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INFLUENCE OF ASPARAGINE MUTATIONS ON ENZYMATIC ACTIVITY AND THERMOSTABILITY OF A RECOMBINANT α-AMYLASE FROM ALKALIPHILIC *Bacillus* SP. STRAIN TS-23

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Abstract- Asparagine (Asn) deamination is one of the critical determinants of protein thermostability. In this study, the potential Asn residues (Asn63, Asn100, Asn180, Asn204, Asn224 and Asn226) involved in the deamination process of a truncated *Bacillus* sp. strain TS-23 α-amylase (BACΔNC) have been selected and substituted with aspartate by site-directed mutagenesis. Parental and mutant enzymes were purified by nickel-chelated chromatography and the purified proteins had a molecular mass of approximately 54 kD. Substitution of Asn63, Asn100 and Asn180 with aspartic acid resulted in a significant reduction in the starch-degrading ability, whereas the enzymatic activity was almost completely abolished by the Asn204, Asn224 and Asn226 mutations. Far-UV circular dichroism spectra were nearly identical for both the parental enzyme and its variants, but they displayed a different sensitivity towards temperature-induced denaturation. BACΔNC had a T_m value of 77.5°C and the value was increased to between 85.5-95.2°C for the mutant enzymes. Collectively, the experimental results indicate that the deamination of these residues may have a deleterious effect on thermostability of BACΔNC.

Keywords- *Bacillus* sp. TS-23, α-amylase, homology modeling, asparagine, site-directed mutagenesis, thermostability

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Introduction

α-Amylase (EC 3.2.1.1) is an endo-acting enzyme that catalyzes the hydrolysis of α-1,4-glycosidic bonds in starch and related substrates [1]. This enzyme is widely distributed in three domains of life and is of great significance in present-day biotechnology [1,2]. Importantly, α-amylases constitute a class of industrial enzymes with approximately 25% of the world biocatalyst market [3]. Microbial α-amylases are of special interest in industrial applications ranging from food, detergent, textile to paper industry [4,5]. With the advent of new frontiers in biotechnology, the application spectrum of α-amylases has expanded into many other fields, including clinical, medicinal and analytical chemistries [2].

Enzymes may undergo essentially irreversible inactivation through deamination of glutamine and asparagine side chains at temperatures above those to which they are ordinarily exposed in their natural environment. Deamination is a hydrolytic reaction that leads to a change in the primary structure and in turn may affect the secondary and tertiary structures of proteins [6]. It has been reported that deamination at amide residues is a dominant pathway for protein denaturation [7] and formation of isoaspartate via deamination of asparaginyl residues plays a major role in protein instability [8]. A study on *Bacillus licheniformis* α-amylase (BLA) has demonstrated that the deamidation of Asn/Gln residues in the primary structure of the enzyme is responsible for the irreversible thermoinactivation [9,10]. Eventually, thermo-stabilization of BLA has been achieved by replacing the critical Asn residues of its primary structure [11].

Earlier, a raw-starch degrading α-amylase gene from *Bacillus* sp. strain TS-23 has been isolated and over-expressed in recombinant *Escherichia coli* M15 cells [12]. A truncated α-amylase (BACΔNC) was further constructed by deletion of both the N-terminal signal peptide and the starch-binding domain from the cloned enzyme [13]. The primary structure of BACΔNC consists of 489 amino acid residues corresponding to a molecular mass of 55.2 kDa, which is closest to the protein size of α-amylases of *Bacillus licheniformis* [14] and *Bacillus stearothermophilus* [15]. In this investigation, the structure-based mutagenesis was employed to probe the role of six Asn residues in thermostability of BACΔNC.

Materials and Methods

Structural Modeling

Computer modeling of BACΔNC was conducted to identify the target Asn residues. The molecular structure of BACΔNC was essentially constructed at SWISS MODEL Server using X-ray crystal structure (PDB code: 3DC0) of *Bacillus* sp. KR-8104 α-amylase,

and the model molecular structure was further energy minimized by the usage of CNS program (http://cns.csb.yale.edu/v1.1/).

Site-directed Mutagenesis, Gene Expression and Protein Purification

Mutations were introduced into plasmid pQE-AMYΔNC [16] using QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and a pair of complementary primers [Table-1]. The success of intended mutations was confirmed by DNA sequencing.

