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Cloning and characterization of two new thermostable and alkalitolerant α-amylases from the *Anoxybacillus* species that produce high levels of maltose

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Abstract Two genes that encode α-amylases from two *Anoxybacillus* species were cloned and expressed in *Escherichia coli*. The genes are 1,518 bp long and encode 506 amino acids. Both sequences are 98% similar but are distinct from other well-known α-amylases. Both of the recombinant enzymes, ASKA and ADTA, were purified using an α-CD–Sepharose column. They exhibited an optimum activity at 60°C and pH 8. Both amylases were stable at pH 6–10. At 60°C in the absence of Ca^{2+}, negligible reduction in activity for up to 48 h was observed. The activity half-life at 65°C was 48 and 3 h for ASKA and ADTA, respectively. In the presence of Ca^{2+} ions, both amylases were highly stable for at least 48 h and had less than a 10% decrease in activity at 70°C. Both enzymes exhibited similar end-product profiles, and the predominant yield was maltose (69%) from starch hydrolysis. To the best of our knowledge, most α-amylases that produce high levels of maltose are active at an acidic to neutral pH. This is the first report of two thermostable, alkalitolerant recombinant α-amylases from *Anoxybacillus* that produce high levels of maltose and have an atypical protein sequence compared with known α-amylases.

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Introduction

The amylase superfamily can be generally divided into two groups, endoamylases (α-amylase) and exoamylases (β-amylase, glucoamylase, and α-glucosidase). Endoamylases hydrolyze the interior of a glucose chain in starch to generate oligosaccharides of various lengths. Exoamylases act at the non-reducing ends of polysaccharides and produce low molecular weight products, e.g., glucose and maltose. Amylases are important in starch-processing industries; endoamylases initiate starch degradation (liquefaction process) to produce maltodextrins, and exoamylases are usually used to further degrade maltodextrins into glucose and maltose (saccharification process) [12].

Alpha-amylases (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) are enzymes that randomly cleave α-1,4-glucosidic linkages in starch and related polysaccharides to produce different length oligosaccharides. Various α-amylases are produced by plants, animals, and microorganisms. However, commercial entities have considerable interest in α-amylases from microbial sources because of advantages such as cost-effectiveness, efficient production, and consistency [29].

There is an ongoing interest in the isolation of α-amylases that are suitable for industrial applications. Great attention has been paid to halophiles [27], thermophiles [1], and alkaliphiles [15]. Halophilic amylases are tolerant of high salt concentrations and certain organic solvents that would easily denature common amylases [27]. Thermostable and alkalitolerant amylases are desirable in the starch and textile industries as well as in detergent

production. A number of thermostable and alkalitolerant α-amylases were isolated from bacteria primarily from the genus *Bacillus*, e.g., *Bacillus* sp. KSM-K38 [13], *B. licheniformis* NH1 [15], and *B. halodurans* [22]. However, the α-amylases from thermophilic and alkalitolerant *Anoxybacillus* species have attracted little attention from researchers. Among the genus *Anoxybacillus*, only *A. flavithermus* [5] and *A. amylolyticus* [24] have been reported to produce α-amylase. Nevertheless, limited information is available on the enzymatic properties and protein sequence from this group of α-amylases, and the α-amylase genes have not been cloned. Thus, there is a great interest in studying α-amylases from *Anoxybacillus* sp. to better understand the molecular biology and enzymatic properties.

There is increasing demand for α-amylases that produce high levels of maltose, as maltose has diverse applications, e.g., food, pharmaceuticals, the biomedical field, and fine chemicals [8]. α-Amylases that produce high levels of maltose are usually active at acidic to neutral pH values [19, 28]. In this study, the two *Anoxybacillus* α-amylases are thermostable and alkalitolerant and produce high levels of maltose. We report herein the isolation of two α-amylase genes from two *Anoxybacillus* sp. through cloning, expression, purification, and biochemical characterization of the recombinant α-amylases, ASKA and ADTA.

The two isolated strains form a sister line of descent with the *Anoxybacillus* species, although they were isolated from two different locations. Both strains had rod-shaped cells that stained Gram positive. However, their pigmentation and colony and individual cell sizes were dissimilar. Additionally, the 16S rDNA sequences, fatty acid profiles, and DNA G+C contents revealed that the strains were distinct [7]. Despite the differences in cell morphology and biochemical characteristics, the sequences of the amylase genes are quite similar. Analysis of the purified ASKA and ADTA showed that they share common characteristics in substrate and product specificity but dissimilarity in thermostability. As this is the first reported amylase gene from *Anoxybacillus* sp., the sequence data presented herein may provide preliminary information upon which to conduct future studies to elucidate the amylase gene variation among closely related isolates.

Materials and methods

Chemicals

The chemicals and solvents used were molecular biology or analytical grade. The restriction endonucleases were purchased from New England Biolabs (MA, USA). The DNA modifying enzymes and ladder were obtained from Promega (WI, USA). The primers used for PCR amplification were synthesized by First BASE Laboratories (Selangor, Malaysia). The Genomic DNA Extraction, SpinPrep Gel DNA, PCR purification, and Plasmid Isolation Kit were purchased from Yeastern Biotech (Taipei, Taiwan) and Novagen (Darmstadt, Germany).

