CHAPTER 1

PURIFICATION, CRYSTALLIZATION AND PRELIMINARY CRYSTALLOGRAPHIC STUDY OF α-AMYLASE FROM *BACILLUS STEAROTHERMOPHILUS*

1.1. Introduction

The enzyme-catalyzed hydrolysis of starch plays a key role in the utilization of storage polysaccharides in many organisms. Bacteria secrete α-amylases into their culture media, and it has been suggested that this favors their survival.

First α-amylase isolated from *Bacillus stearothermophilus* had molecular weight of 15.6 kDa and consisted of two polypeptide chains linked by two disulfide bridges. An enzyme postulated to have a semi-random coil structure with any secondary structure presenting due to high proline content and due to the disulfide bonds. This unusual structure was speculated as the reason for the thermostability of this enzyme. (Manning *et al.*, 1961).

Since then different α-amylases have been characterized from different strains of *B. stearothermophilus* (Pfueller & Elliott, 1969; Ogasahara *et al.*, 1969; Mielenz, 1983; Tsukagoshi *et al.*, 1985; Nakajima *et al.*, 1985; Sohma *et al* ., 1985; Brosnan *et al.*, 1989; Vihinen & Mäntsäla, 1990; Moon-Ju Cho *et al.*, 1998; Zbigniew *et al.*, 1999).

All these amylases could be grouped into the three groups on the basis of their enzymatic activity and three dimensional structure: 1. α-amylases which strictly catalyze internal α-1,4 glycosidic linkage. Postulated to possess basic structure for family 13 α-amylase enzymes. (Pfueller & Elliott, 1969; Ogasahara *et al.*, 1969; Mielenz, 1983; Tsukagoshi *et al.*, 1985; Nakajima *et al.*, 1985; Sohma *et al* ., 1985; Brosnan *et al.*, 1989; Vihinen & Mäntsäla, 1990);

2. α-amylase with several type activity, such as dual hydrolysis of the α-1,4- and α-1,6-bonds and a transglycosylation activity of α-1,4- to α-1,6-

bond. Displays five-domain organization, extremely similar to that of the family 13 CGTases (Moon-Ju Cho *et al.*, 1998);

3. α-amylase, which exhibits no specificity for polymer chain ends and produces maltose as an end product of enzymatic digestion. Amylase forms dimer from monomers, composed from three domains: domain A ($(\beta/\alpha)_8$-barrel), domain C (greek key motif) and domain N (Zbigniew *et al.*, 1999).

The α-amylases from the first group, to which belongs an α-amylase used in this study, show high (>90%) homology in their amino acid sequences and possess common features. All have molecular weight in the range of 48-61 kDa, requires Ca ion for enzyme activity and stability, inhibited by acarbose or its derivative, have their temperature optima in the range of 60-80 degree and shows high homology (60-70%) to the other liquefying α-amylases from *B. amyloliquefaciens* and *B. licheniformis*.

Number of mutation works have been done regarding their enzymatic activity and thermal stability (Vihinen *et al.*, 1990; Holm *et al.*, 1990; Takase, 1993, Declerck *et al.*, 1997; Igarashi *et al.*, 1998 & Declerck *et al.*, 2000).

Our laboratory has been dealing with two bacterial α-amylases, *B. subtilis* and *B. stearothermophilus* α-amylases. Recently we reported the crystal structure of BSUA complexed with a substrate, which revealed the active site structure interacting with the natural substrate and provided possibility to analyze the role of catalytic residues in catalytic process (Fujimoto *et al.*, 1998).

In contrast to mesophilic *Bacillus subtilis* α-amylase, BSTA is thermostable enzyme. An α-amylase used in this research is α-amylase

from *B. stearotermophilus* strain A631 cloned in an α-amylase-deficient strain *B. subtilis* 207-25. It has molecular weight of 59 kDa (Sohma *et al.*, 1987).

Several mutant variants were obtained in our previous studies, that have altered their specific activity, temperature/activity profile, or pH/activity profile (Takase, 1993). To understand and explain these results we started structure analysis of BSTA. 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. In chapter 1 of these thesis we describe purification of those forms, discuss N-terminal processing, and report crystallization and preliminary crystallographic data.

1.2. Materials and methods

Enzyme purification

Bacillus subtilis cells bearing the BSTA A631 gene in the plasmid pTUB617 (Sohma *et al.*, 1987) were grown at 37°C for 27h in Luria-Bertani broth containing 2mM $CaCl_2$ as described previously (Takase *et al.*, 1988; Takase, 1993). The enzyme was purified from the culture supernatant by ammonium sulfate fractionation (90% saturation at 273K), hydrophobic chromatography using Toyopearl HW-55F (TOSOH, a linear gradient elution with 25% to 0% saturated ammonium sulfate solution), and gel filtration chromatography using Toyopearl HW-55F (TOSOH) as described before (Takase, 1993). The purity of the protein was checked by SDS-PAGE and isoelectricfocusing (IEF). Several components were distinguished by IEF and were resolved into two major fractions with pI 7.3 and 8.2 by chromatofocusing on MonoP HR 5/20 column (Pharmacia) using Polybuffer96 (Pharmacia) pH gradient ranging from pH 6.0 to pH 9.4. (Figure 1-1, 1-2). These three samples were subjected for 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* leader sequence and the linker sequence used for cloning and expression in pTUB617 (Figure 1-3). The fractions with pI 8.2 and pI 7.3 were used in crystallization experiment.

