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Purification, crystallization and preliminary X-ray crystallographic study of α-amylase from *Bacillus stearothermophilus*

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A recombinant α-amylase from *Bacillus stearothermophilus* was found to be produced as several isoforms arising from different N-terminal processing. Some of those isoforms were purified to homogeneity and crystallized at 293 K using the hanging-drop vapour-diffusion method under the following conditions: 35 m*M* sodium acetate (pH 4.6), 6.25%(*v*/*v*) 2-propanol, in the presence of 1.23%(*w*/*v*) acarbose (a pseudo-oligosaccharide inhibitor) in the drop. The crystals diffracted beyond 2.0 Å resolution using synchrotron radiation at the Photon Factory, Tsukuba. They belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 53.7 (2), b = 92.9 (4), c = 53.2 (2) Å, β = 109.4 (1)°.

1. Introduction

α-Amylase (α-1,4-glucan-4-glucanohydrolase; E.C. 3.2.1.1) catalyzes the hydrolysis of α-D-(1,4)-glucosidic linkages in starch and other linear and branched polysaccharides. This enzyme is distributed in most living organisms and plays an important role in the utilization of polysaccharides. To date, α-amylases from various sources have been isolated and studied for their structure and function. Similarity in amino-acid sequence among species is found only in the four short stretches of sequence which make up the active site (Nakajima *et al.*, 1986). Our laboratory has been dealing with two bacterial α-amylases, *B. subtilis* α-amylase and *B. stearothermophilus* α-amylase (BSTA). We recently reported the crystal structure of the catalytic site mutant of *B. subtilis* α-amylase (Takase *et al.*, 1992) complexed with a substrate, which revealed the active-site structure interacting with the natural substrate and the possible roles of the catalytic residues in catalysis (Fujimoto *et al.*, 1998). In contrast to mesophilic *B. subtilis* α-amylase, BSTA is a thermostable enzyme. We have created site-directed mutants of BSTA in order to study the effect of mutation of an amino-acid residue near the catalytic site on the activity and have obtained some mutant enzymes that have altered specific activity, temperature/activity profile or pH/activity profile (Takase, 1993). However, the crystal structure of BSTA is not known and thus the structure determination is awaited for the correct interpretation of the results of the mutation experiments. Solution of the structure of BSTA will also be useful for understanding the mechanism of its thermal stability. In the present study, we have successfully obtained crystals of recombinant wild-type BSTA suitable for X-ray diffraction analysis. This was made possible by resolving several molecular forms with differently processed N-terminal residues. Here, we report purification of these forms, N-terminal processing, crystallization and preliminary crystallographic data.

2. Materials and methods

2.1. Enzyme purification

B. subtilis cells bearing the BSTA gene in the plasmid pTUB617 (Sohma *et al.*, 1987) were grown at 310 K for 27 h in Luria–Bertani broth containing 2 m*M* $CaCl_2$, as described previously (Takase *et al.*, 1988; Takase, 1993). The enzyme was purified from the culture supernatant by ammonium sulfate fractionation (90% saturation at 273 K), hydrophobic chromatography using Toyopearl HW-55F (TOSOH) (linear gradient elution from 25 to 0% saturated ammonium sulfate solution) and gel filtration using Toyopearl HW-55F, as described previously (Takase, 1993). The purity of the protein was checked by SDS–PAGE and isoelectric focusing (IEF). Several components were distinguished by IEF and were resolved into two major fractions with pI 7.3 and 8.2 by chromatofocusing on a Mono P HR 5/20 column (Pharmacia). These samples were subjected to N-terminal amino-acid sequencing (PE Applied Biosystems protein sequencer Procise-492 was used) and it was found that they contained additional sequences derived from the *B. subtilis* α-amylase leader sequence and the linker sequence used for cloning and expression in pTUB617 (Fig. 1). The fractions with pI 8.2 and pI 7.3 were used in crystallization experiments.

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses refer to the outermost shell.

Crystal system	Monoclinic
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 53.7 (2), b = 92.9 (4), c = 53.2 (2), β = 109.4 (1)
Total No. of reflections	183694
No. of unique reflections	29849
Resolution range (Å)	20–2.0 (2.09–2.00)
$I/\sigma(I) > 3$ (%)	81.4 (54.1)
Completeness (%)	89 (73)
$R_{\rm merge}$† (%)	7.4 (22.1)
Multiplicity	6.2

† $R_{\rm merge} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $\langle I(h)\rangle$ is the average intensity of the i observations of reflection h.

2.2. Crystallization and data collection

The protein was concentrated using an Amicon PM10 membrane and Ultrafree-CL filters (Millipore). Protein concentrations were estimated from absorbance at 280 nm by assuming an A_{280} of 2.89 for 1 mg ml^{-1} solution (Ogasahara *et al.*, 1970). Crystallization experiments were performed using the hanging-drop vapour-diffusion method (McPherson, 1982). The drops were prepared on siliconized cover slips and were equilibrated over 1 ml reservoir solution containing 35 m*M* sodium acetate (pH 4.6), 6.25%(*v*/*v*) 2-propanol in Linbro trays at 293 K. The pI 8.2 enzyme was crystallized using the same solution in the presence of 1.23%(*w*/*v*) acarbose (a pseudo-oligosaccharide inhibitor kindly provided by Bayer as a gift) in the drop. X-ray intensity data were collected at room temperature using the Weissenberg camera for macromolecules (Sakabe, 1991) at the BL-6A station at the synchrotron facility, Photon Factory, Tsukuba. The wavelength used was 1.00 Å and incident beam was collimated to 0.1 mm in diameter. The radius of the film cassette was set to 286.5 mm and the imaging-plate size was 200 × 400 mm. The diffraction data were collected to 2.0 Å resolution over 180° with an oscillation range of 5°. Data were processed with the program *DENZO* and were merged with the program *SCALEPACK* (Otwinowski & Minor, 1997).

