

Influence of Calcium Ions on the Thermal Characteristics of α -amylase from Thermophilic *Anoxybacillus* sp. GXS-BL

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Abstract: Background: α -Amylases are starch-degrading enzymes and used widely, the study on thermostability of α -amylase is a central requirement for its application in life science and biotechnology.

Objective: In this article, our motivation is to study how the effect of Ca^{2+} ions on the structure and thermal characterization of α -amylase (AGXA) from thermophilic *Anoxybacillus* sp. GXS-BL.

Methods: α -Amylase activity was assayed with soluble starch as the substrate, and the amount of sugar released was determined by DNS method. For AGXA with calcium ions and without calcium ions, optimum temperature (T_{opt}), half-inactivation temperature (T_{50}) and thermal inactivation (half-life, $t_{1/2}$) was evaluated. The thermal denaturation of the enzymes was determined by DSC and CD methods. 3D structure of AGXA was homology modeled with α -amylase (5A2A) as the template.

Results: With calcium ions, the values of T_{opt} , T_{50} , $t_{1/2}$, T_m and ΔH in AGXA were significantly higher than those of AGXA without calcium ions, showing calcium ions had stabilizing effects on α -amylase structure with the increased temperature. Based on DSC measurements AGXA underwent thermal denaturation by adopting two-state irreversible unfolding processes. Based on the CD spectra, AGXA without calcium ions exhibited two transition states upon unfolding, including α -helical contents increasing, and the transition from α -helices to β -sheet structures, which was obviously different in AGXA with Ca^{2+} ions, and up to 4 Ca^{2+} ions were located on the inter-domain or intra-domain regions according to the modeling structure.

Conclusion: These results reveal that Ca^{2+} ions have pronounced influences on the thermostability of AGXA structure.

Keywords: α -amylase, calcium ions, circular dichroism, differential scanning calorimetry, homology modeling, thermostability.

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1. INTRODUCTION

Thermophilic bacteria are extremophiles adapted to life at high temperatures (optimum growth is observed at temperatures above 45°C) [1]. They inhabit separate permanently hot ecological niches, such as areas with geothermal and volcanic activity [2]. Their enzymes (thermozymes) have unique characteristics that include increased temperature, chemical, and pH stability [3]. Among these interesting characteristics thermostability is an important consideration for proteins, particularly for those which are involved in biological processes at elevated temperatures [4].

α -Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α -1,4-*O*-glycosidic bonds in polysaccharides while retaining the α -anomeric configuration of the products [5]. They mostly belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes [6, 7]. α -Amylases can be obtained from a variety of organisms, including plants, animals and microbes [8]. Bacterial α -amylases, especially those from the *Bacillus* species, are used widely in the food, pharmaceuticals, textile, paper, detergent and bioenergy industries [9]. Thermostable α -amylases are especially available from certain thermophilic *Bacillus* species of bacteria, such as *Geobacillus stearothermophilus*, *Anoxybacillus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus alvei*, *Bacillus cereus*, and *Bacillus globisporus* [10]. Compared to the well-studied genera *Geobacillus* or *Bacillus*, *Anoxybacillus* was a new genus first proposed by Pikuta in the year 2000 [11]. To date, a total of 22 species and two subspecies of *Anoxybacillus*

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lus with validly published names have been reported (<http://www.bacterio.net>). Most reported data have revealed that members of *Anoxybacillus* produce biotechnologically important enzymes that are thermostable and tolerant of alkaline pH conditions [12].

Calcium ions often have crucial roles in structure, function, and stability of α -amylases and are especially crucial in thermophilic α -amylases [13, 14]. They are considered to be important for maintaining protein structures in their correct conformations and for resisting thermal inactivation of enzymes [15, 16]. It has been shown that the removal of calcium ions from barley α -amylase irreversibly inactivate the enzyme, whereas the bacterial α -amylases restored its activity after the addition of calcium ions [17]. Some reports indicate that the role of calcium ions in α -amylases is mainly structural, as their catalytic sites are far from the calcium-binding sites [18, 19]. Although most α -amylases are Ca^{2+} -dependent, there are reports of Ca^{2+} -independent α -amylases [20–22], and there are also some α -amylases that are inhibited by Ca^{2+} ions [23, 24]. The study of the effect of calcium ions on the activity and stability of α -amylases from thermophiles may be helpful to determine a mechanism of Ca^{2+} -binding protein in the thermal environment, furthermore to wider application range of the enzymes. We have previously reported that alkalitolerant α -amylase (AGXA) from thermophilic *Anoxybacillus* sp.GXS-BL was moderately thermostable and did not require Ca^{2+} for its activity [25]. In the present study, we investigate the impact of calcium ions on the thermal characterization of the α -amylase.