Isolation of microorganisms

Two bacterial strains were isolated from water samples that were taken from two hot springs in Malaysia, Sungai Klah (SK) and Dusun Tua (DT), which are located at 3°59′47.88″N, 101°23′35.17″E and 3°8′ 13.5″N, 101°50′5.3″E, respectively. The hot springs are separated by approximately 120 km. The temperatures and pH of the samples collected from SK were 70°C and pH 7.3 and those from the DT hot spring were 75°C and pH 7.5. The isolates were screened for amylolytic activity on a 1% (w/v) soluble starch agar plate that contained 0.8% (w/v) polypeptone, 0.4% (w/v) yeast extract, and 0.2% (w/v) sodium chloride and then were incubated at 50°C. The plates were then flooded with an iodine solution [1% (w/v) iodine and 10% (w/v) potassium iodide]. The formation of halozones around the colonies was observed.

Amplification of amylase genes

Degenerate primers were designed on the basis of the conserved regions among bacterial amylases. PCR amplification was performed using the forward primer 5′- GGN GGN GAY TGG CAR GGN -3′ and reverse primer 5′- CAT RTC RTG RTT RTC DAT RAA -3′. The deduced amino acid sequences of the amplicons were searched for in the National Center for Biotechnology Information (NCBI) database using blastp. Next, to obtain the full-length gene sequences, oligonucleotides were designed on the basis of the nucleotide sequences retrieved from NCBI GenBank. The forward primer 5′- AAA CGT GTA TTT CGC GCG C -3′ and reverse primer 5′- TGA TGC TTT TCG TTT ACG CGC -3′ were used. PCR amplification included an initial denaturation at 95°C for 5 min. The first reaction cycle proceeded as follows: 1 min at 95°C, 50 s at 55°C, and 1.5 min at 72°C. After a total of 30 cycles, an additional 5-min extension at 72°C was performed. The gene was cloned into the pGEM®-T vector (Promega, WI, USA) and sent to First BASE Laboratories for sequencing. The open reading frame (ORF) was predicted using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The putative ribosome binding site was identified by a sequence comparison with the corresponding regions of other α-amylase genes. The promoter regions were predicted using the BPROM program (http://linux1.softberry.com/berry.phtml) and verified manually on the basis of the

basic requirements for promoter elements [6]. Multiple sequence alignments were performed with the Clustal W program [18]. Conserved regions were identified by comparing the sequences with the known amylase sequences [16]. A phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1,000 replicates using MEGA 5.0 software [31]. The sequence alignment was performed using the Clustal W program embedded within the MEGA 5.0 software.

Subcloning and expression of amylase genes

The mature amylase gene with *Eco*RI and *Xho*I restriction sites was then subcloned into the pET-22b(+) vector (Novagen, Darmstadt, Germany) and transformed into *E. coli* BL21 (DE3) for protein expression. The recombinant *E. coli* was cultured overnight in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin at 37°C with shaking. The overnight culture was transferred into fresh media and incubated until the 600-nm absorbance intensity reached 1.5, which was read using a 100 UV–Vis spectrophotometer. Subsequently, expression was induced by adding 0.01 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was incubated at 25°C with shaking for 48 h.

Purification of amylases

The culture was centrifuged (10,000×*g*, 20 min, 4°C), and the culture-free supernatant was concentrated by ammonium sulfate precipitation at 70% saturation and stirred for 16 h at 4°C. The pellet was collected by centrifugation and dissolved in a 20 mM sodium phosphate buffer (pH 7.5). The resulting supernatant was dialyzed overnight against the same buffer using SnakeSkin dialysis tubing (10,000 MWCO, Thermo Fisher Scientific, Rockford, USA).

The enzyme was purified by a single step purification using an in-house α-CD–expoxy-activated Sepharose 6B column. The enzyme was loaded onto the column at a 0.5 ml/min flow rate and washed with nine column volumes of 20 mM sodium phosphate buffer (pH 7.5) at a 0.9 ml/min flow rate. The bound enzyme was eluted with 1% α-cyclodextrin (α-CD) supplemented with 500 mM NaCl in the same buffer. The fractions with active enzymes were pooled and dialyzed against 100 mM Tris–HCl buffer (pH 8).

Enzyme assay

Amylase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method described by Miller [21] with a slight modification. The standard reaction contained 0.1 ml of an appropriately diluted enzyme solution and 1.0 ml Tris–HCl buffer (100 mM, pH 8) with 1% soluble starch as a substrate. After incubation at 60°C for 10 min, the reaction was stopped by adding 1.0 ml DNS solution followed by 0.02 ml of 0.1 N NaOH. The mixture was then boiled for 5 min, and the absorbance intensity was measured at 540 nm. One unit of α-amylase was defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute per milliliter under the assay conditions specified. Maltose was used as the assay standard. The protein concentration was determined using the Lowry method [20] and bovine serum albumin as the standard.

Gel electrophoresis and activity staining

The molecular mass and purity of the enzymes were estimated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% (w/v) acrylamide gel. Enzyme activity staining (zymogram) was performed using native PAGE as described by Yang et al. [35] except that the incubation temperature was set at 60°C and the starch solution was prepared in 100 mM Tris–HCl buffer (pH 8).

Effects of reaction conditions

Effects of pH and temperature on enzyme activity and stability

The effects of pH on the purified enzyme's activity and stability were determined for a pH range of 4–11. The following buffer systems were used (100 mM of each buffer): sodium acetate buffer (pH 4–5), sodium phosphate buffer (pH 6–7), Tris–HCl buffer (pH 8–9), and glycine–NaOH buffer (pH 10–11). To measure the pH stability, the enzyme was incubated with the various buffers at 60°C for 30 min, and the residual activity was determined under the standard assay conditions.

