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Crystallization and preliminary X-ray crystallographic study of disproportionating enzyme from potato

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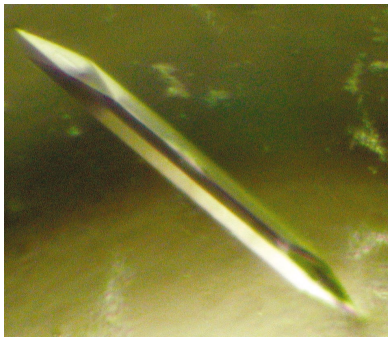

Disproportionating enzyme (D-enzyme; EC 2.4.1.25) is a 59 kDa protein that belongs to the α-amylase family. D-enzyme catalyses intramolecular and intermolecular transglycosylation reactions of α-1,4 glucan. A crystal of the D-enzyme from potato was obtained by the hanging-drop vapour-diffusion method. Preliminary X-ray data showed that the crystal diffracts to 2.0 Å resolution and belongs to space group $C222_1$, with unit-cell parameters a = 69.7, b = 120.3, c = 174.2 Å.

1. Introduction

Disproportionating enzyme (D-enzyme, 4-α-glucanotransferase; EC 2.4.1.25) is present in plants. It was first found in potato tubers by Peat *et al.* (1956) and has since been found in many plant tissues (Lin & Preiss, 1988). A similar 4-α-glucanotransferase is also present in various bacteria and is called amylomaltase (Takaha & Smith, 1999). D-enzyme and amylomaltase have received interest because they catalyze glucan-chain transfer not only from one α-1,4 glucan molecule to another (1), but also within a single linear glucan molecule to produce a cyclic glucan (2),

$$(\alpha\text{-1,4-glucan})_m + (\alpha\text{-1,4-glucan})_n \rightarrow (\alpha\text{-1,4-glucan})_{m-x} + (\alpha\text{-1,4-glucan})_{n+x}, \quad (1)$$

$$(\alpha\text{-1,4-glucan})_m \rightarrow (\alpha\text{-1,4-glucan})_{m-x} + \text{cyclic}(\alpha\text{-1,4-glucan})_x. \quad (2)$$

Although the cyclization reaction has been observed for cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19), these enzymes differ in the smallest cyclic glucan produced. CGTase produces cyclic α-1,4-glucan with a degree of polymerization (DP) of 6–8, which are often referred to as cyclodextrins, while potato D-enzyme and amylomaltase from *Thermus aquaticus* produce cyclic α-1,4-glucan with DPs starting from 17 (Takaha *et al.*, 1996) and 22 (Terada *et al.*, 1999), respectively. Additionally, these three enzymes differ in their reaction specificities. CGTase and amylomaltase from *T. aquaticus* mainly catalyze transglycosylation reactions, but also show a weak but significant level of hydrolytic activity. However, potato D-enzyme exclusively catalyzes transglycosylation reactions and thus appears to be the 4-α-glucanotransferase with the lowest level of hydrolytic activity (Takaha & Smith, 1999).

D-enzyme, amylomaltase and CGTase all belong to the α-amylase family, which includes about 20 different enzymes. The α-amylase family enzymes catalyze the hydrolysis and/or transglycosylation of α-1,4 and/or α-1,6 glucosidic linkages at the conserved anomeric centre (Janecek, 1995, 1997; Janecek *et al.*, 1997; Kuriki & Imanaka, 1999). A number of crystal structures of α-amylase family enzymes have been investigated and have revealed that the enzymes have a common structural feature consisting of three domains and some enzymes have additional domain(s). The core of the protein structure consists of a $(\beta/\alpha)_8$ barrel (TIM-barrel) in all members of the α-amylase family.

Crystal structures of CGTase from several sources (Harata *et al.*, 1996; Knegtel *et al.*, 1996; Lawson *et al.*, 1994; Kubota *et al.*, 1991; Klein & Schulz, 1991) and amylomaltase from *T. aquaticus* (Przylas *et al.*, 2000) have already been determined, but the structure of potato

D-enzyme is still not available. In order to investigate the differences in product specificity and reaction specificity of these three enzymes at the molecular level, we have initiated a three-dimensional structure analysis of D-enzyme from potato. In this paper, we report the crystallization and preliminary X-ray analysis of this enzyme from potato.

2. Materials and methods

2.1. Protein preparation

A DNA fragment containing the potato D-enzyme structural gene was amplified by the 'megaprimer' PCR method (Sarkar & Sommer, 1990) with 5′-TTTACCATGGCCGTTCCTGCTGTAGGTG-3′ as the sense primer, 5′-CGAAGCTTTTACAACCGCCCATAAGTTG-3′ containing *Nco*I and *Hin*dIII restriction sites as the antisense primer, 5′-GATTGGAAAGCGATGGAGAAGGATGG-3′ as the megaprimer and pKK pKK388-DPE2 (Takaha *et al.*, 1993) as the template. The amplified DNA fragment (1.5 kbp) was inserted into the *Nco*I and *Hin*dIII restriction-enzyme sites of pET-21d (Novagen) to construct an expression plasmid, pDPEW.

To label the D-enzyme with SeMet, the *Escherichia coli met*$^-$ auxotrophic strain B834 (DE3) (Novagen) containing pDEPW was grown at 310 K in LB media containing 1%(*w*/*v*) glucose. When the $A_{600\,\mathrm{nm}}$ of the broth reached 0.6, cells were harvested by centrifugation at 4000*g* for 10 min at 277 K and washed twice with double-distilled water. The harvested cells were re-suspended in M9 media containing 0.1%(*w*/*v*) galactose and were grown again at 310 K for 30 min and then at 291 K with the inducer IPTG (1 m*M*).