It is reported that the α -amylases from thermophilic *Anoxybacillus* species (ASKA and ADTA) and *Geobacillus thermoleovorans* (Pizzo, GTA, and Gtamy II), and *halophilic Bacillus aquimaris* (BaqA) have been proposed as a novel subfamily of the α -amylase family, which exhibiting (1) high maltose production; (2) the ability to degrade raw starch; (3) a long C-terminal sequence containing five conserved aromatic residues; (4) dual tryptophan residues between CSR-V and CSR-II; and (5) a unique stretch of amino acids (LPDIX) in CSR-V [26]. The crystal structure of GTA (PDB ID: 4E2O) provided the first insight into the overall structure and Ca^{2+} binding sites of this new GH13 subfamily of α -amylases [27]. To increase understanding of the unique GH13 subfamily, the homologous structures of ASKA (PDB ID: 5A2A, 5A2B and 5A2C) from *Anoxybacillus* in the novel GH13 subfamily were published recently [28]. The results show that there are four Ca^{2+} binding sites in all three ASKA structures. And sequence alignment suggests that all the residues that interact with the four Ca^{2+} ions are conserved in most α -amylases from *Anoxybacillus* spp. In this article, we just want to study how the effect of Ca^{2+} ions on the structure and thermal characterization of AGXA using homology modeling, Differential Scanning Calorimetry (DSC) and Circular Dichroism (CD) techniques. To our knowledge, this is the first report on the calorimetric and spectroscopic characteristics of the novel GH13 subfamily. As revealed in this article, the roles of Ca^{2+} ions on the structure, activity and thermostability of AGXA are interesting and profound. We think these results may be helpful to deep insight into the mechanisms of Ca^{2+} -binding α -amylase adapting to the thermal environment.

2. MATERIALS AND METHODS

2.1. Materials

Anoxybacillus sp.GXS-BL was utilized as the α -amylase gene donor strain, which was isolated from the Tengchong hot spring of Yunnan province of China (GenBank Accession No. JF831203). *Escherichia coli* DH5 α and *E. coli* M15 [pREP4] from Novagen (WI, USA) were used as cloning and expression hosts, respectively. The plasmid vector used for cloning and expression was pQE30 (Novagen, USA). Iso-propyl- β -D-thiogalactopyranoside (IPTG), ampicillin and kanamycin were prepared from Sangon (Shanghai, China). Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices were obtained from Qiagen (CA, USA). HiTrap desalting G-25 and Superdex 75 10/300GL were procured from GE Health (Uppsala, Sweden). Amicon Ultra15 Centrifugal Filter Units (MWCO, 10 kDa) and D-tube dialyzers (MWCO, 12 kDa) were procured from Millipore (MA, USA). Soluble potato starch, calcium chloride and other used chemicals were analytical-grade and obtained from Sigma-Aldrich (St. Louis, USA). All solutions were prepared in Milli-Q (Millipore, USA) water. After treatment with EDTA purified α -amylase samples in 50 mM Tris-HCl buffer (pH 8.0) were dialyzed extensively in the same buffer to remove EDTA. All Ca^{2+} -removal and following steps were performed in plastic vessels to avoid any Ca^{2+} contamination from any glass. For Ca^{2+} -added proteins, small aliquots of a 1 M Ca^{2+} solution was added to Ca^{2+} -free proteins to achieve a final Ca^{2+} concentration of 5 mM.

2.2. Protein Estimation

The purified protein samples were concentrated by an Amicon centrifugal filter unit (MWCO, 10 kDa), enzyme protein concentration was measured using a Bradford assay using Bovine Serum Albumin (BSA) as the standard [29].

2.3. α -Amylase Activity

α -Amylase activity was assayed with soluble starch as the substrate, and the amount of sugar released was determined by using the 3,5-dinitrosalicylic acid (DNS) method [30]. Ten μl of diluted purified Ca^{2+} -added and Ca^{2+} -free enzyme (0.1 mg/ml) were added to 490 μl of Tris-HCl buffer (50 mM, pH 8.0) at 1% (w/v) soluble-starch. After a 10-min incubation at the specified temperature, the reaction was stopped by adding 500 μl of DNS reagent, the absorbance was recorded at 540 nm using a DU800 UV-Visible spectrophotometer (BeckmanCoulter, Germany), and the reducing sugar equivalent was determined using a maltose standard. All enzyme activity measurements were performed in triplicate.

2.4. Homology Modeling

Three-dimensional structure of the α -amylase was homology modeled using BIOVIA Discovery Studio 2016 software. The crystal structure of α -amylase (5A2A, at 1.9 Å resolution) from *Anoxybacillus* sp. SK3-4 was used as the template [28]. After aligning the model sequence to the template, a 3D model was built using Modeller 9.15 [31]. Prior to the modeling, the ligand glucose and crystallographic wa-

ter molecules were removed from the template structure. Ten AGXA models were generated. Models with the lowest energy value (DOPE score) [32] were selected and evaluated by the Root-Mean Square Deviation (RMSD), Ramachandran plot [33] and Profiles-3D protocols [34]. A model with the best scores was selected for refinement and validation.

2.5. Thermal Stabilities

Optimum temperature (T_{opt}) for activity was determined in a range of 40°C to 80°C for 10 min in 50 mM Tris-HCl buffer (pH 8.0). The highest activity at a specified temperature was designated 100%. Half-inactivation temperature (T_{50}) was the temperature where the enzyme retains 50% of its initial activity after 30 min of incubation. The α -amylase was incubated at temperatures between 50-80°C for 30 min, followed by cooling at 4°C for 10 min, and residual activities were then measured at standard conditions. Half-life ($t_{1/2}$) was the time at which the enzyme was reduced to 50% of its initial activity at a set temperature. Thermal inactivation of the α -amylase (0.1 mg/ml) was determined at 70°C by taking samples at regular intervals which were then cooled on ice, and residual activity was measured as described above. All reactions were performed in triplicate.