The effects of temperature on the enzyme activity and stability were determined at 30–90°C at the optimum pH of 8. To determine thermal stability, the residual activity of the enzyme was determined under the standard assay conditions after incubation at different temperatures for 30 min with and without 5 mM calcium chloride. Thermostability was also studied up to 48 h; samples were taken at certain time intervals, and the remaining activities were assayed under the standard conditions.

Effects of metal ions and chemical reagents

The influence of various metal ions (at a 5-mM concentration) on amylase activity was investigated. The enzymes were assayed in the presence of chloride salts of Fe^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, Na^{+}, K^{+}, Mg^{2+}, and Ca^{2+}. The effects

of different surfactant concentrations (SDS, Triton X-100, and Tween 20) and chemical reagents (EDTA, β-mercaptoethanol, phenylmethanesulfonyl fluoride, and urea) on enzyme stability were examined after incubation of the purified enzyme with the additive at 60°C for 30 min. The remaining activity was measured under the standard enzyme assay conditions. The enzyme activity without the additive was defined as 100%.

Substrate specificity

The purified enzyme was reacted with 1% (w/v) substrate (soluble starch, amylose, amylopectin, glycogen, pullulan, and α-CD) in 100 mM Tris–HCl buffer (pH 8), and the activity was assayed under standard conditions. The enzyme activity with soluble starch was used as a reference for comparison.

Analysis of hydrolysis products

Purified amylases were incubated with 1% (w/v) of various starches (soluble, corn, tapioca, wheat, potato, rice, and sago) in 100 mM Tris–HCl buffer (pH 8) at 60°C. The potato starch mixtures were reacted at different time intervals and then boiled for 15 min to stop the enzymatic reaction. The insoluble particles were then filtered using a 0.45-μm nylon syringe filter (Whatman, Kent, England). The reaction products were analyzed using a Waters HPLC system with a 4.6 × 250 mm Zorbax carbohydrate analysis column (Agilent, CA, USA) and a 2414 refractive index detector (Waters, MA, USA), both of which were maintained at 30°C. Acetonitrile–water (65:35, v/v) was used as the mobile phase, and the flow rate was 1.4 ml/min. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (Sigma–Aldrich, MO, USA) were used as standards in the reaction product analyses. The starch solutions without the enzymatic reaction were used as controls.

Statistical analysis

The results were analyzed using SYSTAT 12 (Systat Software Inc., CA, USA). The data were compared using a Student's *t* test. A probability level of *P* less than 0.05 was used to test all hypotheses.

Results and discussion

Isolation of microorganisms

Two *Anoxybacillus* strains, SK3-4 and DT3-1, respectively, were isolated from water samples that were taken from the SK and DT hot springs in Malaysia [7]. The NCBI accession numbers for the 16S rDNA sequences are GQ184213 and GU129931. Upon the addition of an iodine solution, a halozone formed around the colony on the starch medium, which indicated the presence of amylolytic activity.

Sequence analysis of amylase genes

Amplification of the genes that encode for the conserved region yielded an amplicon approximately 800 bp long. The subsequent amplification yielded the full amylase genes. The amylases from the strains SK3-4 and DT3-1 are ASKA and ADTA, respectively. The full-length gene is 1,518 bp long and has an ATG start codon and a TGA stop codon. A predicted ribosome binding site was found 10 nucleotides upstream of the start codon in both sequences, which comprised the sequences GGAGAG and GGGGAG for ASKA and ADTA, respectively. The predicted ribosome binding site of ASKA was found to be completely conserved with the corresponding region of the α-amylase gene of *Bacillus amyloliquefaciens* [23]. A putative promoter for ASKA included TTACTA (at position −54 bp), which corresponds to the −35 region, and TATATT (at position −40 bp), which corresponds to the −10 region. For ADTA, the putative promoter sequences are TTACTA (at position −53 bp), which corresponds to the −35 region, and TATATT (at position −39 bp), which corresponds to the −10 region. The gene sequences have been deposited in the GenBank database with the accession numbers JF932307 (ASKA) and JF932308 (ADTA).

The amino acid sequence deduced from the nucleotide sequence consisted of 506 amino acids for both enzymes. The sequence includes a putative signal peptide with 23 residues preceding the mature enzyme N-terminus, as predicted by using SignalP 3.0 Server [4]. By performing a multiple sequence alignment of the amino acid sequences with other known α-amylases, we compared the seven highly conserved amylase regions labeled I–VII (Online Resource 1). Important amino acids, such as the hypothetical active site residues, were conserved and are located at Asp-190, Glu-219, and Asp-287 (Online Resource 1). The two calcium ion binding sites were conserved with TAKA. The first calcium binding site residues include Asn-116, Glu-150, Asp-159, and His-194, whereas the second calcium binding site residues include Asp-190 and Glu-219.

The amino acid sequences of the mature ASKA are 98% similar to ADTA, which contains 17 different residues (Online Resource 1). From the blastp result, the most similar characterized α-amylase is from *Anaerobranca gottschalkii*, AmyA (68% similarity). Alternatively, a sequence comparison of ASKA and ADTA with well-known

α-amylases, such as those from *Aspergillus oryzae*, TAKA (47 and 48% similarity, respectively), *Bacillus licheniformis*, BLA (37% similarity for both), and *Bacillus stearothermophilus*, BSTA (49 and 44% similarity, respectively), revealed that both *Anoxybacillus* α-amylases are unique and distinct from the publically available sequences.