Crystallization and data collection

The protein was concentrated by Amicon equipment (PM10 membrane) and Ultrafree-CL filters (Millipore). Protein concentrations were estimated from OD_{280} by assuming an OD_{280} of 2.89 for 1mg/ml solution (Ogasahara *et al*, 1990). Crystallization experiments were performed using the vapor-diffusion hanging drop method (McPherson, 1982). The drops were prepared on siliconized cover slips and equilibrated over the reservoir solutions of 1ml in Linbro trays at 293K. The enzyme of pI 8.2 has been crystallized under the condition: 0.035M Na-acetate (pH 4.6), 0.035M $CaCl_2$, 6.25% (v/v) 2-propanol, 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 at the BL-6A station in the synchrotron facility, Photon Factory, High Energy Accelerator Research Organization, Tsukuba, Japan, using the Weissenberg Camera for Macromolecules (Sakabe, 1991). The wavelength used was 1.00Å and incident beam was collimated to 0.1mm in diameter. The radius of the film cassette was set to 286.5 mm and the imaging plate size was 200x400 mm^2. The diffraction data were collected to 2.0Å resolution over 180° in oscillation range of 5°.

Data processing and scaling

Data were processed with the program DENZO and merged using SCALEPACK (Otwinowski & Minor, 1997) on workstation Indigo2 R10000 (Silicon Graphics Inc.). Unit cell parameters and space group were determined using same programs.

1.3. Results and discussion

While we examined the purity of the BSTA sample and conditions for crystallization, we found that the sample purified as described by Takase (1993) consisted of at least three components with pI 8.2, 7.3 and 6.0 (see also Materials and methods). The N-terminal amino acid sequence analysis revealed N-terminal extensions (Figure 1-3), which suggests that they are N-terminal processing intermediates. On the other hand, Sohma *et al.* (1987) obtained the 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 BSUA (Ohmura *et al.*, 1984). Previously we showed that BSUA could be produced as the N40 form which had its N-terminus at the same position as BSTA pI 7.3 form (Takase *et al.*, 1988). The N40 form was further processed to the N42 form, the mature enzyme by removal of two amino acid residues from the N-terminus. Thus, it is likely that BSTA pI 8.2 form (and the mature form reported by Sohma *et al.*, (1987)) were produced by a similar processing mechanism via pI 7.3 form.

For initial trials of crystallization, we used the BSTA sample before purification on chromatofocusing. Crystals were obtained under two conditions of fast screening kit I (Hampton Research; Table 1-1). Good shaped plate-like crystals were obtained at 0.2M Na_3-citrate, 0.1M Na-cacodylate pH 6.5, 30% v/v 2-propanol at 293K after six months. The crystals diffracted to 2.2Å, but appeared to be twin in the structure, which caused troubles in data processing. The crystals obtained under another condition were inter grown or too thin to collect diffraction data. On the

other hand, using the pI 8.2 enzyme a cluster of small crystals were obtained under condition: 0.035M Na-acetate (pH 4.6), 0.035M $CaCl_2$, 6.25% (v/v) 2-propanol, after three months, in the presence of 1.23% (w/v) acarbose in the drop. Further refinement of this and other conditions did not give any good results. Unlike the 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 crystals with dimensions of approximately 0.1x0.05x0.02 mm^3 was separated carefully from the cluster (Figure 1-4) and used for data collection. Though the size of the crystal was very small, it diffracted to 2.0Å (Figure 1-5). The data collection statistics are summarized in Table 1-2. The crystals belong to the monoclinic $P2_1$, with unit cell dimensions of a=53.5, b=92.8, c=53.0Å, β=109.4°. Assuming a molecular mass of 59 kDa for BSTA in the asymmetric unit, the V_m value is 2.2$Å^3Da^{-1}$ (Matthews, 1968), which indicates a solvent content of 41%.

1.4. Summary

A recombinant α-amylase from *Bacillus stearothermophilus* was purified to homogeneity and crystallized at 293K using the hanging-drop vapor diffusion method under the condition: 0.035M Na-acetate (pH 4.61), 0.035M $CaCl_2$, 6.25% (v/v) 2-propanol, in the presence of 1.23% (w/v) acarbose (pseudo-oligosaccharide inhibitor) in the drop. The crystals diffracted beyond 2.0Å resolution using a synchrotron radiation in Photon Factory, Tsukuba. They belong to the monoclinic space group $P2_1$, with cell dimensions of a=53.5, b=92.8, c=53.1Å and β =109.4°. The calculated packing density of 2.2$Å^3Da^{-1}$ indicates an estimated solvent content of 41%, assuming a molecular mass of 59 kDa for BSTA in the asymmetric unit.