2.6. DSC Measurements

The thermal denaturation was determined by differential scanning calorimeter (Microcal VP Capillary DSC system, Malvern). Ca^{2+} -added and Ca^{2+} -free protein samples (0.80 mg/ml) in 50 mM Tris-HCl buffer (pH 8.0) were degassed with a Microcal thermovac instrument (GE Healthcare). Then, 400 μ l of protein and buffer were loaded into the sample and reference cells, respectively. Measurements were performed by increasing the temperature from 10°C to 120°C at 1°C/min scan rate. Protein sample scans were corrected by subtraction of the respective buffer from the baseline values. Molar excess heat capacities (C_p) were obtained by normalizing to the sample concentration. The data were analyzed according to a non-two state model using Origin

7.0 software (Microcal). The apparent T_m was determined as the temperature corresponding to maximum C_p , the calorimetric enthalpy ΔH corresponded to the area under the peak of the heat capacity versus temperature graph, and the van't Hoff enthalpy ΔH_v was determined by the shape of the transition peak. The test was repeated three times.

2.7. CD Spectroscopy Measurements

The Chirascan instrument (Applied Photophysics) was also utilized to conduct the thermal denaturation experiments. The spectrometer was equipped with a Peltier-type temperature controller attached to a water bath. Ca^{2+} -added and Ca^{2+} -free protein samples (0.28 mg/ml) in 5 mM Tris-HCl buffer (pH 8.0) were degassed as described above. Next, 300 μ l of a protein sample was pipetted into Hellma quartz cuvettes with a path length of 0.1 cm. The CD spectra were observed in the 201-260 nm range in 1 nm steps, and the time-per-point was 0.7 s. The bandwidth was set to 0.8 nm, and the temperature was varied from 35°C-85°C in step with a ramp rate of 1°C/min. The sample temperature was measured directly using an external probe immersed in the sample. The data was processed by Global3 software.

3. RESULTS AND DISCUSSION

In this work, the recombinant α -amylase AGXA was overexpressed in *E. coli* M15 [pREP4] and purified by Ni-NTA and size-exclusion chromatography. The AGXA purity was showed by SDS-PAGE and the molecular mass was about 55 kDa (Figure 1).

3.1. Homology Modeling

Searching sequences by similarity, results showed that the highest similarity with AGXA was observed in 5A2A (97%), followed by 4E2O (72%), with the others showing less than 35% similarity. A 3D model of AGXA was constructed using Discovery Studio 2016 software, with 5A2A used as the template. As shown in Figure 2, the model structure consists of three domains containing characteristic of

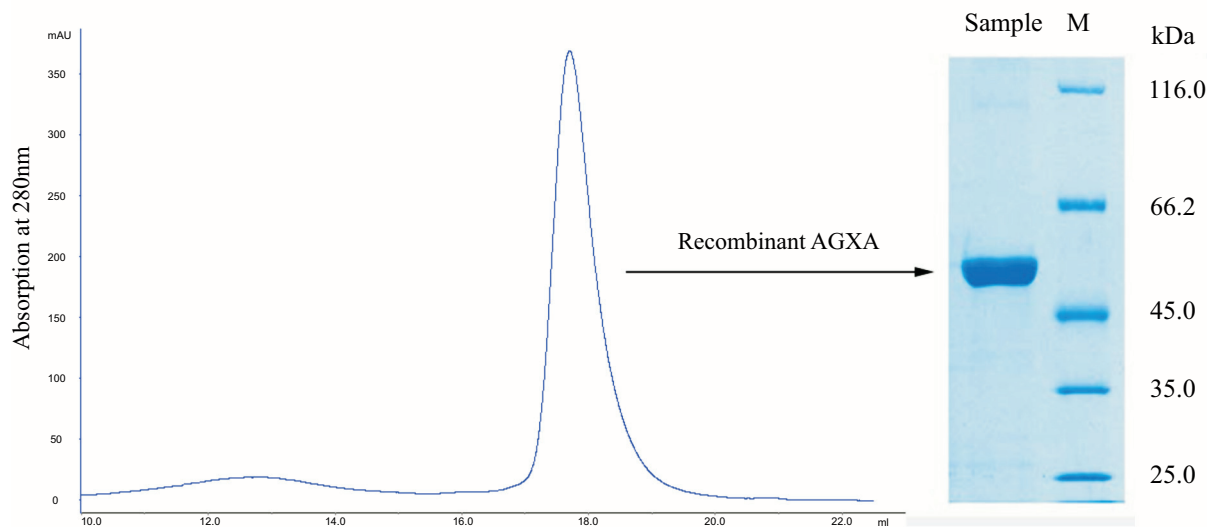


Figure 1. Recombinant α -amylase AGXA purification by gel filtration. Size-exclusion chromatography (left) showed a single narrow peak, and the SDS-PAGE analysis (right) also showed a single band, indicating sample homogeneity.

