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Crystallization and preliminary X-ray analysis of β-amylase from *Bacillus polymyxa*

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A truncated β-amylase (E.C. 3.2.1.2) from *Bacillus polymyxa* has been crystallized using the hanging-drop vapour-diffusion method at 277 K. The crystals belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions a = 64.6, b = 141.9, c = 155.1 Å and diffract to 2.5 Å resolution. The asymmetric unit containing three protein molecules was derived from an electron-density map calculated at 4 Å resolution using MIR phases. This gives a V_m value of 2.36 Å^3 Da^{-1}.

1. Introduction

β-Amylase (E.C. 3.2.1.2) is an exo-type enzyme which hydrolyzes the α-1,4-glucosidic linkages from the non-reducing end of α-1,4-glucans such as starch and liberates β-anomeric maltose. β-Amylase is present in certain bacteria as well as in higher plants. The amino-acid sequence homology is 43–53% among bacterial β-amylases and is below 32% between bacterial and plant β-amylases (Nanmori *et al.*, 1993). However, highly conserved amino-acid sequences are found in three homologous regions of β-amylase (Uozumi *et al.*, 1991), one of which contains the active site (Mikami *et al.*, 1994).

Crystal structures of β-amylase from higher plants have been determined in order to understand their structure–function relationships; for example, soybean β-amylase complexed with α-cyclodextrin (Mikami *et al.*, 1993), soybean β-amylase reacted with β-maltose and maltal (Mikami *et al.*, 1994) and sweet potato β-amylase (Cheong *et al.*, 1995). These structure analyses have revealed that β-amylase comprises an $(\alpha/\beta)_8$ barrel domain which is characteristic of the tertiary structure of α-amylase (Matsuura *et al.*, 1984; Buisson *et al.*, 1987), although the spatial arrangement of the active sites of β-amylase differs to that of α-amylase (Mikami *et al.*, 1994). In soybean β-amylase, a small domain constructed with three long loops, L3 (the loop between strand β3 and helix α3), L4 and L5, forms the active site, together with the C-terminal side of the barrel.

B. polymyxa produces a 130 kDa precursor protein which shows both β- and α-amylase activities (Kawazu *et al.*, 1987; Uozumi *et al.*, 1989). The 130 kDa precursor protein is proteolytically cleaved into multiform β-amylases (of approximate molecular weights 70, 56 and 42 kDa) and a 48 kDa α-amylase. The N termini of these β-amylases coincide with the N terminus of the precursor protein. A 51 kDa β-amylase from *B. polymyxa*, corresponding to amino-acid residues 1–471 of the precursor protein, was expressed in *B. subtilis* (Uozumi *et al.*, 1991). The recombinant 51 kDa β-amylase contained the N-terminal region corresponding to both the barrel and small domains but lacked the C-terminal fragment, and showed enzymatic activity. The optimal pH of 7.5 is in the alkaline region and the optimal temperature of 308–318 K is the lowest of the bacterial β-amylases, although compared with β-amylases from plants, the bacterial β-amylases are generally unstable at higher temperatures.

Bacterial β-amylases could be applied industrially in the direct production of maltose from raw starch if their thermal stability could be improved. The alkaline adaptation mechanism of M-protease in the subtilisin family was elucidated on the basis of both the crystal structures and the amino-acid sequences of subtilisin family serine proteases by Shirai *et al.* (1997). Recently, we began investigating the structure of bacterial β-amylases in order to clarify the molecular basis of adaptation to non-physiological temperature conditions. Here, the structure analysis of the 51 kDa β-amylase from *B. polymyxa* is reported.

2. Materials and methods

2.1. Crystallization and search for isomorphous derivatives

The purification of β-amylase was performed as described previously (Uozumi *et al.*, 1991). For crystallization, the protein was concentrated to 10 mg ml^{-1} in 50 m*M* sodium phosphate buffer (pH 7.0). The initial screening for determination of the crystal-

Table 1
Summary of processed data.

Figures in parentheses refer to reflections with $I > 3\sigma(I)$.

Data set	Native-1	Hg-1	Pt-1
Resolution (Å)	3.5	4.0	3.5
Total reflections	98353	62848	109338
Independent reflections	16926 (15359)	11684 (9818)	17463 (15055)
R_{merge} (%)	6.3 (5.9)	13.3 (11.5)	6.3 (5.5)
Completeness (%)	91.0 (82.6)	92.9 (78.1)	92.7 (79.9)
Completeness of final shell (%)	90.5 (79.0)	81.4 (71.5)	87.5 (67.4)

Table 2
Refined heavy-atom parameters using *MLPHARE* (*CCP*4 program package).

Site	*x*	*y*	*z*	*B* (Å^2)	Occupancy†	Anomalous occupancy†
Hg-1 derivative						
Hg1	0.792	0.719	0.102	26.2	0.275	0.048
Hg2	0.137	0.911	0.129	32.4	0.281	0.036
Hg3	0.388	0.307	0.029	36.4	0.275	0.033
Pt-1 derivative						
Pt1	0.702	0.155	0.037	65.2	0.229	0.071

† Arbitrary scale.

lization conditions was carried out using Crystal Screen I and II from Hampton Research (California) and the hanging-drop vapour-diffusion method. 3 µl of the protein solution was mixed with an equal volume of a reservoir solution. The volume of the reservoir solution was 1 ml in all experiments. Under the best reservoir solution conditions, 2.05 *M* ammonium sulfate and 2.0%(*w*/*v*) polyethylene glycol 400 in 0.1 *M* HEPES buffer (pH 8.0) at 277 K, crystals of dimensions less than 0.1 mm were obtained within a week.