Most α-amylases belong to family 13 of the glycoside hydrolases (GH13). As reported in a large-scale phylogenetic analysis published in 2006 [30], family GH13 is further divided into 35 subfamilies. To date, a total of 36 subfamilies are found in the Carbohydrate-Active enZyme (CAZy) database (http://www.cazy.org/). Among the subfamilies, GH13_1, GH13_5, GH13_6, GH13_7, GH13_15, GH13_19, GH13_24, GH13_27, GH13_28, GH13_32, and GH13_36 exhibit α-amylase activities. Figure 1 shows an evolutionary tree of α-amylases, constructed using representatives from each of the subfamilies as well as the *Anoxybacillus* α-amylases. The tree clearly illustrates that the *Anoxybacillus* α-amylases do not cluster with the existing GH13 subfamilies, suggesting that both enzymes are distinct from other α-amylases.

Cloning, expression, and purification of amylases

To elucidate the biochemical properties of the *Anoxybacillus* amylase, the gene encoding the mature protein was subcloned into the vector pET-22b(+) and expressed in *E. coli* BL21 (DE3). During the cloning process, the native signal peptide DNA sequence was excluded, as the pET-22b(+) contains a pelB leading sequence. Recombinant amylase was produced by inducing with IPTG, and the post-induction culture temperature was set at 25°C. The amylases were purified by a single step of affinity chromatography using α-CD coupled with an expoxy-activated Sepharose 6B column after concentration by ammonium sulfate precipitation. The purified enzyme has a single protein band on a denatured SDS-PAGE and a clear band on a native polyacrylamide gel when stained with iodine solution (Fig. 2). The size of the amylase is approximately

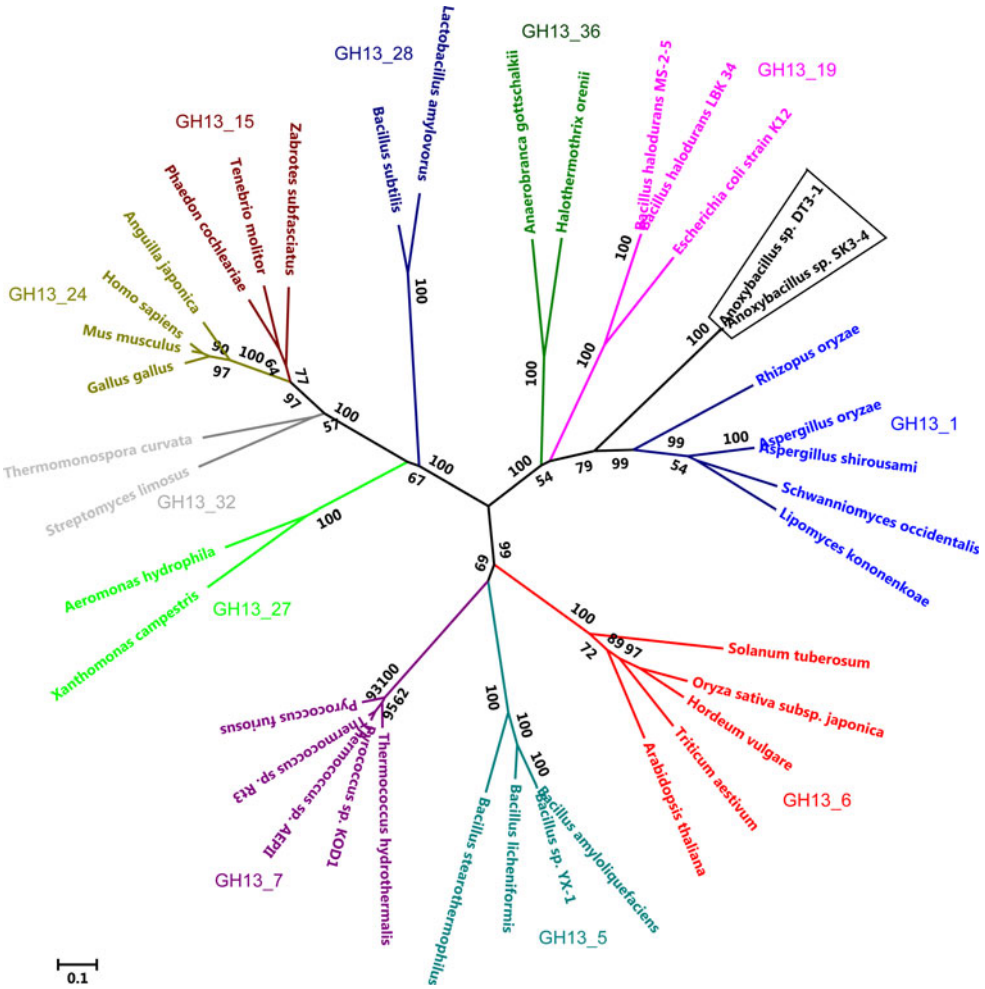

Fig. 1 Evolutionary tree of family GH13 α-amylases. The taxonomical origin of the individual organisms and the accession numbers for the corresponding α-amylases are given in Online Resource 2. The tree was constructed using neighbor-joining method based on the sequence alignment performed using Clustal W embedded within the MEGA 5.0 software. The GH13 α-amylases are divided into subfamilies. The tree illustrates that the two *Anoxybacillus* α-amylases do not cluster with any GH13 subfamilies