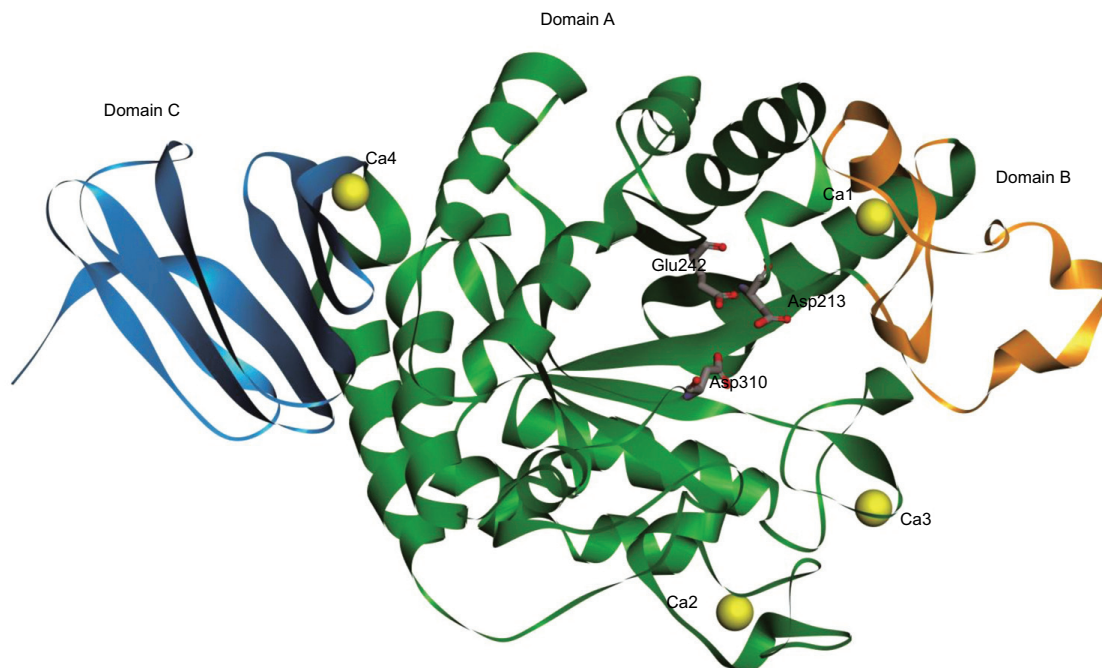


Figure 2. Model structure of AGXA. The model structure of AGXA predicted by homology modeling based on the crystal structure of *Anoxybacillus* sp. SK3-4 α -amylase (PDB code: 5A2A). The three conserved active site residues are labeled by sticks. Domains A, B, and C are shown in green, orange, and blue, respectively. The calcium ions are shown in yellow.

GH13 α -amylase [35]: central domain A (residues 26–139 / 187–393 and the three catalytic residues of D213, E242 and D310) made up of a $(\alpha/\beta)_8$ TIM barrel; domain B (residues 140–186); and domain C (residues 394–475). The overall structure of the AGXA model was compact and similar to *Geobacillus thermoleovorans* CCB US3 UF5 α -amylase (GTA) [27], *Bacillus* KSM-1378 α -amylase (LAMY) [36], and a truncated *Bacillus* sp. strain TS-23 α -amylase (BACANC) [37]. There were four Ca^{2+} ions (Ca_1 –4) bound to the model structure. Ca_1 was in the region between domain A and domain B, coordinated by the N139, D182, H217, and E173 residues and three water molecules. Ca_2 and Ca_3 were both located in domain A, with Ca_2 coordinated by the N44, N46, N49, D50, G63, and D65 residues and a water molecule, and Ca_3 coordinated by the N92, E109, and E110 residues and three water molecules. Ca_4 was in the region between domain A and domain C, coordinated by the E400 residue and five water molecules. All the identified Ca^{2+} -ion locations were consistent with what was been observed in 5A2A [28].

3.2. Effects of Calcium Ions on the Thermal Stability of Recombinant AGXA

Optimum temperature of maximal enzymatic activity (T_{opt}) was an important parameter in characterizing the thermal adaptation process [38]. T_{opt} of the recombinant AGXA with calcium ions and without calcium ions were determined by evaluating their activity at different temperatures (40–80 °C). As the recombinant AGXA was stable at pH 6.0–9.5 (above 70% of its highest activity) [25], and so in the above temperature range, the change in pH of buffer Tris-HCl was small and negligible. The results showed that

the recombinant AGXA without calcium ions was active from 40°C to 70°C, with the T_{opt} observed at 60°C, which was similar to the values previously reported for the alkaliphilic α -amylases from *Bacillus* sp. KSM-K38 [39] and in agreement with the 60°C and pH 8.0 reported for *Anoxybacillus* sp. SK3-4 and DT3-1 α -amylases (ASKA and ADTA, respectively) [40], which were the closest α -amylases to AGXA by sequence comparisons. The recombinant AGXA was active over a wide range of temperatures with calcium ions from 40 to 80°C and maintained more than 80% of its maximum activity between 55 and 75°C, with the maximal enzymatic activity observed at 70.0°C (Figure 3A). This value was similar to what has been reported for α -amylase from *A. beppuensis* TSSC-1 (70°C and pH 5.5) [41] and GTA (70 °C and pH 6.0) [27].

Half-inactivation temperature (T_{50}) of the recombinant AGXA was individually assessed at different incubation temperatures in the absence and presence of Ca^{2+} . After heat treatment from 60 to 80°C (with 2 °C steps) for 30 min, the residual activity of the α -amylase was assessed. As showed in Figure 3B, the T_{50} value of AGXA without calcium ions was 63.5°C, while 73.8°C with calcium ions, which was 10.3°C higher than that observed in AGXA without calcium ions. As T_{50} approaches or reaches the critical denaturation temperature, using the thermostable curve, we could speculate that the denaturation temperatures of the α -amylase with and without Ca^{2+} were likely to be approximately 65°C, and 75°C, respectively. These values were slightly higher than those reported for α -amylase in *Aspergillus oryzae* (TAKA) [42].

