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

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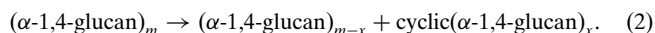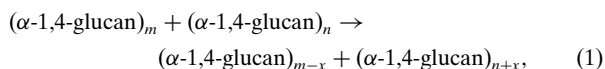nitta@biochem.osakafu-u.ac.jp

Disproportionating enzyme (D-enzyme; EC 2.4.1.25) is a 59 kDa protein that belongs to the $\alpha$-amylase family. D-enzyme catalyses intramolecular and intermolecular transglycosylation reactions of $\alpha$-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-$\alpha$-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-$\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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-$\alpha$-glucanotransferase with the lowest level of hydrolytic activity (Takaha & Smith, 1999).

D-enzyme, amylomaltase and CGTase all belong to the $\alpha$-amylase family, which includes about 20 different enzymes. The $\alpha$-amylase family enzymes catalyze the hydrolysis and/or transglycosylation of $\alpha$-1,4 and/or $\alpha$-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 $\alpha$-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 $\alpha$-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
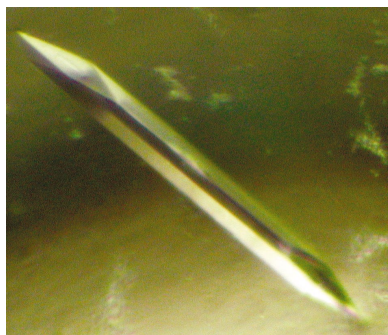
D-enzyme is still not available. In order to investigate the differences in product specificity and reaction specificity of these three enzymes at the molecular level, we have initiated a three-dimensional structure analysis of D-enzyme from potato. In this paper, we report the crystallization and preliminary 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 $A_{600\,nm}$ of the broth reached 0.6, cells were harvested by centrifugation at 4000g 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 000g 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 000g 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–
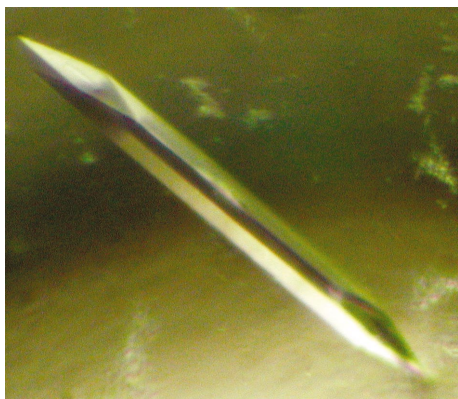
**Table 1**
X-ray data-processing statistics.

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 $I/I(I)$ (%) | 24.5 (8.2) | 24.6 (5.7) | 26.8 (8.2) |
| $R_{merge}$ (%) | 5.4 (19.4) | 5.2 (24.5) | 4.7 (18.8) |

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).

### 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* CaCl₂ 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* CaCl₂ 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 ($V_M$; Matthews, 1968) was calculated to be 3.10 Å³ 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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