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Purification, crystallization and preliminary X-ray crystallographic analysis of xylose reductase from *Candida tropicalis*

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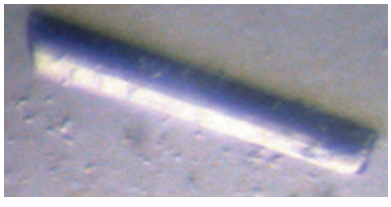

Xylose reductase (XR), which requires NADPH as a co-substrate, catalyzes the reduction of D-xylose to xylitol, which is the first step in the metabolism of D-xylose. The detailed three-dimensional structure of XR will provide a better understanding of the biological significance of XR in the efficient production of xylitol from biomass. XR of molecular mass 36.6 kDa from *Candida tropicalis* was crystallized using the hanging-drop vapour-diffusion method. According to X-ray diffraction data from *C. tropicalis* XR crystals at 2.91 Å resolution, the unit cell belongs to space group $P3_1$ or $P3_2$. Preliminary analysis indicated the presence of four XR molecules in the asymmetric unit, with 68.0% solvent content.

1. Introduction

The global energy crisis has worsened owing to the scarcity of the fossil fuel oil. The ethanolic fermentation of lignocellulosic biomass is a sustainable option for the production of bioethanol (Bettiga *et al.*, 2008). Hemicellulose is present in many crop residues and treatment with diluted acids degrades it to a mixture of monosaccharides that contains pentoses, with xylose as the main component together with arabinose, mannose and galactose (Beck, 1989). At present, two distinct paths are available in nature to convert xylose to xylulose: those based on reduction and oxidation and those based on isomerization (Bettiga *et al.*, 2008). In pentose-growing yeasts, xylose is first reduced by xylose reductase (XR) to xylitol, which in turn is oxidized to xylulose by xylitol dehydrogenase (XDH; Almeida, Modig *et al.*, 2008; Almeida, Röder *et al.*, 2008; Laadan *et al.*, 2008). In bacteria, some anaerobic fungi and a few yeasts such as *Rhodotorula gracilis* (Hofer *et al.*, 1971) and *Candida utilis* (Tomoeda & Horitsu, 1964), xylose isomerase (XI) is responsible for the direct conversion of xylose to xylulose. Xylulose is eventually phosphorylated to xylulose 5-phosphate, an intermediate compound in the pentose-phosphate pathway, by xylulose kinase (XK; Chang & Ho, 1988). As most yeasts lack XI, XR is the first key enzyme in D-xylose metabolism.

XR is commonly found in yeasts and filamentous fungi, usually with several isozymes in one species (Yokoyama *et al.*, 1995; Mayr *et al.*, 2000; Nidetzky *et al.*, 2003). In general, XR is specific for NADPH, whereas in some fungi the enzyme utilizes both NADPH and NADH (Verduyn *et al.*, 1985) and in at least one fungus it prefers NADH over NADPH (Lee *et al.*, 2003). XRs have attracted attention because of their importance in the production of cellulosic ethanol from biomass and in the generation of xylitol, a five-carbon sugar alcohol used as a low-calorie sweetener in food products (Hyvonen *et al.*, 1982). The improvement of the metabolic utilization of xylose, for which XR activity is essential, is of considerable interest. XR belongs to the aldose reductase (ALR) family (EC 1.1.1.21) and the aldo–keto reductase (AKR) enzyme superfamily (Lee, 1998; Petrash, 2004). Characterization of XRs from various organisms, such as *Neurospora crassa*, *Candida intermedia*, *C. parapsilosis*, *C. tropicalis*, *C. tenuis*, *Pachysolen tannophilus*, *Pichia stipitus* and *Saccaromyces cerevisiae*, has revealed that most XRs function as noncooperative tightly associated dimers with a subunit molecular mass of 33–38.4 kDa and an optimal pH of 5.5–7.0 (Lee, 1998; Rizzi *et al.*, 1988; Kuhn *et al.*, 1995; Yokoyama *et al.*, 1995; Woodyer *et al.*, 2005).