Thermal inactivation of the recombinant AGXA with calcium ions and without calcium ions was evaluated by

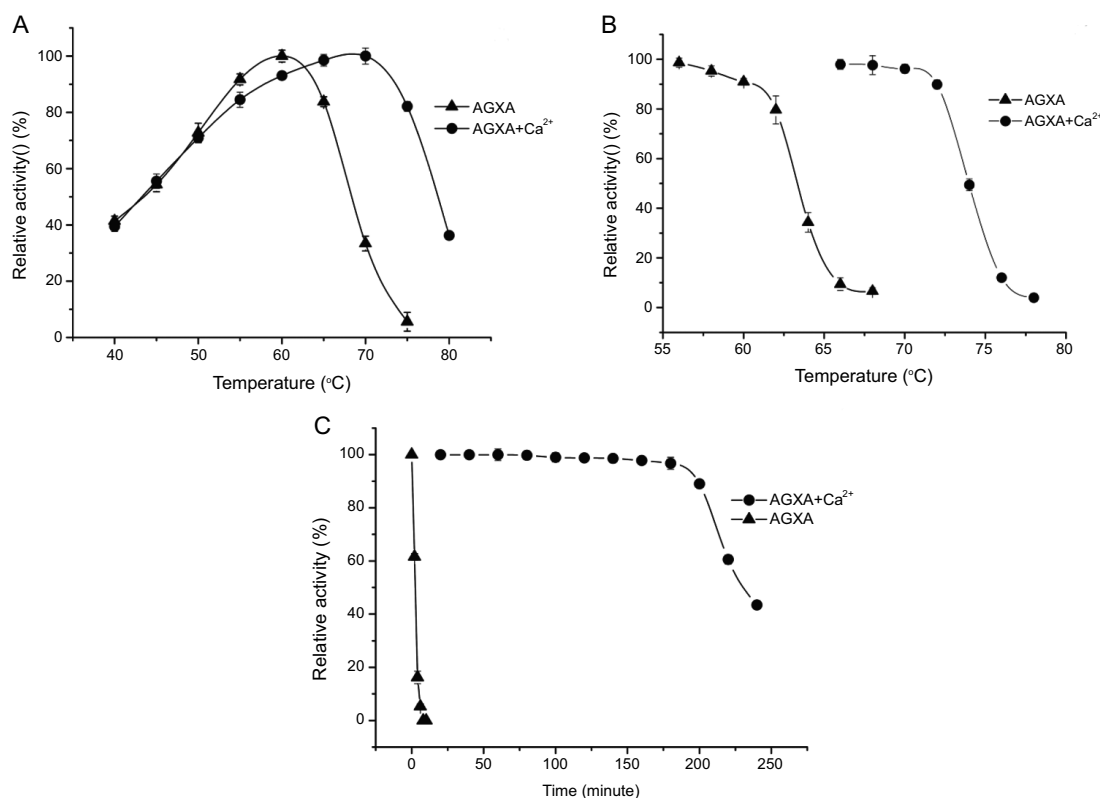


Figure 3. The optimal temperature curves (A), and stability curves (B), the inactivation curves at 70 °C of AGXA with Ca²⁺ and without Ca²⁺ ions (C). The optimal temperature curves were determined by measuring the enzymatic activities at a temperature range of 40–80 °C with 5 °C intervals. The stability curves were determined by measuring the enzymatic activity after treatment from 60 to 80 °C (with 2 °C steps) for 30 min following a 10-min incubation on ice. Inactivation curves were determined by incubating enzymes at 70 °C and enzymatic activities were measured at various time points. Error bars were obtained from the standard deviation of triplicates.

incubating the enzymes at 70°C and residual activity was measured at various incubation times. As shown in Figure 3C, without calcium ions, recombinant AGXA lost its activity completely at 70°C after 4 min of incubation, while with Ca²⁺, more than 90% of the activity was retained even after 200 min of incubation at the same temperature. It was indicated that, with Ca²⁺, the α -amylase was 50-fold more thermostable than without calcium ions at 70°C. Similar results were shown for GTA [27], ASKA and ADTA [40]. GTA without Ca²⁺ lost its activity after 15 min, whereas GTA with 2.0 mM Ca²⁺ retained 85% of its activity even after 72 h of incubation at 70°C [34]. With Ca²⁺, ASKA and ADTA could remain stable for up to 48 h at 70°C [40]. In comparison, the thermostability in ASKA and ADTA were greater than in AGXA.