Table 1- Oligonucleotide primers used for site-directed mutagenesis

Enzyme	Nucleotide sequence (5' to 3')[a]	Change
N63D	AATGAAGCCGCAGATCTTTCTTCGCTC	AAT→GAT
N100D	CTTGGGGAATTTGATCAAAAAGGAACG	AAT→GAT
N180D	CCCGGTCGGGGGGACACATACTCGAGT	AAC→GAC
N204D	AGCCGAAAATTAGATCGGATTTACAAA	AAT→GAT
N224D	GTCGATACAGAAGACGGAAACTATGAT	AAC→GAC
N226D	ACAGAAAACGGAGACTATGATTATTTA	AAC→GAC

[a] *Only the sense sequences are shown.*

Expression and one-step purification of BACΔNC and its variants were performed as described previously [13].

Protein Analyses

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS - PAGE) was performed on 12% acrylamide slabs using a Mini-Protean III Cell (Bio-Rad). The proteins were stained with Coomassie Brilliant Blue R-250 (CBR) dissolved in 50% methanol - 10% acetic acid, and destained with a solution of 30% methanol and 10% acetic acid. The protein markers includes phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), chicken egg albumin (45.0 kDa), and carbonic anhydrase (29 kDa).

Protein concentrations were determined using the Bio-Rad protein assay reagent and bovine serum albumin as a standard protein.

Amylolytic Activity Assay

The assay mixture contained 250 ml of soluble starch in 50 mM Tris -HCl buffer (pH 8.0) and 250 ml of approximately diluted enzyme solution. The reaction was performed at 60°C for 10 min and stopped by the addition of 0.5 ml of 3,5-dinitrosalicyclic reagent [16]. The enzyme activities were obtained from a calibration curve prepared by following the same procedure with D-glucose as the standard. One unit of amylase activity is defined as the amount of enzyme that releases the amount of reducing sugar equivalent to 1 mmol of glucose per min under the assay conditions.

Circular Dichroism

Circular dichroism (CD) analysis of the purified proteins was carried out with the established procedures [17].

Results and Discussion

The molecular structure of BACΔNC was essentially constructed at SWISS MODEL Server using the crystal structure of α-amylase from *Bacillus* sp. KR-8104 (PDB code: 3DC0). As shown in [Fig-1], the three-dimensional structure of BACΔNC shows the overall topology characteristic of α-amylases and related amylolytic enzymes that belong to glycosyl hydrolase (GH) family 13 [18]. The molecular structure is made up of a central $(\alpha/\beta)_8$ barrel (domain A), a small B domain, and a C-terminal domain containing a Greek key motif (domain C). It has been established that deamination of asparagine and glutamine residues is mainly responsible for the irreversible inactivation of proteins at high temperatures [19]. Besides, asparagine has approximately 10-fold higher propensity to deamidate than glutamine [20]. In this regard, we have chosen six Asn residues that potentially could be important determinants of BACΔNC thermostability [Fig-1]. SDS - PAGE analysis showed that the molecular mass of the purified proteins was approximately 54 kD [Fig-2A]. The purified BACΔNC had a specific activity of 171.5 ± 12.8 U/mg protein. As compared with parental enzyme, the relative activity was decreased to 63.1, 40.4, 62.9, 1.3, 1.2 and 2.4% for N63D, N100D, N180D, N204D, N224D and N226D, respectively [Fig-2B]. These observations indicate that the mutations do impair the catalytic activity of the recombinant enzyme. A generally accepted mechanism for GH family 13 is that it proceeds via α-retaining double displacement process in which a glutamate residue acts as the acid-base catalyst and an aspartate residue functions as the nucleophile [21]. As shown in [Fig-1], the corresponding residues of BACΔNC are Asp233 and Glu264. These two residues locate within the central $(\alpha/\beta)_8$ barrel and stay away from the microenvironment of Asn63, Asn100 and Asn180. In this regard, it can be explained why the introduced mutation at 63, 100 and 180 positions does not dramatically affect the enzyme function. In contrast, Asn204, Asn224 and Asn226 are part of B domain loop, which very closely situates to the catalytic residues Asp233 and Glu264 of BACΔNC. Any change in the region might consequently disturb the catalytic process of the enzyme.