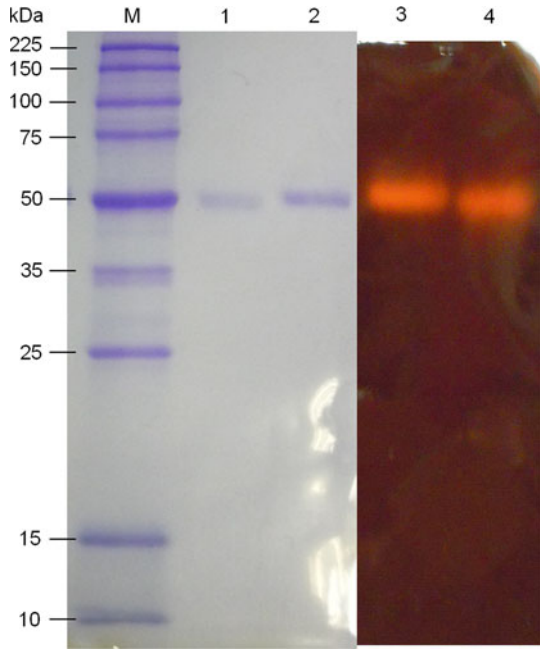

Fig. 2 SDS-PAGE and zymogram analysis of the purified ASKA and ADTA. Protein bands from SDS-PAGE (10% acrylamide gel) were stained with the Imperial™ Protein Stain (Thermo Fisher Scientific, Rockford, USA). A zymogram was performed on a native PAGE (10% acrylamide gel) that was stained with an iodine solution, as described in section "Gel electrophoresis and activity staining". *M* protein marker; *lanes 1* and *2*, SDS-PAGE of ADTA and ASKA, respectively; *lanes 3* and *4*, zymogram of ASKA and ADTA, respectively

50 kDa, which is smaller than the theoretical calculated size, 56 kDa.

Biochemical properties of the amylases

Effects of pH on enzyme activity and stability

The optimum activity of the purified amylases was examined in buffers at pH 4–11 and 60°C. The maximum activity of both enzymes was observed at pH 8, which is similar to that reported for the alkaliphilic α-amylases from *Bacillus* sp. KSM-K38 [13] and *Anaerobranca gottschalkii* [3]. ASKA and ADTA exhibited high activity (above 60%) in the pH range 5–10 (Fig. 3). This range is slightly broader than for AmyA of *A. gottschalkii* (pH 6.0–9.5), which is the closest α-amylase using a sequence comparison [3]. The *Anoxybacillus flavithermus* α-amylase had an optimum pH of 5.5–6.0 [5], and this suggests that ASKA and ADTA could be different from the reported amylase, although they are from the same genus source.

ASKA and ADTA were stable at pH 6–10 for 30 min, and the activity was at least 80% of the initial enzyme activity (Fig. 3a, b). Both enzymes were relatively stable (near 65–75%) upon incubation at pH 11. These results indicate that they are alkalitolerant enzymes. Alkalitolerant

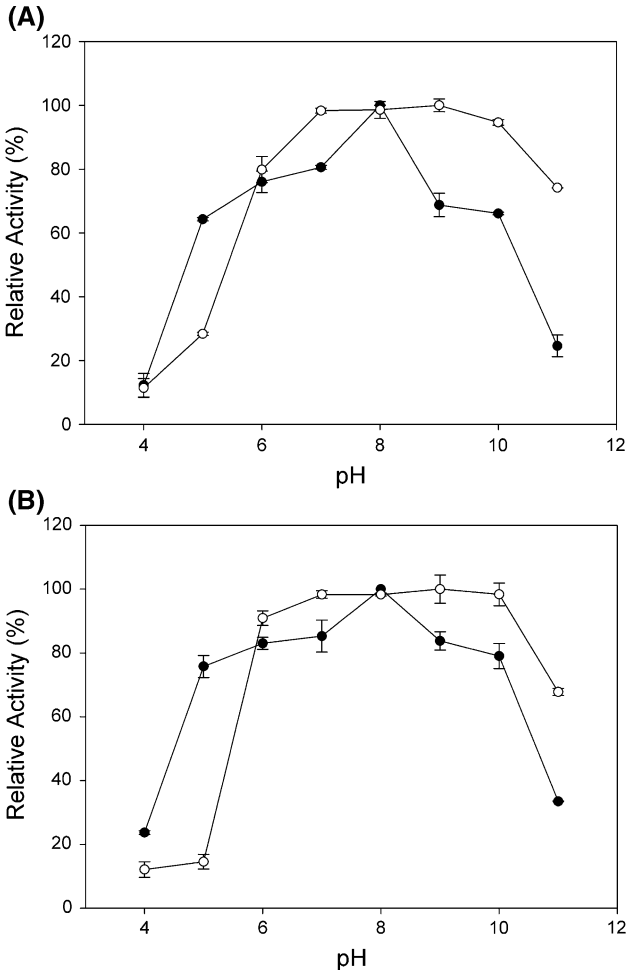

Fig. 3 Effects of pH on the activity (*filled circles*) and stability (*open circles*) of **a** ASKA and **b** ADTA. The buffer systems used (100 mM of each buffer) were sodium acetate (pH 4–5), sodium phosphate (pH 6–7), Tris–HCl (pH 8–9), and glycine–NaOH buffer (pH 10–11). Values are the mean ± standard error from triplicate analyses

