GENERAL INTRODUCTION

Amylases (EC 3.2.1) are enzymes which hydrolyze starch and at present are classified into the three groups based on their action modes and the end product from starch degradation. They are α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2) and α-glucan glucosidases (glucoamylase; EC 3.2.1.3) (The Amylase Research Society of Japan, 1995). Glucoamylases and β-amylases are exo-type enzymes cleaving glucose and maltose units, respectively from the non reducing end of starch materials by hydrolyzing the α-1,4-glycosidic bonds (Cho *et al.*, 1998; Schema 1).

α-Amylase (α-1,4-glucan-4-glucanohydrolase EC 3.2.1.1) catalyzes the hydrolysis of an internal α-D-(1,4)-glycosidic linkages in starch and other linear and branched polysaccharides (Schema 1). This enzyme is distributed in most living organisms and is an essential enzyme, playing an important role in energy metabolism (The Amylase Research Society of Japan, 1995). To date α-amylases from various sources have been isolated and studied for their structure and function.

Although all α-amylases share the same catalytic function, their amino acid sequences are quite varied, that is, sequence identity among α-amylases from bacteria, fungi, plants and animals is as low as 10%. Four highly conserved regions are found in the amino acid sequences of all α-amylases. These regions are thought to be necessary to build up the active site and the conserved calcium ion- binding site (Nakajima *et al.*, 1986; Figure 1).

Despite low sequence identity, crystal structures of fungal, plant, animal and bacterial α-amylases such as α-amylase from *A. oryzae*, called Taka-amylase A (TAA), (Matsuura *et al.*, 1984), porcine pancreatic α-

amylase (PPA) (Buisson *et al.*, 1993), an acid α-amylase from *A. niger* (Boel *et al..*, 1990) human saliva (Rydberg *et al.*, 1999), yellow meal worm (Strobl *et al.*, 1998), and *Alteromonas haloplanctis* (Aghajari *et al.*, 1998) bacterial α-amylases from *B.subtilis* (BSUA) (Fujimoto *et al.*, 1998) and *B. licheniformis* (BLA) (Machius *et al.*, 1998) show relative similarity in their three dimensional structure.

α-Amylases from class EC 3.2.1.1 are monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains A, B and C, and belong to the $(\beta/\alpha)_8$-barrel protein family (Farber & Petsko, 1990). Active site of the enzymes with this $(\beta/\alpha)_8$-barrel motif is composed from the residues, situated at the C-terminal side of the β-strands of the central β-barrel. Domain A represents a compact core structure comprising a $(\beta/\alpha)_8$-barrel. Domain B shows a variable structure in α-amylases, small in TAA (Matsuura *et al.*, 1984), PPA (Buisson *et al.*, 1993), BSUA (Fujimoto *et al.*, 1998) and slightly complexed in BLA (Machius *et al.*, 1998) and BSTA. A third C domain forms a greek-key motif and stabilized with a calcium ion in BLA (Machius *et al.*, 1998) and BSTA.

Amylases, specially those produced by bacteria and fungi, are widely used in starch processing, paper manufacture and pharmacology industries. (Fogarty *et al.*, 1983; Takagi *et al.*, 1971; Thoma *et al.*, 1971). Bacterial α-amylases from genus *Bacillus* are most industrially significant and best studied amylases (Kindle, 1983).

Bacterial α-amylases are divided into two types from the specificity: one is starch liquefying and the other, saccharifying type (The Amylase Research Society of Japan, 1995).

Saccharifying type α-amylase is secreted by aerobic, spore forming, mesophilic bacteria *Bacillus subtilis*. It hydrolyzes amylose to produce

glucose, maltose and maltotriose. On a long incubation, the enzyme hydrolyzes amylose to produce glucose, maltose and maltotriose. Also it hydrolyses α-1,4-linked glucosidic chains existing outside the anomalous linkage of amylopectin, glycogen and produces glucose, maltose and maltotriose. (The Amylase Research Society of Japan, 1995; Schema 1).

Liquefying α-amylase are produced by *Bacillus amyloliquefacines*, *Bacillus stearothermophilus* and *Bacillus licheniformis* and it hydrolyzes amylose to produce glucose, maltose, maltotriose, -tetraose, -pentaose and -hexaose. (The Amylase Research Society of Japan, 1995; Schema 1).

Traditionally, starch hydrolysis was carried out using acid and high temperatures (Shildneck and Smith, 1967). Enzymatic hydrolysis of starch has now almost replaced acid hydrolysis of the starch due to many advantages it has (Norman, 1979; Fogarty, 1983). At present the enzyme-catalyzed hydrolysis of starch is fundamental to many important industrial reactions and is the basis of both the liquefaction and saccharification processes during the production of high-glucose syrups from corn starch. Starch-active enzymes are also frequently utilized in the textile and detergent industries and are increasingly playing a role in the baking industry (Dauter *et al.*, 1999).