3.3. DSC Measurements

Thermal denaturation of recombinant AGXA was investigated using DSC. DSC is a tool used to study thermodynamic properties of macromolecules and protein unfolding processes that gives the unfolding temperature (T_m) and enthalpy (ΔH) of a protein simultaneously [43]. After data processing, including buffer correction, normalization, and baseline subtraction, the results are shown in Figure 4. The transition curves of recombinant AGXA with Ca²⁺ and without Ca²⁺ were fitted using a non-two-state model, and the values of $\Delta H/\Delta H_v$ were approximately equal to 1.0, showing the α -amylases underwent thermal denaturation following a

two-state irreversible model. In the absence of calcium ions, the T_m value of recombinant AGXA was 67.3°C, and in the presence of calcium ions, the thermal denaturation curve was found to be shifted to a higher temperature range, with the T_m value at 77.8°C, where the increased temperature was 10.5°C. T_m was the midpoint temperature of transition, where the folded and the unfolded states of the protein were in equilibrium. The higher the T_m of a protein, the higher its thermal stability [44]. It has been reported that, with saturated Ca²⁺ and without Ca²⁺, differences in unfolding temperatures (ΔT_m) were large for α -amylase from *Bacillus licheniformis* (BLA) and α -amylase from *Bacillus amyloliquefaciens* (BAA), for which the values were 50°C, and 48°C, respectively [45]. However, for the *Alteromonas haloplantis* α -amylase (AHA), *Bacillus halmopalus* α -amylase (BHA), *Aspergillus oryzae* α -amylase (TAKA) and pig pancreatic α -amylase (PPA), the differences were relatively smaller, showing ΔT_m values of 0°C, 5°C, 14°C, and 17°C, respectively [42, 46, 47], as shown in Table 1.

The thermal stability of the α -amylase AGXA with calcium ions, also reflected in a higher ΔH , reached 290.7 kcal.mol⁻¹ in the presence of calcium ions and reached 238.3 kcal.mol⁻¹ in the absence of calcium ions. The ΔH value for AGXA without Ca²⁺ was similar to the reported ΔH for AHA without Ca²⁺ (238 kcal.mol⁻¹) [47]. The ΔH value for AGXA with Ca²⁺ was larger but still less than the values reported for BHA (581.7 kcal.mol⁻¹) [46], TAKA (535.9 kcal.mol⁻¹) [42], BAA(336.2 kcal.mol⁻¹) and BLA (362.1

Table 1. The effect of calcium ions on thermostability in terms of melting temperatures of various α -amylases.

Enzymes	T_m (Ca ²⁺ -with) (°C)	T_m (Ca ²⁺ -without) (°C)	ΔT_m (°C)	Reference
AHA	44	44	0	[46]
PPA	65	48	17	[46]
TAKA	71	57	14	[41]
BHA	94	89	5	[45]
BAA	86	40	46	[44]
BLA	103	52	51	[44]
AGXA	78	67	11	present study

kcal.mol⁻¹) [45]. ΔH was the energy required to denature proteins, and a higher ΔH demonstrated that more energy was required to unfold the protein.

Generally, irreversible denaturation of a protein has been suggested to occur according to the Lumry and Eyring model [48]:



where N is the native state, U is the unfolded state and F is the final state [48, 49]. When a native, functional protein underwent irreversible alteration processes (aggregation, proteolysis, strong interactions with other macromolecules, and other similar events), it would eventually end up in the nonfunctional, final state [50]. When most of the unfolded molecules were converted to final state, the thermal denaturation could be regarded as a one-step process by a first-order irreversible process:



in which the conversion from N to F was determined by the temperature-dependent, first-order rate constant (*k*) [50, 51]. This model has been used successfully to describe the irreversible unfolding processes of various proteins [52-55]. In the present study, the ratio of calorimetric and van't Hoff enthalpies ($\Delta H/\Delta H_v$) was near 1.0, suggesting that AGXA with Ca²⁺ and without Ca²⁺ adopts a two-state thermal denaturation process. Similarly, it has been reported that thermal denaturation of *Bacillus* α -amylases has been characterized in terms of a two-state irreversible denaturation model [49, 56-58]. Nazmi *et al.* [59] reported on the apparent thermodynamic and kinetic properties of BLA renatured with CaCl₂ using DSC, CD and DLS studies, indicating that partially-renatured BLA could be represented well through superposition of two irreversible processes, each of which following the two-state irreversible model.

3.4. CD Measurements

The effect of calcium ions on the thermal unfolding of recombinant AGXA has also been studied by recording CD spectra as a function of temperature. As shown in Figure 5, at low temperature (<40°C), the CD spectra of recombinant

AGXA with Ca²⁺ and without Ca²⁺ were equivalent minima near 208 and 222 nm, which is the characteristic in α -amylase that suggests a predominant α -helix structure [60]. As temperature increased α -amylases with Ca²⁺ and without Ca²⁺ underwent different denaturation processes. For α -amylase without Ca²⁺ there were two significant changes: the first change was between 40 and 50°C, with the CD spectra of two deeper negative ellipticities, indicating the α -helical contents of the structure increase; the second change was observed above 60°C, with the emergence of a new peak with a minima ellipticity at approximately 215 nm, demonstrating the structural transition from an α -helix to a β -sheet structure, and the skeleton structure became loose. For the α -amylase with Ca²⁺, when the temperature was between 40°C and 60°C, there were few changes in the spectra; when the temperature increased to 70°C and 80°C, the spectra showed a gradual diminution of a decrease in α -helix structures. With temperatures over 80°C, the ellipticity of the spectra tended to have a value of 0, indicating aggregation and even precipitation of the α -amylase. As an excellent method for rapidly evaluating the secondary structure and folding properties of proteins [61], CD has been widely used to study enzyme structure stability, thermodynamics, thermal unfolding and its mechanism [62, 63]. CD spectra have examined thermal stability and catalytic activity of α -amylase in the presence of different concentrations of CaCl₂, and the results show that the enzyme displays optimum catalytic activity in the presence of 1.0-2.0 mM CaCl₂, while further addition of CaCl₂ leads to enzyme inhibition [64]. Recently, the stability and changes in the secondary structure of α -amylase from thermophilic *Bacillus* sp. TSSC-3 had been reported and discovered to have a reduced α -helix content and increased amount of β -sheets after denaturation [65]. Far-UV CD studies revealed α -helical structures were shifted to 215 nm in porcine pancreatic α -amylase upon AgNP binding, with structural conformation changes with peak shifts confirmed through FTIR spectroscopy [66].