α-amylases from the *Bacillus* sp. strain TSCVKK, *Bacillus halodurans* LBK 34, and *Chromohalobacter* sp. TVSP 101 were stable (more than 75% activity) in a pH range of 6.0–9.5 [17], 8–10 [14], and 7–10 [25], respectively. In contrast to other alkalitolerant α-amylases, ASKA and ADTA exhibited a broader pH stability range. In addition, the commercially available BLA showed the same pH stability range as that of ASKA and ADTA [26]. Owing to their wide pH stability range and high alkalitolerant nature, both amylases show potential for application in the detergent and textile industries.

Effects of temperature on enzyme activity and stability

The optimum reaction temperature for ASKA and ADTA is 60°C (Fig. 4a, b), which is similar to those reported for the *Anoxybacillus flavithermus* α-amylase [5] and the alkaliphilic α-amylases from *Bacillus* sp. KSM-K38 [13] and

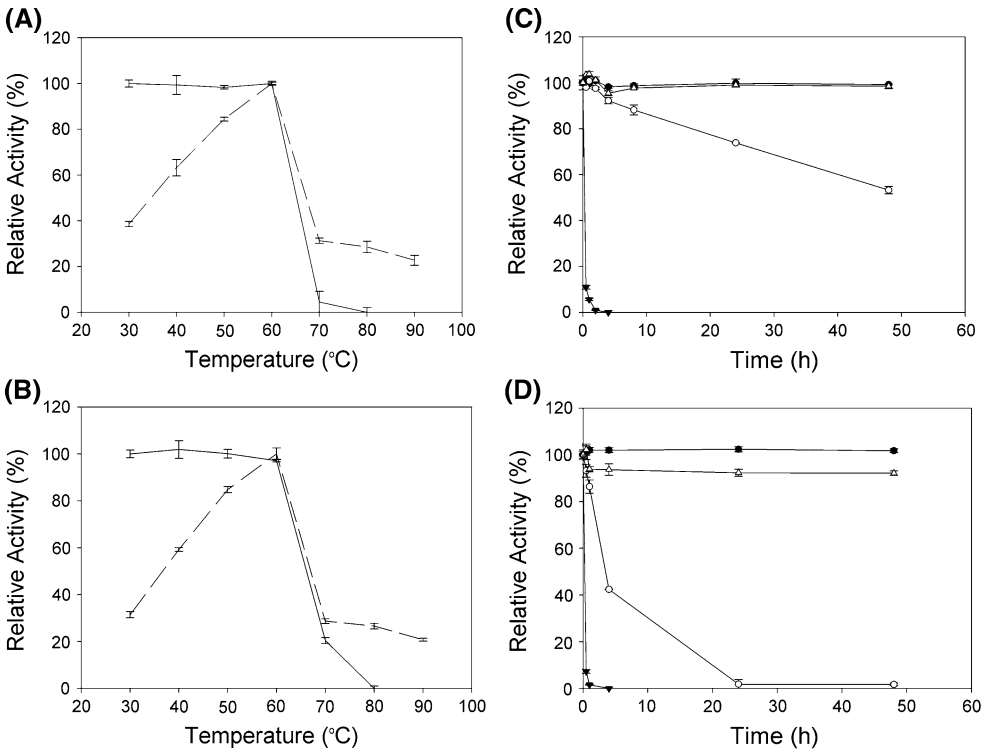

Fig. 4 Effects of temperature on the activity (*dashed line*) and stability (*solid line*) of **a** ASKA and **b** ADTA. The effects of a long-term incubation at 60°C (*filled circles*), 65°C (*open circles*), and 70°C (*inverted filled triangles*) without $CaCl_2$ and at 70°C (*open triangle*) with 5 mM $CaCl_2$ on **c** ASKA and **d** ADTA. Both enzymes retained their full activity at 60 and 65°C for 46 h in the presence of 5 mM $CaCl_2$ (for clarity purposes, these data are not shown because a negligible drop in activity was observed). Values are the mean ± standard error of triplicate analyses

B. halodurans LBK 34 [14]. In a thermostability study, both enzymes retained full activity in the range of 30–60°C for 30 min (Fig. 4a, b). The thermostability of the two enzymes was further studied for 48 h. Both enzymes were highly stable (maintaining 100% activity) at the optimum temperature (60°C) for up to 48 h (Fig. 4c, d). When incubated at an elevated temperature of 65°C, the half-life of ASKA was approximately 48 h, whereas it was approximately 3 h for ADTA. ASKA is more thermotolerant than certain other alkaliphilic *Bacillus* α-amylases, e.g., the α-amylases from *Bacillus* sp. KSM-K38 [13] and *B. halodurans* LBK 34 [14].

The result indicates that ASKA outperformed ADTA in thermostability. The ASKA and ADTA primary sequences contained only 17 different amino acids, and some of these residues may be involved in stabilizing the ASKA structure. To date, BLA is the most well-studied α-amylase for thermostability. Through numerous mutagenesis studies, more than a dozen residues were determined to be significantly important for structural stabilization, including His-133, Asn-172, Asn-188, Asn-190, and Ala-209 of BLA, which are all clustered in domain B and interface with domain A [9–11]. Interestingly, none of the locations for these ASKA–ADTA invariant amino acids have been studied previously in BLA. Currently, there is no indication as to which residues contribute to the ASKA conformational stability.