The pelleted cells were suspended in 50 m*M* Tris–HCl pH 7.0 and disrupted by sonication. The cell debris was pelleted by centrifugation (12 000*g* for 20 min at 277 K). The supernatant was collected and ammonium sulfate was added to a final concentration of 600 m*M*. After keeping the solution at 277 K for 30 min, it was centrifuged at 12 000*g* for 10 min at 277 K and the supernatant was loaded onto a Phenyl-Toyopearl 650M (Tosoh) column (1.6 × 8 cm) and eluted with a linear gradient of 600–0 m*M* ammonium sulfate in the same buffer. Active fractions were pooled and dialyzed against 50 m*M* Tris–HCl pH 7.0. The dialysate was loaded onto a DEAE-Toyopearl 650S (Tosoh) column (0.9 × 8 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0–200 m*M* NaCl in the same buffer. Active fractions were pooled and dialyzed against 50 m*M* Tris–HCl pH 8.0. The dialysate was loaded onto a DEAE-Toyopearl 650S (Tosoh) column (0.9 × 8 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 50–150 m*M* NaCl in the same buffer. The active fractions were concentrated to 10 mg ml^{-1} and the buffer was exchanged to 5 m*M* Tris–HCl pH 7.5 using an Amicon Centricon YM-30 (Millipore).

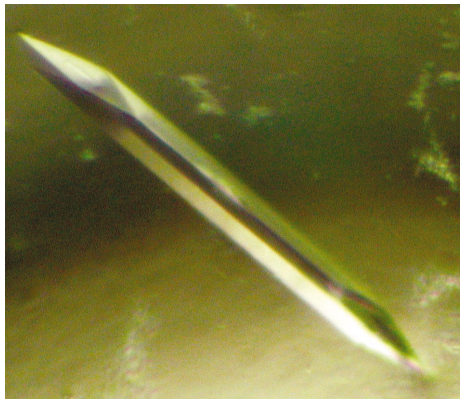

Figure 1
Crystal of D-enzyme from potato. The size of this crystal is approximately 0.1 × 0.1 × 0.9 mm.

Table 1
X-ray data-processing statistics.

Values in parentheses are for the highest resolution shell.

	Peak	Edge	Remote
Unit-cell parameters (Å, °)	a = 69.7, b = 120.3, c = 174.2, $\alpha = \beta = \gamma$ = 90		
Space group	$C222_1$		
Wavelength (Å)	0.9791	0.9794	1.0000
Resolution range (Å)	50–2.03 (2.10–2.03)	50–1.96 (2.03–1.96)	50–2.00 (2.07–2.00)
Observed reflections	342034	371974	349274
Unique reflections	47324 (4693)	52631 (5179)	49370 (4872)
Completeness (%)	100 (99.9)	99.9 (99.2)	99.9 (99.4)
Multiplicity	7.2 (6.4)	7.1 (5.7)	7.1 (5.8)
Mean $I/(I)$ (%)	24.5 (8.2)	24.6 (5.7)	26.8 (8.2)
$R_{\rm merge}$ (%)	5.4 (19.4)	5.2 (24.5)	4.7 (18.8)

2.2. Crystallization and X-ray data collection

Crystals of D-enzyme from potato were grown using the hanging-drop vapour-diffusion method at 277 K by mixing 2 µl protein solution (10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and an equal volume of reservoir solution [100 m*M* HEPES pH 7.6, 100 m*M* $CaCl_2$ and 9%(*w*/*v*) PEG 8000]. The crystals appeared within a week and grew to average dimensions of approximately 0.1 × 0.1 × 0.4 mm (Fig. 1).

A crystal was transferred to a cryoprotectant solution [27%(*v*/*v*) glycerol, 100 m*M* HEPES pH 7.6, 100 m*M* $CaCl_2$ and 9%(*w*/*v*) PEG 8000], picked up in a nylon loop and then flash-cooled at 100 K in a nitrogen-gas stream. A multiple-wavelength anomalous dispersion data set was collected from a selenomethionine-labelled crystal at wavelengths of 1.0000 Å (remote), 0.9794 Å (peak) and 0.9791 Å (edge) at 100 K on beamline BL5A at the Photon Factory (Tsukuba, Japan) using a Quantum 315 CCD detector (ADSC, USA). The crystal-to-detector distance was 200.0 mm. A total of 360 rotation images for each wavelength were collected with an oscillation angle of 0.5°, with an exposure time of 5 s for each image. The data were indexed and scaled with the *HKL*2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

A complete data set was collected to a resolution of 2.0 Å using a single crystal. Detailed data-processing statistics are shown in Table 1. From the autoindexing and scaling by *HKL*2000, the space group was determined to be $C222_1$, with unit-cell parameters a = 69.7, b = 120.3, c = 174.2 Å, $\alpha = \beta = \gamma = 90°$. Assuming that one D-enzyme molecule is contained in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$; Matthews, 1968) was calculated to be 3.10 Å^3 Da^{-1}; the estimated solvent content is thus 59.9%, which is in the range typically found for protein crystals.

The amino-acid sequence of D-enzyme is 40% homologous with that of amylomaltase from *T. aquaticus*, the crystal structure of which has already been solved (Przylas *et al.*, 2000). Therefore, extensive attempts were made using the molecular-replacement method (MR) with coordinates of amylomaltase from *T. aquaticus* as the model. However, all promising MR solutions did not result in interpretable electron-density maps. This is probably because of conformational differences or the low amino-acid sequence similarity between the D-enzyme from potato and amylomaltase from *T. aquaticus*. Therefore, the crystal structure of D-enzyme from potato will be solved by

the multiwavelength anomalous dispersion method. A search for Se-atom sites is now in progress.

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