In order to improve crystals for X-ray diffraction experiments, a macroseeding technique was applied. Seed crystals were transferred to fresh drops composed of equal volumes of protein and reservoir solutions. The concentration of ammonium sulfate in the reservoir solution was reduced to 1.8 *M*, resulting in growth of crystals of dimensions up to 0.30 × 0.25 × 0.14 mm (Fig. 1) in one week. The crystals belong to orthorhombic space group $P2_12_12_1$ and have cell dimensions a = 64.6, b = 41.9 and c = 155.1 Å.

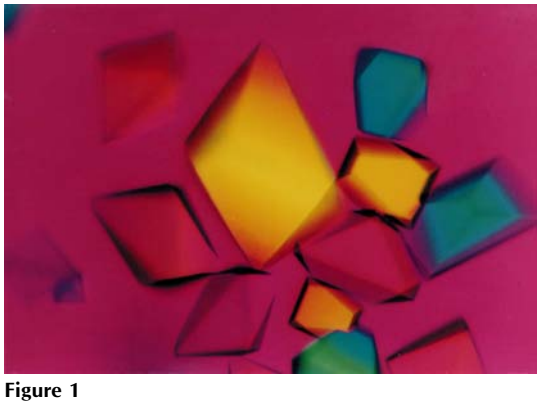

Figure 1
Crystals of the 51 kDa β-amylase. The maximum dimension of the crystals obtained by macroseeding was approximately 0.3 mm.

To solve the crystal structure by molecular replacement, the programs *FASTROT* (Crowther, 1972) and *AMoRe* (Navaza, 1994) were applied at first. However, the search model, the 2.2 Å refined structure of soybean β-amylase with R = 0.169 (Protein Data Bank entry 1BYA), could give no appropriate solutions. Heavy-atom derivatives were therefore searched for. Two isomorphous derivatives which appeared to be suitable were prepared by soaking crystals for 1 d in a 1 m*M* solution of either $Hg(CH_3COO)_2$ or K_2PtCl_4.

2.2. X-ray data collection and analysis

X-ray diffraction data for the native and two isomorphous derivative crystals, *i.e.* Native-1, Hg-1 [$Hg(CH_3COO)_2$ derivative] and Pt-1 (K_2PtCl_4 derivative), respectively, were collected by the oscillation method on a DIP-100 imaging-plate detector system (MacScience Co.) with focusing mirror optics mounted on an RU-300 Rigaku rotating-anode generator at 293 K. Only one crystal was required for each set of data.

Oscillation images were processed with programs *DENZO* (Otwinowski, 1993) and *SCALEPACK* (Minor, 1993). Scaling between native and derivative data and calculation of Patterson and Fourier maps were carried out with the program system *PROTEIN* (Steigemann, 1993). A summary of processed data is shown in Table 1.

3. Results and discussion

The major factor which affected the nature of crystals was pH. Crystals grown at pH 7.5 and 277 K were so fragile at room temperature that it was very difficult to mount them in capillaries, and they were easily soluble when kept at room temperature. On the other hand, crystals grown at pH 8.0 were easier to handle and less soluble when kept at room temperature. In addition, crystals were produced with good reproducibility at pH 8.0 using a macroseeding technique, compared with crystals produced at pH 7.5.

The three Hg sites of the Hg-1 derivative, Hg1, Hg2 and Hg3, were determined by the vector-search method (Steigemann, 1993) in several difference Patterson maps at different resolutions from 6.0 to 4.0 Å (Fig. 2). The Pt-site, Pt1, and the common origin of the two derivatives were fixed from a difference Fourier map at 6 Å resolution, which was phased using single isomorphous replacement with anomalous scattering from the Hg-1 derivative. All heavy-atom positions outlined in Table 2 and relative scale factors between the native and derivative crystals were refined with the program *MLPHARE* (Collaborative Computational Project, Number 4, 1994). Protein phases calculated with *MLPHARE* and phase calculation statistics were: mean figure of merit, 0.36; phasing power, 1.28 for 8908 acentric reflections for the Hg-1 derivative, 0.59 for 9044 acentric reflections for the Pt-1 derivative.

The molecular boundaries were clearly distinguished from solvent regions on an electron-density map calculated with X-ray data from 30.0 to 4.0 Å. Three molecules were easily identified in the asymmetric unit. This resulted in a V_m (Matthews, 1968) of

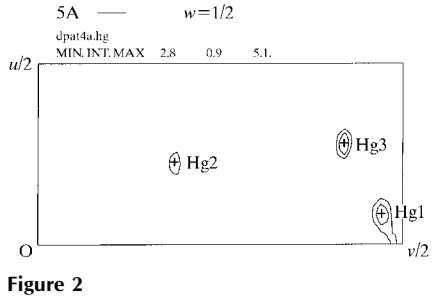

Figure 2
A Harker section (w = 0.5) of the difference Patterson map for the Hg-1 derivative at 4 Å resolution. Three heavy-atom sites, Hg1, Hg2 and Hg3, are shown. Contours are drawn at every σ level starting from the 3σ level.

2.36 Å^3 Da^{-1}. The protein contained three cysteine residues, one of which had a free SH group of high reactivity. The map showed that one mercury site is located in each protein molecule and that the binding site may be the free SH group. On the other hand, in the Pt-1 derivative only one platinum site with a rather low occupancy could be assigned to one of the three protein molecules in an asymmetric unit. The conditions for derivative preparation are currently being refined and data collection beyond 3 Å resolution is under way.

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