The addition of 5 mM $CaCl_2$ significantly enhanced the thermostability of ASKA and ADTA. They remained fully active at 60 and 65°C for up to 48 h of incubation. At 70°C, they remained stable (more than 90% active) for up to 48 h (Fig. 4c, d). This result indicates that both amylases contain calcium binding sites, as shown in the sequence alignment (Online Resource 1). The structural integrity improved when Ca^{2+} was bound to these sites, which was also shown for most of the α-amylases including those from *Bacillus licheniformis* NH1 [15], *Bacillus* sp. strain TSCVKK [17], *Bacillus halodurans* MS-2-5 [22], and *Pseudoalteromonas* sp. MY-1 [33]. The high thermostability of both in-house enzymes enhances their value for industrial applications.

Effect of metal ions and chemical reagents

In addition to enhancing the thermostability of the amylase by maintaining the structural integrity, Ca^{2+} ion addition can increase the relative activity of starch hydrolysis [33]. Ca^{2+} (5 mM) significantly increased the enzymatic activity of ASKA and ADTA (259% and 176%, respectively; $P < 0.05$) (Table 1). However, at a higher Ca^{2+} concentration (10 mM), the activity decreased by 23 and 9% for ASKA and ADTA, respectively (Table 1). A similar decrease was observed in α-amylase from *Bacillus halodurans* LBK 34 [14]. This finding could be from interference of Ca^{2+} ions that bind the secondary binding site of the substrate binding cleft [32].

Among the various metal ions tested, K^+, Na^+, and Fe^{2+} enhanced ASKA activity (17–44% activation)

Table 1 Effects of metal ions and chemical reagents on the activity of ASKA and ADTA

	Relative activity (%)	
	ASKA	ADTA
None	100 ± 1.2	100 ± 1.5
Metal ions (5 mM)		
Ca^{2+}	259 ± 2.9	176 ± 4.8
Ca^{2+} (10 mM)	199 ± 2.2	160 ± 2.9
K^{+}	144 ± 4.4	121 ± 4.8
Na^{+}	117 ± 3.6	91 ± 3.8
Fe^{2+}	119 ± 0.1	112 ± 3.0
Fe^{3+}	44 ± 2.1	13 ± 3.6
Mg^{2+}	59 ± 3.8	74 ± 2.7
Co^{2+}	45 ± 3.0	9 ± 0.4
Cu^{2+}	2 ± 0.1	1 ± 0.2
Chemical reagents		
Triton X-100 (5%, v/v)	119 ± 0.4	100 ± 1.1
Tween 20 (5%, v/v)	106 ± 0.2	99 ± 0.4
β-Mercaptoethanol (50 mM)	67 ± 2.2	75 ± 0.2
Urea (8 M)	53 ± 0.7	71 ± 1.4
SDS (1%, w/v)	3 ± 3.0	14 ± 2.8
EDTA (5 mM)	4 ± 3.4	7 ± 0.2

All of the metal ions were used at 5 mM. Calcium ions significantly increased the activity of both amylases. Values are the mean ± standard error from triplicate analyses

(Table 1). A similar effect was observed for ADTA, but Na^{+} had a smaller effect on enzyme activity. The activity of the *Bacillus* sp. Ferdowsicous α-amylase was also enhanced by Fe^{2+}, but no effect was observed with K^{+} or Na^{+} [2]. ASKA and ADTA were inhibited by Fe^{3+}, Mg^{2+}, Co^{2+}, and Cu^{2+}. Similar results were shown for the *Bacillus halodurans* LBK 34 α-amylase, except for a slight decrease in activity with Co^{2+} [14]. The metal ions might interact with some important amino acid residues that are involved in the active site or substrate binding sites, which result in a conformational change that leads to a decrease in activity. ASKA and ADTA had a similar sensitivity to chemical reagents (Table 1); the enzymes tolerated non-ionic surfactants, such as Triton X-100 and Tween 20, up to a concentration of 5% (v/v), were slightly inhibited by β-mercaptoethanol (50 mM) and urea (8 M), and were inhibited by SDS and EDTA. The effects of chemical reagents on the enzyme activity vary widely among the α-amylases [1, 14, 15]. For instance, the *B. halodurans* LBK 34 α-amylase was completely inhibited by 5 mM EDTA [14], but *Bacillus* sp. A3-15 α-amylase remained stable at the same concentration of EDTA [1].

Substrate specificity

In addition to a high activity on starch, both enzymes were significantly active with amylose, but they had a low activity with amylopectin, pullulan, and glycogen (Table 2). These results indicate that both amylases hydrolyze α-1,4-glycosidic linkages in starch, but they have no or low hydrolytic activity for the α-1,6-glycosidic linkage. No hydrolytic activity was detected in either enzyme with α-CD. The substrate specificity for both enzymes was consistent with the common features of typical α-amylases [12].