A panel of structures of wild-type and mutant XRs from *C. tenuis* has been obtained for functional studies (Kavanagh *et al.*, 2002, Petschacher *et al.*, 2006). Of the various *Candida* species, *C. tropicalis* exhibits the greatest activity in terms of the yield and rate of xylitol production (Oh & Kim, 1998), but the structure of XR from *C. tropicalis* is not yet available. In order to further understand the biological significance of XR in the efficient production of xylitol, it is important to determine the three-dimensional structure of XR from *C. tropicalis*. A comparison of XR structures and amino-acid sequences between *C. tropicalis* and other species, especially that of *C. tenuis*, which has 78% sequence identity (Kavanagh *et al.*, 2002), is expected to provide useful information to address the differences in activity and function, which may be applicable to biomass utilization. The gene encoding XR from *C. tropicalis* (*Ct*XR) was cloned and expressed in *Escherichia coli*. Here, we report the purification, crystallization and X-ray crystallographic characterization of *Ct*XR, which comprises 358 amino acids.

2. Materials and methods

2.1. Molecular cloning

To amplify the *Ct*XR coding sequence by the polymerase chain reaction (PCR), we used cDNA that had been prepared from genomic DNA of *C. tropicalis* as the template, for which the forward primer was 5′-ATGTCTACTACTCCTACTATT-3′ and the reverse primer was 5′-TTAAACAAAGATTGGAATGTT-3′. An open reading frame (ORF) that contained 975 nucleotides and codes for a polypeptide chain of 324 amino acids was identified. Sequence analysis indicated a protein with an estimated molecular mass of approximately 36.6 kDa and an isoelectric point at pH 5.1.

2.2. Protein expression and purification

The *C. tropicalis* XR gene (GenBank accession No. FJ804147) was subcloned into the expression plasmid pET28a(+) and subsequently transformed into *E. coli* BL21(DE3)pLysS cells for recombinant protein expression and purification. The putative plasmid containing the *Ct*XR fragment was then further confirmed by nucleotide sequencing. For expression of *Ct*XR, isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration 0.5 m*M*) was added to the culture for induction and incubation was continued at 310 K for 10 h with rotary shaking. The whole cells were then harvested by centrifugation (10 000*g*) at 277 K for 30 min. The medium was discarded and the cell pellet was resuspended in binding buffer (30 ml) containing 0.25 *M* NaCl and 20 m*M* Tris–HCl pH 7.9, and then subjected to cell disruption by ultrasonication using a pulsation cycle of 1 s on/5 s off with a total duration of 5 min sonication at 40% energy on ice. The suspension was collected by centrifugation (12 000*g*) at 277 K for 30 min. The soluble protein extract was then passed through a His-Tag Ni^{2+}–NTA column (length 10 cm; General Electric) that was pre-equilibrated with binding buffer (50 ml 0.25 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). The column was washed with binding buffer (200 ml) containing 20 m*M* imidazole and XR protein containing an extra six histidines at the N-terminus was eluted with binding buffer containing stepwise increasing concentrations of imidazole: 50, 100, 150, 200 and 500 m*M*. The eluted fractions with XR enzymatic activity (Almeida, Modig *et al.*, 2008) were collected and dialyzed against 20 m*M* Tris–HCl buffer pH 7.9 before crystallization. The protein sample was concentrated on Centricon (10 000 Da molecular-weight cutoff; Amicon Ultra, Millipore). The yield of the protein was approximately 5 mg per litre and the purity was greater than 95% as analysed using 12% SDS–PAGE (Fig. 1) with Coomassie Brilliant Blue R-250 staining.

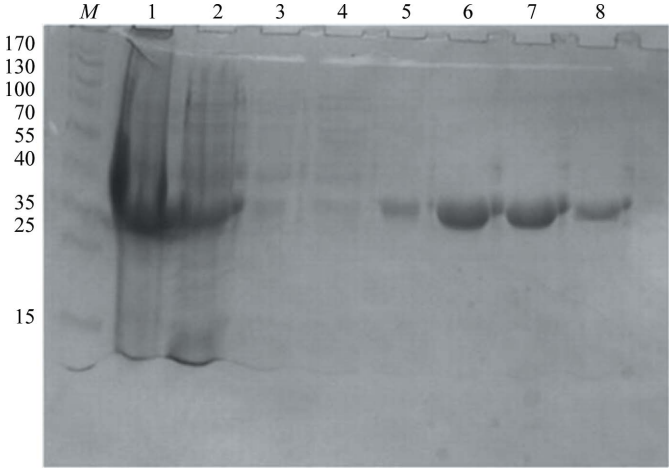

Figure 1
Coomassie Blue-stained 12% Tricine SDS–PAGE of pools from the purification of *Ct*XR. Purification fractions containing XR activity were collected at each step. All samples were boiled at 373 K for 10 min in 5× sample buffer. Lane 1, pool from supernatant after centrifugation; lane 2, pool from flowthrough; lane 3, pool from 0 m*M* imidazole wash; lane 4, pool from 50 m*M* imidazole wash; lane 5, pool from 100 m*M* imidazole wash; lane 6, pool from 150 m*M* imidazole elution; lane 7, pool from 200 m*M* imidazole elution; lane 8, pool from 500 m*M* imidazole wash; lane *M*, molecular-weight markers (kDa).