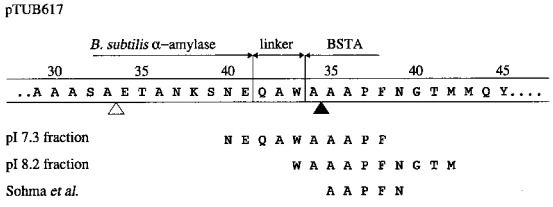

Figure 1
N-terminal amino-acid sequence of BSTA fused with *B. subtilis* α-amylase leader sequence in pTUB617. Amino acids are numbered from the initiator Met of each protein. N-terminal amino-acid sequences of the fractions with pI 7.3 and pI 8.2 purified in this study and that reported by Sohma *et al.* (1987) are aligned. Open triangles, signal-peptide cleavage site of *B. subtilis* α-amylase (Takase *et al.*, 1988); filled triangles, signal-peptide cleavage site of BSTA (Sohma *et al.*, 1987). Ala35 of the precursor protein of BSTA corresponds to the N-terminal residue (Ala1) of mature BSTA.

3. Results and discussion

When we examined the purity of the BSTA sample and the conditions for crystallization, we found that the sample purified as described by Takase (1993) consisted of at least three components, with pIs of 8.2, 7.3 and 6.0 (see also §2). The N-terminal amino-acid sequence analysis revealed N-terminal extensions (Fig. 1), which suggests that they are N-terminal processing intermediates. On the other hand, Sohma *et al.* (1987) obtained an N-terminal amino-acid sequence starting exactly from the N-terminus of mature BSTA and did not find the molecular forms reported here. The expression plasmid pTUB617 utilizes the promoter and signal peptide of *B. subtilis* α-amylase (Ohmura *et al.*, 1984). Previously, we showed that *B. subtilis* α-amylase could be produced as the N40 form, which has its N-terminus at the same position as the pI 7.3 BSTA form (Takase *et al.*, 1988). The N40 form was further processed to the N42 form, the mature *B. subtilis* α-amylase, by removal of two amino-acid residues from the N-terminus. Thus, it is likely that the pI 8.2 BSTA form (and the mature form reported by Sohma *et al.*, 1987) were produced by a similar processing mechanism *via* the pI 7.3 form. The N-terminal processing depends on the culture conditions (Takase *et al.*, 1988), which may reconcile the discrepancy between our results and those of Sohma *et al.* (1987).

For initial crystallization trials, we used the BSTA sample before purification by chromatofocusing. Crystals were obtained under two conditions from the fast screening kit I (Hampton Research). Well shaped plate-like crystals were obtained at 100 m*M* sodium cacodylate (pH 6.5), 30%(*v*/*v*) 2-propanol at 293 K after six months. The crystals diffracted to 2.2 Å resolution, but appeared to be twinned in the structure, which caused problems in data processing. Crystals obtained under the other set of conditions were intergrown or were too thin to collect diffraction data. On the other hand, using the pI 8.2 enzyme, a cluster of small crystals were obtained after three months under the following conditions: 35 m*M* sodium acetate (pH 4.6), 6.25%(*v*/*v*) 2-propanol, in the presence of 1.23%(*w*/*v*) acarbose in the drop. Further refinement of this and other conditions did not give any useful results. Unlike α-amylase from *B. licheniformis* (Suzuki *et al.*, 1990), the addition of chelators such as EDTA to the drop did not improve crystallization. Therefore, a small piece of crystal with approximate dimensions 0.1 × 0.05 × 0.02 mm was separated carefully from the cluster (Fig. 2) and used for data collection. Though the size of the crystal was very small, it diffracted beyond 2.0 Å resolution. The data-collection statistics are summarized in Table 1. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 53.7 (2), b = 92.9 (4), c = 53.2 (2) Å, β = 109.4 (1)°. Assuming one molecule in the asymmetric unit and using a molecular mass of 59 kDa for BSTA (Sohma *et al.*, 1987), the V_m value is 2.2 Å^3 Da^{-1} (Matthews, 1968), which indicates a solvent content of 41%. The structural analysis of BSTA by the molecular-replacement method using the structure of *B. licheniformis* α-amylase (PDB code 1bli; Machius *et al.*, 1998) as a search model is in progress.

Synchrotron radiation was used for this work with the approval of the Photon Factory Advisory Committee and High Energy Accelerator Research Organization, Tsukuba (proposal number 98 G156). We

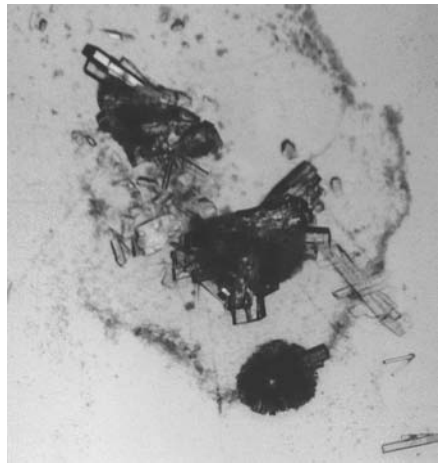

Figure 2
Cluster of BSTA crystals. A piece of approximate dimensions 0.1 × 0.05 × 0.02 mm cut out of the cluster was used for data collection.

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