Table 2 Relative activities of ASKA and ADTA toward various substrates

Substrates (1%, w/v)	Relative activity (%)	
	ASKA	ADTA
Soluble starch	100 ± 0.9	100 ± 0.6
Amylose	104 ± 3.2	120 ± 1.6
Amylopectin	49 ± 1.1	66 ± 1.2
Pullulan	14 ± 2.6	8 ± 0.6
Glycogen	6 ± 0.2	6 ± 0.1
α-CD	ND	ND

Values are the mean ± standard error from triplicate analyses

ND not detected

Analysis of hydrolysis products

The distribution of the end products from hydrolysis of different types of starch was determined by HPLC. Oligosaccharides of various chain lengths were produced from random hydrolysis at the interior of the starch molecules. On the basis of this hydrolytic pattern, both enzymes are classified as endo-acting α-amylases [34]. For ASKA, potato starch was the best substrate for maximum total

Table 3 Total oligosaccharides produced by the hydrolysis of different starches with ASKA and ADTA

Starches (1%, w/v)	Total oligosaccharides (mg/ml)	
	ASKA	ADTA
Soluble	6.2 ± 0.3	7.3 ± 0.4
Potato	7.4 ± 2.2	7.4 ± 0.8
Sago	7.1 ± 3.9	7.5 ± 3.3
Tapioca	7.0 ± 0.5	7.2 ± 2.6
Rice	6.8 ± 2.7	6.9 ± 2.7
Wheat	6.3 ± 1.2	6.6 ± 1.8
Corn	4.3 ± 1.6	4.6 ± 1.5

Enzymes were incubated with 1% (w/v) starch in a 100 mM Tris–HCl buffer (pH 8) at 60°C for 24 h. The produced oligosaccharides were analyzed and quantified using an HPLC system. Values are the mean ± standard error from triplicate analyses

oligosaccharide production among the starches tested (Table 3). For ADTA, oligosaccharide production with potato starch was slightly lower than with sago starch.

To compare the hydrolytic profiles for ASKA and ADTA, potato starch was chosen as a substrate, as the total yields of oligosaccharides from both amylases were similar (Table 3). The oligosaccharides formed from potato starch hydrolysis were monitored over a period of 46 h. From Fig. 5, it can be deduced that the mode of hydrolytic action for both amylases is similar. At the beginning of the reaction, starch was hydrolyzed to form oligosaccharides of various lengths that ranged from maltose to maltopentaose. In a prolonged reaction, some of the maltotetraose and maltopentaose oligosaccharides were further degraded into maltose (69%) and eventually accumulated as the final primary product. Under the experimental conditions, negligible glucose levels were detected.

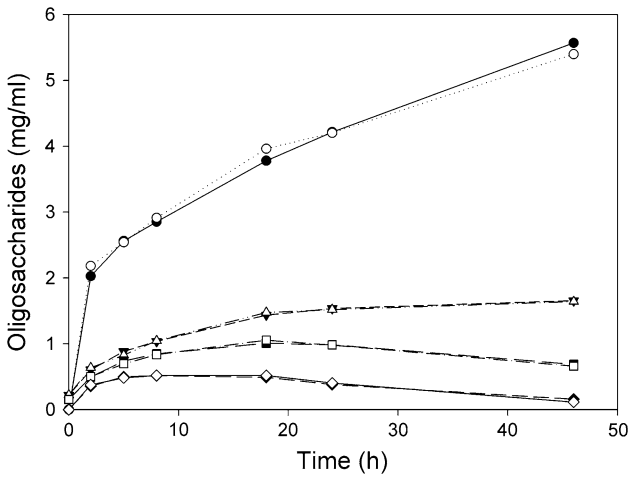

Fig. 5 Time course for the hydrolysis of 1% (w/v) gelatinized potato starch by ASKA and ADTA in a 100 mM Tris–HCl buffer (pH 8) at 60°C. The oligosaccharides that were formed by the action of ASKA (G2 *filled circles*, G3 *filled inverted triangle*, G4 *filled squares*, and G5 *filled diamond*) and ADTA (G2 *open circles*, G3 *open triangles*, G4 *open squares*, and G5 *open diamonds*). Maltose is the main product for both enzymes. Values are the mean ± standard error of triplicate analyses

The abilities of ASKA and ADTA to produce high levels of maltose suggest that they may be useful in the baking industry and maltose syrup production. The commonly used industrial *A. oryzae* α-amylase produced syrups of 50–60% maltose, whereas the recently published high maltose-forming α-amylase from *Bacillus acidicola* produced 67% maltose [28]. Under the experimental conditions, 69% maltose was produced by both *Anoxybacillus* α-amylases, and this percentage is comparable to those of the *A. oryzae* and *Bacillus acidicola* amylases. It was known that most of the high maltose-forming α-amylases are active at acidic to neutral pH values and that some have a low thermostability, especially those of fungal origin [19]. Hence, the alkalitolerant and thermostable features of ASKA and ADTA provide a new alternative for the industry.

Conclusion

The two reported α-amylases (ASKA and ADTA) from the *Anoxybacillus* strains SK3-4 and DT3-1 suit the requirements for wide industrial applications that include the food, pharmaceutical, biomedical, and detergent industries. Both enzymes were highly stable in the presence of calcium. However, even in the absence of calcium, they remained stable at 60°C for at least 48 h and were stable over a wide pH range of 6–10. Both of the *Anoxybacillus* α-amylases could produce high levels of maltose and possess atypical protein sequences compared with other α-amylases. These two *Anoxybacillus* α-amylases are only different at 17 amino acids; however, their thermostabilities clearly differ. This result enhances the level of interest in the protein structure and function of these new amylases. Structure predictions, dynamics simulations, and mutagenesis experiments will be conducted in the near future.

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