2.3. Protein crystallization

Prior to crystallization trials, the purified protein sample was concentrated to 5 mg ml^{-1} in 20 m*M* Tris–HCl buffer pH 7.9. Crystallization was performed using the hanging-drop vapour-diffusion method at 293 K with Crystal Screen kits in 48-well plates (Hampton Research).

2.4. X-ray data collection and processing

The protein crystals were initially tested and characterized using synchrotron radiation on SPXF beamlines BL13B1 and BL13C1 equipped with CCD detectors (Q315 and Q210, ADSC) at the National Synchrotron Radiation Research Center (NSRRC), Taiwan and on beamline BL12B2 equipped with a CCD detector (Quantum-4R, ADSC) at SPring-8, Japan. The crystal was transferred from a crystallization drop into cryoprotectant solution (5 µl) containing 16%(*w*/*v*) PEG 8000, 0.1 *M* $MgCl_2$ and 15%(*v*/*v*) glycerol in 0.1 *M* Tris buffer pH 8.3 for a few seconds, mounted on a synthetic nylon loop (0.2–0.3 mm, Hampton Research) and then flash-cooled in liquid nitrogen. For complete data collection, 180° of rotation was measured with 1.0° oscillations using an X-ray wavelength of 1.00 Å

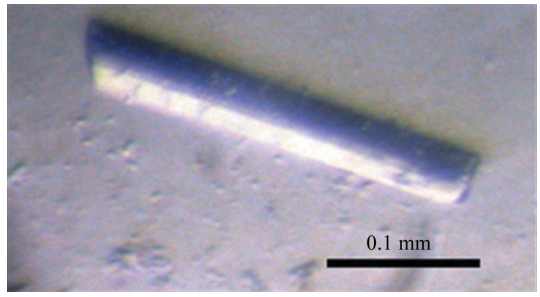

Figure 2
Single crystals of *Ct*XR grown by the hanging-drop method.

Table 1
Crystal diffraction statistics for *Ct*XR.

Values in parentheses are for the highest resolution shell (3.01–2.91 Å).

Wavelength (Å)	1.00
Temperature (K)	110
Resolution range (Å)	30.0–2.91
Space group	$P3_1$ or $P3_2$
Unit-cell parameters (Å)	a = 123.52, c = 128.01
Unique reflections	44533
Completeness (%)	93.4 (80.7)
$\langle I/\sigma(I)\rangle$	16.6 (2.7)
Average redundancy	4.8
$R_{\rm merge}$† (%)	8.8 (32.5)
Mosaicity (°)	1.83
No. of molecules per ASU	4
Matthews coefficient (Å^3 Da^{-1})	3.85 (2.57)
Solvent content (%)	68 (52)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations i of reflection hkl.

on beamline NSRRC_BL13C1 with an exposure duration of 60 s and a crystal-to-detector distance of 300 mm at 110 K in a nitrogen stream generated by a cryosystem (X-Stream, Rigaku/MSC Inc.). All data were indexed, integrated and scaled using the program *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Under SDS denaturating and reducing conditions, the SDS–PAGE showed a single band corresponding to a molecular mass of about 37 000 Da (Fig. 1), which is in agreement with the approximate molecular mass of XR according to the amino-acid sequence. Small crystals were observed from a condition consisting of 20%(*w*/*v*) PEG 8000 and 0.2 *M* magnesium chloride in 0.1 *M* Tris buffer pH 8.5 (Crystal Screen I condition No. 3) within 5 d of setup. This condition was further refined to produce larger XR crystals using 2 µl hanging drops containing 1 µl protein solution and 1 µl reservoir solution equilibrated against 200 µl reservoir solution containing 16%(*w*/*v*) PEG 8000 and 0.1 *M* $MgCl_2$ in 0.1 *M* Tris buffer pH 8.3. Protein crystals appeared after 5 d and continued to grow to final dimensions of 0.25 × 0.05 × 0.05 mm with a rectangular shape over two weeks in an incubator at 293 K. Crystals of satisfactory quality for diffraction were used for data collection (Fig. 2). The protein crystals were sensitive to variation of the precipitant concentration during transfer to the cryoprotectent solution containing 15%(*v*/*v*) glycerol. Crystals of satisfactory quality were identified by careful screening and selection for data collection, as they typically exhibited fairly high mosaicity (>1.5°). Radiation damage was observed after protracted exposure during data collection, which caused a decrease in $I/\sigma(I)$ and an increase in $R_{\rm merge}$. Although 270° of data were collected, after a preliminary inspection of data statistics with regard to crystal decay only the first 180° of data were selected for final data processing. Analysis of the diffraction pattern indicated that these crystals exhibited trigonal symmetry; systematic absences indicated the space group to be $P3_1$ or $P3_2$. Assuming the presence of four or six XR molecules per asymmetric unit, the Matthews coefficient was estimated to be 3.85 or 2.57 Å^3 Da^{-1}, corresponding to a solvent content of 68% or 52%, respectively (Matthews, 1968); either value is within the general range for protein crystals. Table 1 presents details of data statistics. The structure determination of *Ct*XR by the molecular-replacement method using the monomer structure of XR from *C. tenuis* (78% sequence identity; PDB code 1jez; Kavanagh *et al.*, 2002) as a search model is in progress.

