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Crystallization and preliminary X-ray analysis of a novel unsaturated glucuronyl hydrolase from *Bacillus* sp. GL1

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Unsaturated glucuronyl hydrolase from *Bacillus* sp. GL1 catalyzes the hydrolytic release of unsaturated glucuronic acids from oligosaccharides produced by the reactions of polysaccharide lyases such as gellan, xanthan, hyaluronate and chondroitin lyases. The enzyme was crystallized at 293 K from a droplet containing 56% MPD, 0.1 *M* NaCl, 0.1 *M* glycine–NaOH pH 8.2 and 0.1 *M* dithiothreitol using the vapour-diffusion method. The crystals were hexagonal and belonged to space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 102.8$, $c = 223.4$ Å. Diffraction data to 2.4 Å were collected from a single crystal.

1. Introduction

A large number of microorganisms have been reported to assimilate polysaccharides by means of polysaccharide lyases (Sutherland, 1995). Almost all polysaccharide lyases recognize uronic acid residues in polysaccharides and produce unsaturated saccharides with C=C double bonds at the non-reducing terminal uronate residues through a β-elimination reaction. Although the properties of polysaccharide lyases have been well documented (Sutherland, 1995; Jedrzejas, 2000; Herron *et al.*, 2000; Wong *et al.*, 2000), there is little information on the enzymes that act on the unsaturated saccharides produced by the reactions of polysaccharide lyases.

Recently, two types of enzymes that degrade unsaturated saccharides were found: lyases and hydrolases. Lyases for oligogalacturonate (Ogl; Shevchik *et al.*, 1999) and oligoalginate (Oal; Hashimoto *et al.*, 2000) are produced by *Erwinia chrysanthemi* and *Sphingomonas* sp. A1, respectively, and attack pectin and alginate oligosaccharides produced through the reactions of pectin and alginate lyases, respectively. On the other hand, the unsaturated glucuronyl hydrolase produced by *Bacillus* sp. GL1 specifically hydrolyzes a glycosidic bond in an unsaturated saccharide between the unsaturated glucuronyl residue at the non-reducing terminus and the saccharide linked to the residue (Hashimoto *et al.*, 1999) (Fig. 1). The released unsaturated saccharide is non-enzymatically converted to an α-keto acid. Since the unsaturated glucuronyl hydrolase is active towards various unsaturated saccharides produced by the reactions of lyases on gellan, xanthan, hyaluronate and chondroitin, it is considered to play an important role in the metabolism of unsaturated oligosaccharides produced by the reactions of polysaccharide lyases in organisms capable of assimilating polysaccharides. For example, *Streptococcus pyogenes* and *S. pneumoniae* have an unsaturated glucuronyl hydrolase gene (Mori *et al.*, 2003). Streptococcal cells cause severe infectious diseases (*e.g.* pneumonia, bacteremia, sinusitis and meningitis) and excrete a hyaluronate lyase as a virulent factor involved in the degradation of glycosaminoglycans (*e.g.* hyaluronan, chondroitin and heparin), which are major components of proteoglycan in the extracellular matrix of human connective tissues (Jedrzejas, 2001). The unsaturated glucuronyl hydrolase may be essential for the assimilation of hyaluronan degradation products. Therefore, structural analysis of the unsaturated glucuronyl hydrolase and polysaccharide lyases is thought to be important for clarification of the sequential reaction mechanisms in polysaccharide depolymerization and for the molecular design of drugs useful for the treatment of infectious streptococcal diseases.

This article deals with crystallization and preliminary X-ray crystallographic analysis of an unsaturated glucuronyl hydrolase which is present at an important position in the polysaccharide lyase metabolic pathway.

2. Methods and results

2.1. Analytical methods

The unsaturated glucuronyl hydrolase was assayed as described previously (Hashimoto *et al.*, 1999). Briefly, the enzyme was incubated at 303 K in 1 ml of a reaction mixture comprising 0.005% gellan tetrasaccharide (ΔGlcA-Glc-Rha-Glc) and 50 m*M* potassium phosphate buffer (KPB) pH 7.0; its activity was then determined by monitoring the decrease in the absorbance at 235 nm arising from the double