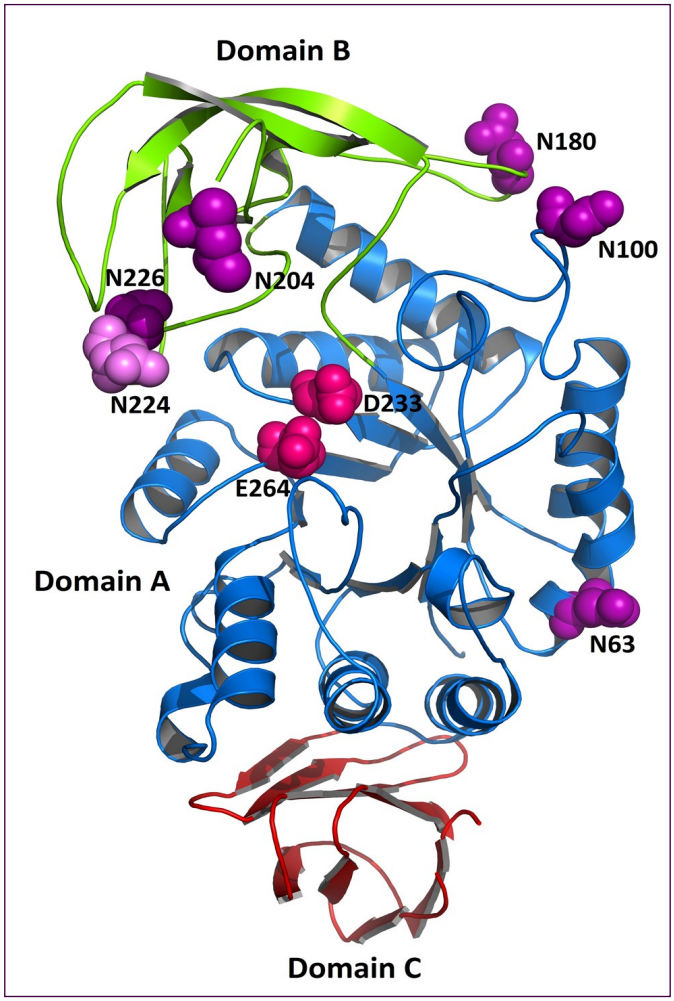

Fig. 1- Ribbon representation of the modeled structure of BACΔNC. The spheres correspond to the selected Asn residues (pink) and the catalytic residues (red).

The far-UV CD spectrum of BACΔNC displays two strong peaks of negative ellipticity at 208 and 222 nm that are indicative of substantial α-helical contents [Fig-3A]. The spectrum was further quantitatively analyzed by the DICHROWEB server [22]. The estimated helical and β-strand contents of BACΔNC are 36 and 25%, respectively. The calculated helical and β-strand contents of mutant proteins were basically consistent with that of the parental enzyme. These data clearly indicate that no significant change in the secondary structural elements of BACΔNC have been occurred as consequence of the introduced mutations. In an attempt to probe the conformation of parental and mutant enzymes, thermal denaturation of these proteins was monitored by following the loss of CD signal at 222 nm. [Fig-3B] shows the transition curves obtained with protein solutions at a heating rate of 0.5°C. A well-defined transition with the melting temperature (T_m) of 77.5°C was observed for BACΔNC. Interestingly, the T_m value for N63D, N100D, N180D, N204D, N224D and N226D was 93.3, 95.2, 86.1, 86.5, 87.9 and 85.5°C, respectively. These data reflect that the mutations have a beneficial effect on the thermostability of the enzyme.