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References

Almeida, J. R. M., Modig, T., Röder, A., Lidén, G. & Gorwa-Grauslund, M. F. (2008). *Biotechnol. Biofuels*, **1**, 12.
Almeida, J. R. M., Röder, A., Modig, T., Laadan, B., Lidén, G. & Gorwa-Grauslund, M. F. (2008). *Appl. Microbiol. Biotechnol.* **78**, 939–945.
Beck, M. J. (1989). *Biotechnol. Bioeng. Symp.* **17**, 617–627.
Bettiga, M., Hahn-Hägerdal, B. & Gorwa-Grauslund, M. F. (2008). *Biotechnol. Biofuels*, **1**, 16.
Chang, S. F. & Ho, N. W. (1988). *Appl. Biochem. Biotechnol.* **17**, 313–318.
Hofer, M., Betz, A. & Kotyk, A. (1971). *Biochim. Biophys. Acta*, **252**, 1–12.
Hyvonen, L., Koivistoinen, P. & Voirol, F. (1982). *Adv. Food Res.* **28**, 373–403.
Kavanagh, K. L., Klimacek, M., Nidetzky, B. & Wilson, D. K. (2002). *Biochemistry*, **41**, 8785–8795.
Kuhn, A., van Zyl, C., van Tonder, A. & Prior, B. A. (1995). *Appl. Environ. Microbiol.* **61**, 1580–1585.
Laadan, B., Almeida, J. R., Radstrom, P., Hahn-Hägerdal, B. & Gorwa-Grauslund, M. (2008). *Yeast*, **25**, 191–198.
Lee, H. (1998). *Yeast*, **14**, 977–984.
Lee, J.-K., Koo, B.-S. & Kim, S.-Y. (2003). *Appl. Environ. Microbiol.* **69**, 6179–6188.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mayr, P. K., Bruggler, K. D., Kulbe, K. D. & Nidetzky, B. (2000). *J. Chromatogr. B*, **737**, 195–202.
Nidetzky, B., Bruggler, K., Kratzer, R. & Mayr, P. (2003). *J. Agric. Food Chem.* **51**, 7930–7935.
Oh, D. K. & Kim, S. Y. (1998). *Appl. Microbiol. Biotechnol.* **50**, 419–425.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Petrash, J. M. (2004). *Cell. Mol. Life Sci.* **61**, 737–749.
Petschacher, B., Leitgeb, S., Kavanagh, K. L., Wilson, D. K. & Nidetzky, B. (2006). *Biochem. J.* **385**, 75–83.
Rizzi, M., Erlemann, P., Bui-Thanh, N. A. & Dellweg, H. (1988). *Appl. Microbiol. Biotechnol.* **29**, 148–154.
Tomoeda, M. & Horitsu, H. (1964). *Arg. Biol. Chem.* **28**, 139–143.
Verduyn, C., Vankleef, R., Frank, J., Schreuder, H., Vandijken, J. P. & Scheffers, W. A. (1985). *Biochem. J.* **226**, 669–677.
Woodyer, R., Simurdiak, M., van der Donk, W. A. & Zhao, H. (2005). *Appl. Environ. Microbiol.* **71**, 1642–1647.
Yokoyama, S.-I., Suzuki, T., Kawai, K., Horitsu, H. & Takamizawa, K. (1995). *J. Ferment. Bioeng.* **79**, 217–223.