The majority of industrial applications of α-amylases require their use at high temperatures (up to 110°C), thus necessitating a search for increasingly thermostable enzymes (Kindle, 1983). Among the thermostable α-amylases, those produced by *B. licheniformis*, *B. subtilis*, and partially *B. stearothermophilus* are being produced on an industrial scale and gradually replacing the traditional mesophilic α-amylase derived from *B. amyloliquefaciens* (BAA) (The Amylase Research Society of Japan, 1995).

Thus far, the search for the thermostable enzymes has been limited to screening α-amylases from thermophilic microorganisms (Kindle, 1983). In order to understand reason why the enzymes with high homology have such different thermostability as well as to design the rational approaches to the thermostabilization of enzymes, a knowledge about the structural basis of the thermostability of the proteins is necessary. Site-directed mutagenesis is one of the strategy for the production and study of the thermostable α-amylases (Tomazic & Klibanov, 1988). Besides its practical importance, the research on the correlation between the amino acid sequence of a protein and the stability of its biologically active conformation is one of the central problems of molecular biology.

Although thermostable α-amylases from *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* display a 20 to 30 degree difference in their thermal stability (Susan *et al.*, 1987; Tomazic & Klibanov, 1988), they show quite high homology in their amino acid sequences, 65% between BSTA and BLA, 67% between BSTA and BAA, and 80% between BLA and BAA (Yuuki *et al.*, 1985; Nakajima *et al.*, 1986). For these reason these three enzymes are very unique natural model for investigation of mechanisms of the thermostability.

Up to now many works has been done on the reversible and irreversible thermoinactivaton of BLA, BAA and BSTA (Tomazic *et al.*, 1987; Tomazic & Klibanov, 1988; Suzuki *et al.*, 1989; Brosnan *et al.*, 1992) as well as numerous mutational experiments to approach the correlation between the conformational changes and the function and stability of the enzymes (Vihinen *et al.*, 1990; Holm *et al.*, 1990; Takase, 1993; Declerck *et al.*, 1997; Igarashi *et al.*, 1998 & Declerck *et al.*, 2000).

According to the thermoinactivation studies, listed above, the main reason of the inactivation of the enzymes at high temperature is unfolding and improper refolding of protein molecules. The other reason is a deamidation process of asparagine and glutamine residues at elevated temperatures, which creates an unfavorable electrostatic interactions.

Mutational experiments on BSTA, BAA, BLA and α-amylase from *Bacillus* sp. KSM-1378 (LAMY) revealed many regions and residues that are critical for the thermal stability in these enzymes. Among them the three mutations have been unambiguously identified as stabilizing attributes unique to BLA. That are: 1. deletion of the two amino acid residues near the insertion site in BAA and LAMY, which corresponds to the Arg179 and Gly180 in BSTA (Figure 2-9). 2. disturbance of Asp121-Arg127 salt bridge in BLA 3. Lys269Ala substitution in BLA and BAA (Declerck *et al.*, 2000). However, as noted by the same author it is unlikely, that these three features alone determine the increased thermostability of BLA. Since the only crystal structure of BLA (but not of BAA) is known (Machius *et al.*, 1998), solution of the structure of BSTA will aid in further understanding the mechanism of thermostability.

Our laboratory has been dealing with two bacterial α-amylases, *Bacillus subtilis* α-amylase (BSUA) and *B.stearothermophilus* α-amylase (BSTA). The crystal structure of BSUA complexed with a substrate was reported, 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).

Previously, the mutant variants for BSTA was created on the basis of the resolved crystal structure of α-amylase from Aspergillus Oryzae, TAKA-amylase (Takase, 1993). In TAA, Asn295 (Asn329 in BSTA) locates

in close proximity to the catalytic residue Asp297 (Asp331 in BSTA). To study the effect of mutation of an amino acid near the catalytic site on the activity, Asn329 was chosen for mutation experiments. Some mutant enzymes that had altered specific activity, temperature/activity profile, or pH/activity profile were obtained (Takase, 1993). Determination of the three dimensional structure of BSTA would help us to interpret these observations.

In this thesis the crystal structure of wild type BSTA is defined and studied in comparison with BLA in order to understand the difference in their termostability. In chapter 1, the crystallization and preliminary X-ray diffraction studies of BSTA are described. In chapter 2, crystal structure of BSTA is described and investigated in comparison with that of BLA.