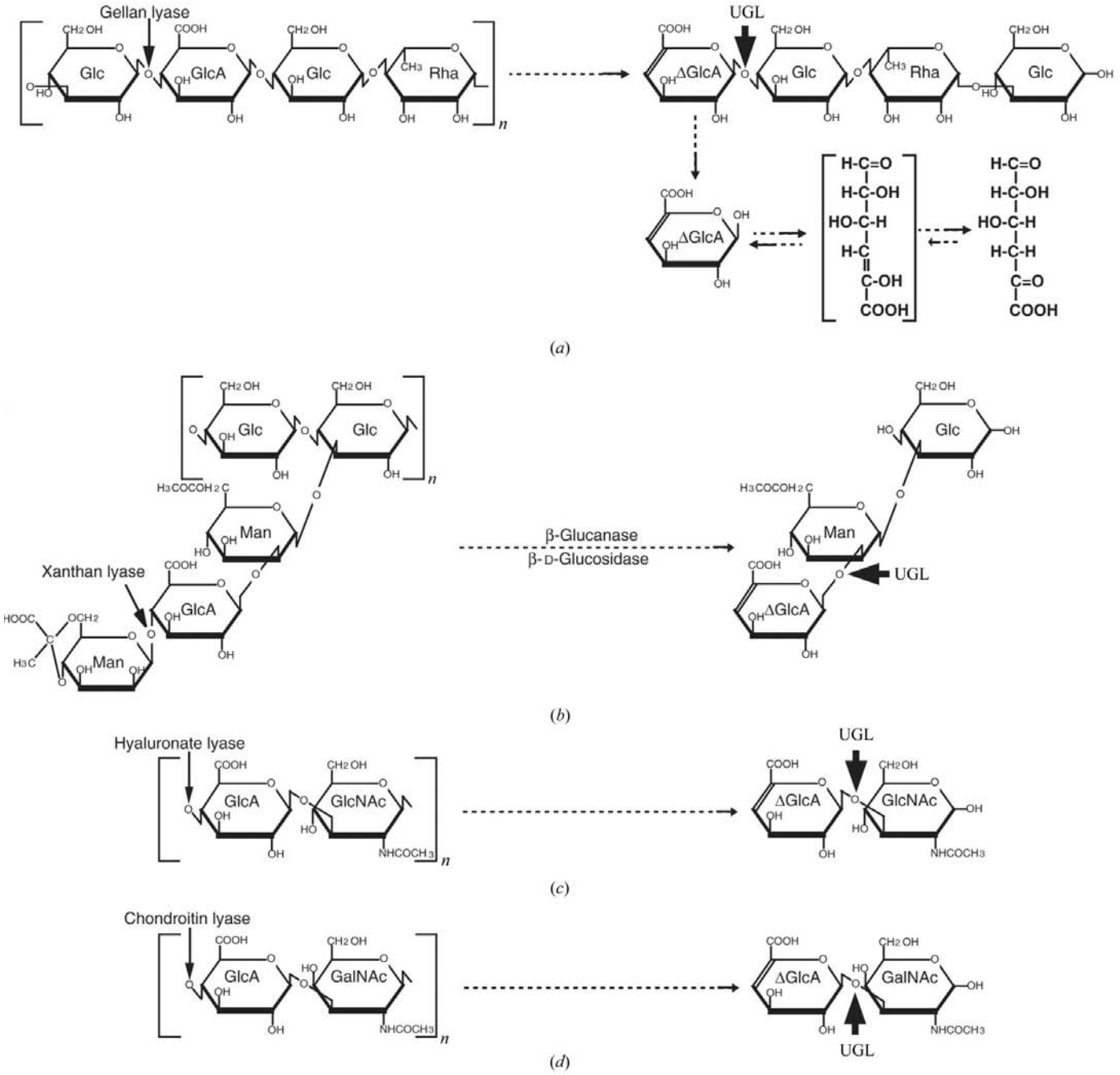

Figure 1
Structures of polysaccharides (left) and unsaturated oligosaccharides (right): (*a*) gellan, (*b*) xanthan, (*c*) hyaluronate, (*d*) chondroitin. Thin and thick arrows indicate the cleavage sites for polysaccharide lyases and the unsaturated glucuronyl hydrolase (UGL), respectively; dotted arrows represent the depolymerization of polysaccharides.

bond in the tetrasaccharide. One unit of enzyme activity was defined as the amount of enzyme required to produce a decrease of 1.0 in the absorbance at 235 nm per min. The protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, or by measuring the absorbance at 280 nm assuming that E_{280} = 1.0 corresponds to 1 mg ml^{-1} protein. The purity of the enzyme was confirmed by SDS–PAGE (Laemmli, 1970) and native gradient PAGE (Andersson *et al.*, 1972).