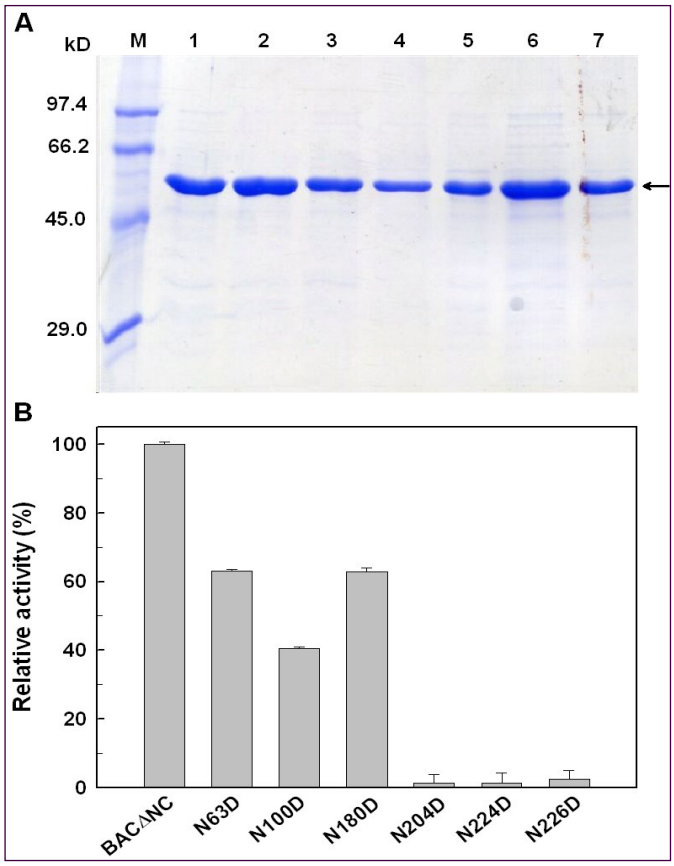

Fig. 2- SDS-PAGE analysis (A) and specific activity (B) of BACΔNC and its variants. Lanes: M, protein size markers; 1, BACΔNC; 2, N63D; 3, N100D; 4, N180D; 5, N204D; 6, N224D; 7, N226D. The position of purified enzymes was indicated by an arrow.

In conclusion, the majority of industrial applications of α-amylases require their performance at high temperatures. Our work reveals that removal of the possible deaminating residues definitely improves the thermostability of BACΔNC enzyme. Although the highly thermostable BLA has been extensively used in starch-processing industry, the drawback of this enzyme is that it is not active in the absence of Ca^{2+} ion. The innovation of this study is the dramatic increase in thermostability of this Ca^{2+}-independent BACΔNC that makes the engineered enzymes more suitable for industrial applications.

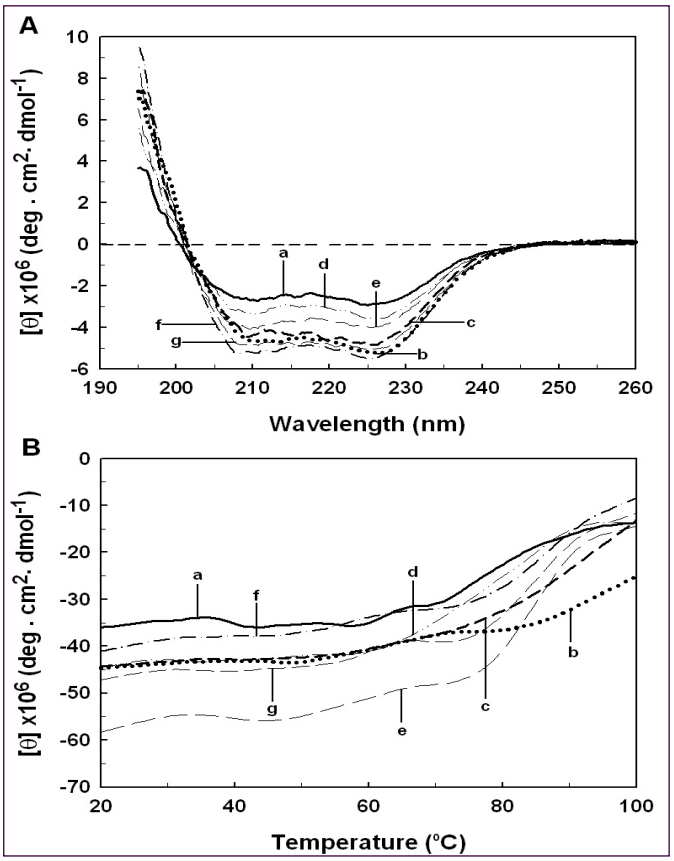

Fig. 3- CD spectra (A) and temperature-induced denaturation (B) of BACΔNC and its variants. The far-UV CD spectra were recorded at 22°C. Thermal unfolding experiment was performed at a protein concentration of 1.2 µg/µl. Lines: a, BACΔNC; b, N63D; c, N100D; d, N180D; e, N204D; f, N224D; g, N226D.

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Conflict of Interest : None Declared

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