. The protein determination was performed using a protein assay (Bio-Rad) with bovine serum albumin as a standard. The activity of α 2,6-STase was measured according to the method of Yamamoto *et al.* (1996).

2.2. Crystallization and data collection

Initial crystallization trials were carried out by the hanging-drop vapour-diffusion method in a 24-well plate at 293 K using the Crystal

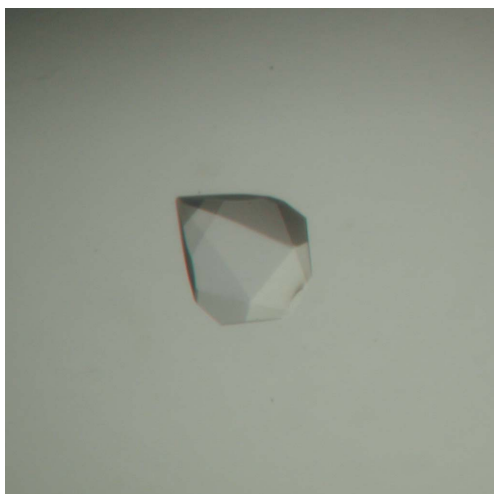


Figure 1

A crystal of ISH224 α 2,6-STase obtained using the hanging-drop vapour-diffusion method. The approximate dimensions of the crystal are 0.3 \times 0.3 \times 0.3 mm.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell (2.59–2.50 Å).

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 90.29$, $c = 204.33$
Beamline	BL38B1, SPring-8
Wavelength (Å)	1.000
Resolution range (Å)	50.0–2.5
No. of observed reflections	184556
No. of unique reflections	34177
Redundancy	5.4 (5.5)
R_{sym}^\dagger	0.082 (0.432)
$I/\sigma(I)$	20.0 (3.78)
Completeness (%)	99.8 (100.0)

$^\dagger R_{\text{sym}} = \sum I - \langle I \rangle / \sum \langle I \rangle$, where I is the intensity measurement for a given reflection and $\langle I \rangle$ is the average intensity for multiple measurements of this reflection.

Screen, Crystal Screen 2 and Index Screen kits (Hampton Research). Each drop was prepared by mixing 1 μ l protein solution (10 mg ml⁻¹) containing 10 mM cytidine monophosphate (CMP) and 10 mM lactose and an equal volume of reservoir solution. After 5 d, a small crystal of α 2,6-STase was observed using a reservoir condition consisting of 0.2 M lithium sulfate monohydrate, 0.1 M Tris-HCl pH 8.5 and 30% (w/v) PEG 4000 (Fig. 1). This result was found to be reproducible. Under these conditions, crystals grew within 10 d to a sufficient size for data collection. Prior to data collection, the crystal was transferred stepwise into cryoprotectant solution consisting of the crystallization condition containing 10% glycerol and flashed-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected using synchrotron radiation (1.000 Å wavelength) with a Jupiter 210 detector (Rigaku/MSC Corporation) at beamline BL38B1 of SPring-8 (Hyogo, Japan). The oscillation angle was 1.0 $^\circ$ and the exposure time was 10.0 s per frame. A total of 180 diffraction images were collected at a camera distance of 150 mm and were processed using *HKL-2000* (Otwinowski, 1993).

3. Results and discussion

The putative mature form of ISH224 α 2,6-STase was expressed in *E. coli* and purified by a combination of chromatographies. The final yield of the pure protein was approximately 24 mg from 300 ml culture, corresponding to 21% recovery. The molecular weight of the α 2,6-STase was determined to be 55 kDa by SDS-PAGE and 59 kDa by gel filtration, suggesting that α 2,6-STase exists as a monomer in solution.

A crystal of ISH224 α 2,6-STase in a complex with CMP and lactose diffracted to 2.5 Å and belongs to the hexagonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 90.29$, $c = 204.33$ Å . The data statistics are summarized in Table 1. We have attempted molecular-replacement methods for phase determination, but have not succeeded. The crystal structure is now being solved by the MAD method with selenium as the anomalous scattering atom using synchrotron radiation.

Recently, Ni and coworkers reported the crystal structure of the multifunctional STase Δ 24PmST1 from *P. multocida* (Ni *et al.*, 2006, 2007). Since ISH224 α 2,6-STase shows only α 2,6-STase activity, structural analysis of the α 2,6-STase would explain the functional difference between Δ 24PmST1 and ISH224 α 2,6-STase. Furthermore, from primary sequence analysis of ISH224 α 2,6-STase, it was revealed that the enzyme is composed of an unknown N-terminal domain and a catalytic C-terminal domain. Structural information on the α 2,6-STase will also provide new information about the function of the N-terminal domain.

We thank Dr Kaori Kojo for her technical assistance in preparation of the ISH224 α 2,6-STase. This study was supported in part by a grant from the National Project on Protein Structural and Functional Analyses from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Angata, T. & Varki, A. (2002). *Chem. Rev.* **102**, 439–469.
- Bozue, J. A., Tullius, M. V., Wang, J., Gibson, B. W. & Munson, R. S. Jr (1999). *J. Biol. Chem.* **274**, 4106–4114.
- Chiu, C. P., Lairson, L. L., Gilbert, M., Wakarchuk, W. W., Withers, S. G. & Strynadka, N. C. (2007). *Biochemistry*, **46**, 7196–7204.
- Chiu, C. P., Watts, A. G., Lairson, L. L., Gilbert, M., Lim, D., Wakarchuk, W. W., Withers, S. G. & Strynadka, N. C. (2004). *Nature Struct. Mol. Biol.* **11**, 163–170.
- Gilbert, M., Brisson, J. R., Karwaski, M. F., Michniewicz, J., Cunningham, A. M., Wu, Y., Young, N. M. & Wakarchuk, W. W. (2000). *J. Biol. Chem.* **275**, 3896–3906.
- Gilbert, M., Watson, D. C., Cunningham, A. M., Jennings, M. P., Young, N. M. & Wakarchuk, W. W. (1996). *J. Biol. Chem.* **271**, 28271–28276.
- Ni, L., Chokhawala, H. A., Cao, H., Henning, R., Ng, L., Huang, S., Yu, H., Chen, X. & Fisher, A. J. (2007). *Biochemistry*, **46**, 6288–6298.
- Ni, L., Sun, M., Yu, H., Chokhawala, H., Chen, X. & Fisher, A. J. (2006). *Biochemistry*, **45**, 2139–2148.
- Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend: Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 55–62. Warrington: Daresbury Laboratory.
- Schauer, R. (2000). *Glycoconj. J.* **17**, 485–499.
- Shen, G. J., Datta, A. K., Izumi, M., Koeller, K. M. & Wong, C. H. (1999). *J. Biol. Chem.* **274**, 35139–35146.
- Tsukamoto, H., Takakura, Y., Mine, T. & Yamamoto, T. (2007). Submitted.
- Vimr, E. R., Kalivoda, K. A., Deszo, E. L. & Steenbergen, S. M. (2004). *Microbiol. Mol. Biol. Rev.* **68**, 132–153.
- Vimr, E. & Lichtensteiger, C. (2002). *Trends Microbiol.* **10**, 254–257.
- Yamamoto, T., Nakashizuka, M., Kodama, H., Kajihara, Y. & Terada, I. (1996). *J. Biochem. (Tokyo)*, **120**, 104–110.
- Yamamoto, T., Nakashizuka, M. & Terada, I. (1998). *J. Biochem. (Tokyo)*, **123**, 94–100.