2.2. Protein expression and purification

The enzyme was purified from recombinant *Escherichia coli* cells as described in Mori *et al.* (2003). Unless specified otherwise, all operations were carried out at 273–277 K. *E. coli* cells harbouring a plasmid (pET3a-UGL) were grown in 15 l of LB medium (1.5 l per flask), collected by centrifugation at 6000*g* and 277 K for 5 min, washed with 20 m*M* KPB pH 7.0 and then resuspended in the same buffer. The cells were disrupted ultrasonically (Insonator Model 201M, Kubota, Tokyo, Japan) at 273 K and 9 kHz for 20 min and the clear solution obtained on centrifugation at 15 000*g* and 277 K for 20 min was used as the cell extract. After supplementation with 1 m*M* phenylmethylsulfonyl fluoride and

0.1 μ*M* pepstatin A, the cell extract was fractionated with ammonium sulfate. The precipitate (30–50% saturation) was collected by centrifugation at 15 000*g*, 277 K for 20 min, dissolved in 20 m*M* KPB pH 7.0 and applied to a DEAE-Sepharose CL-6B column (4.2 × 30 cm; Pharmacia Biotech Co., Uppsala, Sweden) equilibrated with 20 m*M* KPB pH 7.0. The enzyme was eluted with a linear gradient of NaCl (0–1.0 *M*) in 20 m*M* KPB pH 7.0 (1 l), a 10 ml fraction being collected every 8 min. The active fractions, which were eluted at around 0.65 *M* NaCl, were combined and saturated with ammonium sulfate (30%). The enzyme was applied to a butyl-Toyopearl 650M column (2.6 × 15 cm; Tosoh Co., Tokyo, Japan) equilibrated with 20 m*M* KPB pH 7.0, saturated with ammonium sulfate (30%) and eluted with a linear gradient of ammonium sulfate (30–0% saturation) in 20 m*M* KPB pH 7.0 (500 ml). A 4 ml fraction was collected every 3 min. The active fractions, which were eluted with about 12% ammonium sulfate, were combined and dialyzed against 20 m*M* KPB pH 7.0; the dialysate was used as the purified enzyme (521 mg protein). For crystallization, the purified enzyme was concentrated to 10 mg ml^{-1} by ultrafiltration with an Amicon Model 8200 (Amicon Co., Beverly, MA, USA).

The purified enzyme gave a single band at a position of 42 kDa on both SDS–PAGE and native gradient PAGE, indicating that it was in a homogeneous monomeric form. The properties, such as optimum pH (6.0) and temperature (318 K) for reactions, thermal stability (below 323 K) and substrate specificity (specific to unsaturated glucuronyl saccharides), of the enzyme purified from *E. coli* cells were almost the same as those of the native enzyme from *Bacillus* sp. GL1 cells (Hashimoto *et al.*, 1999; Mori *et al.*, 2003).

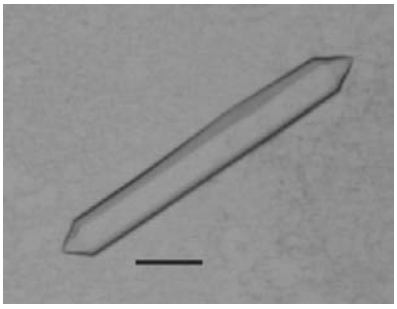

Figure 2
Crystal of the unsaturated glucuronyl hydrolase of *Bacillus* sp. GL1. The scale bar is 0.1 mm long.

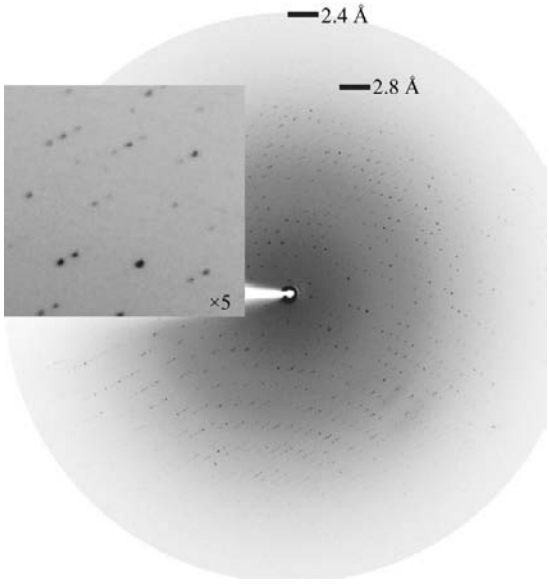

Figure 3
X-ray diffraction image of the crystal of the unsaturated glucuronyl hydrolase.