Global 3 was a package software to calculate structural and thermodynamic data developed by Applied Photophysics. After processing with Global 3 software the calculated spectra are shown in Figure 6, and the unfolding temperatures (T_m) were 67.5°C and 77.5°C for AGXA without Ca²⁺ and with Ca²⁺, respectively, which were consistent with the

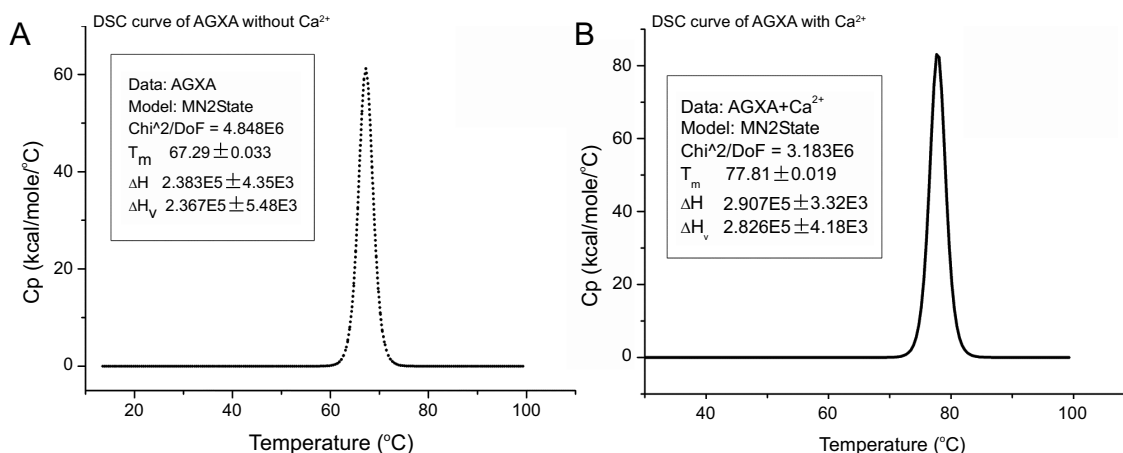


Figure 4. DSC curves of AGXA without Ca²⁺ (A) and with Ca²⁺ (B) were fitted using a non-two-state model. The T_m , ΔH and ΔH_v for AGXA without Ca²⁺ were 67.3 °C, 238.3 kcal.mol⁻¹ and 236.7 kcal.mol⁻¹, respectively. The T_m , ΔH and ΔH_v for AGXA with Ca²⁺ were 77.8 °C, 290.7 kcal.mol⁻¹ and 282.6 kcal.mol⁻¹, respectively. Ca²⁺ ions caused a thermal increase (~10 °C and ~52 kcal.mol⁻¹) in T_m and ΔH , and the values of $\Delta H/\Delta H_v$ were approximately equal to 1.0 for AGXA both with Ca²⁺ and without Ca²⁺ ions.

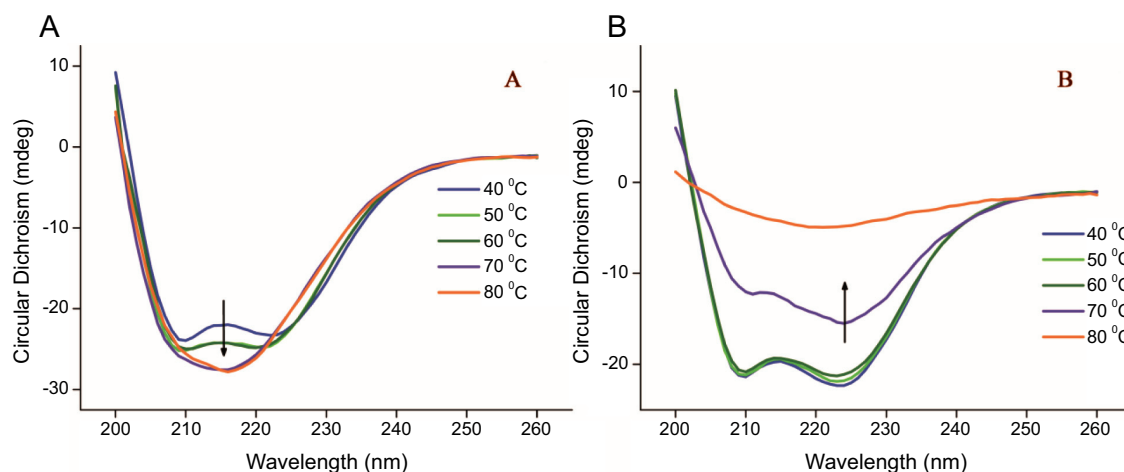


Figure 5. The far UV-CD spectra of AGXA without Ca²⁺ (A) and with Ca²⁺ (B) from 40 °C to 80 °C. As temperature increased, AGXA without Ca²⁺ and with Ca²⁺ underwent different denaturation processes.