2.3. Crystallization

The unsaturated glucuronyl hydrolase from *E. coli* cells was crystallized at 293 K using the hanging-drop vapour-diffusion method. The crystallization droplet solution was prepared on a siliconized cover slip by mixing 3 μl of the protein solution (10 mg ml^{-1}) with 3 μl of the precipitant solution. Since few crystals of the enzyme were initially found by sparse-matrix screening using commercial crystallization kits from Hampton Research (Laguna Niguel, CA, USA), crystallization of the enzyme was attempted under a large number of conditions with ammonium sulfate, polyethylene glycol or 2-methyl-2,4-pentanediol (MPD) as the major precipitant. Small rod-shaped crystals of the enzyme were found in a droplet consisting of 10 mg ml^{-1} enzyme, 60% MPD, 0.1 *M* glycine–NaOH pH 9.0 and 0.1 *M* dithiothreitol. After improvement of the conditions, the solution most suitable for crystallization was determined to be a mixture consisting of 10 mg ml^{-1} enzyme, 56% MPD, 0.1 *M* NaCl, 0.1 *M* glycine–NaOH pH 8.2 and 0.1 *M* dithiothreitol. Crystals of the enzyme grew in these droplets in three weeks at 293 K (Fig. 2).

2.4. X-ray analysis

A crystal of the enzyme picked up from a droplet using a mounted nylon loop (Hampton Research, Laguna Niguel, CA, USA) was placed directly into a cold nitrogen-gas stream at 100 K. X-ray diffraction images were collected from the crystal at 100 K under the nitrogen-gas stream using a MAR CCD detector with synchrotron radiation of wavelength 0.9 Å at the BL-41XU station of SPring-8 (Japan) (Fig. 3). The distance between the crystal and the detector was set to 200 mm and 1.0° oscillation images were recorded with an exposure time of 2 min. The diffraction data for the crystal were obtained in the resolution range 50–2.4 Å and were processed using *HKL*2000 (*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). The space group of the crystal was estimated to be $P6_122$ or $P6_522$ (hexagonal), with unit-cell parameters $a = b = 102.8$, $c = 223.4$ Å. Of the total reflections measured (598 875), 28 181 independent reflections were obtained, with an $R_{\rm merge}$ value of 10.4%. The data set was 99.9% complete at the resolution limit of 2.4 Å. The preliminary X-ray crystallographic properties of the enzyme are summarized in Table 1. The $V_{\rm M}$ value (Matthews, 1968), the crystal volume per unit of protein molecular weight, was calculated to be 1.99 Å^3 Da^{-1}, assuming two molecules of the enzyme to be present in an asymmetric unit, and the solvent content was 38%. The $V_{\rm M}$ value and solvent content lie within the ranges usually found for protein crystals.

Table 1
Data-collection statistics for a crystal of the unsaturated glucuronyl hydrolase.

Values in parentheses refer to the highest resolution shell.

Wavelength (Å)	0.9
Resolution (Å)	50–2.40 (2.49–2.40)
Crystal system	Hexagonal
Space group	$P6_122$ or $P6_522$
Unit-cell parameters (Å)	$a = b = 102.8$, $c = 223.4$
Total observations	598875
Independent reflections	28181
Completeness (%)	99.9 (99.9)
$I/\sigma(I)$	6.0 (2.3)
$R_{\rm merge}$ (%)	10.4 (26.8)

A selenomethionine derivative of the unsaturated glucuronyl hydrolase has already been obtained and crystallization of the derivative is now in progress. The phase problem will be solved by multiwavelength anomalous dispersion (MAD) experiments and/or the multiple isomorphous replacement (MIR) method, as the enzyme shows no homology with proteins of known structure.

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References

Andersson, L.-O., Borg, H. & Mikaelsson, M. (1972). *FEBS Lett.* **20**, 199–202.
Hashimoto, W., Kobayashi, E., Nankai, H., Sato, N., Miya, T., Kawai, S. & Murata, K. (1999). *Arch. Biochem. Biophys.* **368**, 367–374.
Hashimoto, W., Miyake, O., Momma, K., Kawai, S. & Murata, K. (2000). *J. Bacteriol.* **182**, 4572–4577.
Herron, S. R., Benen, J. A., Scavetta, R. D., Visser, J. & Jurnak, F. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 8762–8769.
Jedrzejas, M. J. (2000). *Crit. Rev. Biochem. Mol. Biol.* **35**, 221–251.
Jedrzejas, M. J. (2001). *Microbiol. Mol. Biol. Rev.* **65**, 187–207.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265–275.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mori, S., Akao, S., Nankai, H., Hashimoto, W., Mikami, B. & Murata, K. (2003). In the press.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Shevchik, V. E., Condemine, G., Robert-Baudouy, J. & Hugouvieux-Cotte-Pattat, N. (1999). *J. Bacteriol.* **181**, 3912–3919.
Sutherland, I. W. (1995). *FEMS Microbiol. Rev.* **16**, 323–347.
Wong, T. Y., Preston, L. A. & Schiller, N. L. (2000). *Annu. Rev. Microbiol.* **54**, 289–340.