values from DSC measurements. From the CD-temperature profiles, there were two transition states (the low temperature changes were minor and the high temperature changes were obvious) for the α -amylase without Ca²⁺, whereas only one transition was observed in α -amylase with Ca²⁺, which were similar with the CD spectra shown above. From homology modeling, this α -amylase was identified to have four calcium ions binding to its structure, one in the region between domain A and B, another in the region between domain A and C, and the other two in domain A. These calcium ions could form extra bridges between the different domains of α -amylase to enhance enzyme stability [45]. For AGXA without Ca²⁺, the spectra changes could be speculated to be due to temperature increases cause higher thermal-induced mobility of domain C with respect to central domain A, which might be related to the first subtransition. As partially unfolded or completely unfolded proteins were much more susceptible to proteolytic degradation [59], β -sheet-like structures of the TIM on domain A lead to a higher probability of forming random inter-domain and intermolecular hydrogen bonds, which increase the chance of conformational

scrambling or of protein aggregation, and in particular the formation of amyloid-like fibrils [67]. Ca²⁺ ions, however, are possibly causing irreversibility of thermal unfolding due to high temperature chemical modifications, and the Ca²⁺ ions act as intermolecular cross-links to adjacent anionic molecules by forming protein-Ca²⁺-protein complexes, exhibiting intramolecular electrostatic shielding of negative charges on the protein, and inducing ion-induced conformational changes leading to altered hydrophobic interactions [68-70]. The increased aggregation tendency then results from hydrophobic residues that became exposed to the solvent and interact preferably with hydrophobic residues from other unfolded protein molecules to minimize their exposure to the solvent [71, 72]. Recently, Uzma *et al.* [73] reported a unique thermostable dimer α -amylase Tp-AmyS, which with a Ca²⁺ ion in each monomer and showing catalytic cooperativity within the dimer. Just as the sequence similarity between AGXA and Tp-AmyS was only 20%, how the role of dimerization in the thermostability of the α -amylase was not involved in AGXA.

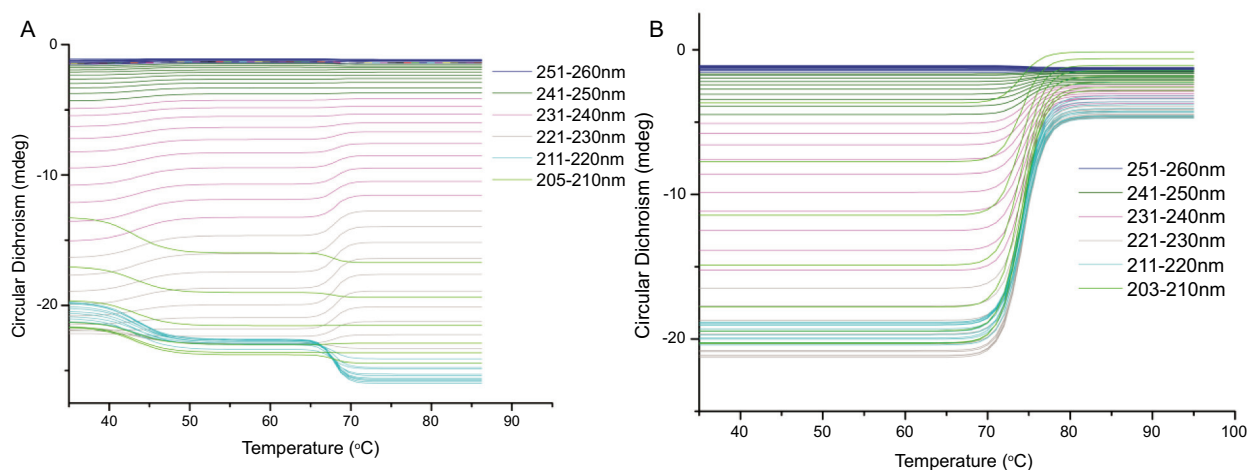


Figure 6. The CD-temperature profiles of AGXA without Ca^{2+} (A) and with Ca^{2+} (B) from 35 °C to 85 °C were calculated with Global 3 software. The spectra with absorbance above 2.0AU were excluded. The results showed that the unfolding temperatures (T_m) were 67.5 °C and 77.5 °C for AGXA without Ca^{2+} and with Ca^{2+} , respectively.

CONCLUSION

In summary, the thermostability of *Anoxybacillus* sp. GXS-BL α -amylase with calcium ions and without calcium ions has been studied. It was found that, in the presence of calcium ions, the values of T_{opt} , T_{50} , $t_{1/2}$, T_m and ΔH in AGXA were significantly higher than those observed in the absence of calcium ions, showing calcium ions had stabilizing effects on α -amylase structure with the increased temperature. Based on DSC measurements AGXA underwent thermal denaturation by adopting two-state irreversible unfolding processes for the $\Delta H/\Delta H_{vh}$ to be close to 1.0. Based on the CD spectra, AGXA without calcium ions exhibits two transition states upon unfolding, including α -helical contents increasing, and the transition from α -helices to β -sheet structures, which was obviously different with AGXA added Ca^{2+} ions. According to the modeled structure up to 4 Ca^{2+} ions were located on the inter-domain or intra-domain regions. These results reveal that Ca^{2+} ions have pronounced influences on the structural features accountable for the thermostability of α -amylase from *Anoxybacillus* sp GXS-BL.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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