A New Extremely Halophilic, Calcium-Independent and Surfactant-Resistant Alpha-Amylase from *Alkalibacterium* sp. SL3

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A new α -amylase-encoding gene (*amySL3*) of glycoside hydrolase (GH) family 13 was identified in soda lake isolate *Alkalibacterium* sp. SL3. The deduced AmySL3 shares high identities (82–98%) with putative α -amylases from the genus *Alkalibacterium*, but has low identities (<53%) with functionally characterized counterparts. *amySL3* was successfully expressed in *Escherichia coli*, and the recombinant enzyme (rAmySL3) was purified to electrophoretic homogeneity. The optimal temperature and pH of the activity of the purified rAmySL3 were determined to be 45°C and pH 7.5, respectively. rAmySL3 was found to be extremely halophilic, showing maximal enzyme activity at a nearly saturated concentration of NaCl. Its thermostability was greatly enhanced in the presence of 4 M NaCl, and it was highly stable in 5 M NaCl. Moreover, the enzyme did not require calcium ions for activity, and was strongly resistant to a range of surfactants and hydrophobic organic solvents. The major hydrolysis products of rAmySL3 from soluble starch were maltobiose and maltotriose. The high ratio of acidic amino acids and highly negative electrostatic potential surface might account for the halophilic nature of AmySL3. The extremely halophilic, calcium-independent, and surfactant-resistant properties make AmySL3 a promising candidate enzyme for both basic research and industrial applications.

Keywords: α -Amylase, glycoside hydrolase family 13, halophilic, surfactant-resistant, calcium-independent, *Alkalibacterium*

Introduction

Starch is composed of linear amylose and branched amylopectin, in which glucose units linked by α -1,4 and α -1,6 glucosidic bonds form the main and branched chains, respectively [1]. Usually, four types of enzymes, endo-amylase, exo-amylase, debranching enzyme, and transferase, are involved in the conversion of starch [2]. α -Amylase (E.C. 3.2.1.1) is a well-known endo-amylase that randomly catalyzes the hydrolysis of the internal α -1,4-glucosidic bonds in starch and related carbohydrates to produce glucose, oligosaccharides, and dextrins [3]. Based on sequence similarities of the catalytic domain, α -amylases have been classified into several glycosyl hydrolase (GH) families (http://www.cazy.org/fam/acc_GH.html): 13, 57,

119, and 126 [4]. The majority of α -amylases belong to GH family 13, which has a catalytic triad in the TIM barrel catalytic domain and four conserved regions [2].

α -Amylases are among the most important industrial enzymes and account for approximately a quarter of the enzyme market [5]. Although α -amylases are universally distributed in plants, animals, and microorganisms, microbial α -amylases have attracted particular attention because of their wide applications in starch saccharification, baking, brewing, laundry, and textiles, as well as in the pharmaceutical industries [6–8]. α -Amylases from extremophiles have been of great interest because they are able to remain active under harsh conditions [9, 10]. In addition, they offer a good starting point to understand how protein structure relates to function, paving the way for the engineering of

other enzymes [11, 12]. To date, α -amylases from diverse extremophiles, including thermophiles, psychrophiles, acidophiles, alkaliphiles, and halophiles, have been reported [9]. Of these, α -amylases from halophiles have attracted increasing interest as they are intrinsically stable and active at high salt concentrations and thus have important potential biotechnological applications in food processing, environmental bioremediation, and pharmaceuticals [13].

Despite their great potential for industrial applications, there have been very limited studies on halophilic α -amylases. To date, no more than 20 halophilic α -amylases have been purified and characterized from halophilic Archaea, bacteria, and fungi [13]. There are even fewer studies on the gene cloning, overexpression, and characterization of halophilic or halotolerant α -amylases [13]. In our previous study, salt-tolerant xylanase and esterase were obtained from soda lake isolate *Alkalibacterium* sp. SL3 [14, 15]. In this study, an extremely halophilic α -amylase-encoding gene (*amySL3*) was cloned from strain SL3. The α -amylase gene was successfully expressed in *Escherichia coli* and purified by Ni-affinity chromatography. Sequence and function analyses suggested that the recombinant enzyme was extremely halophilic and had some superior properties over the known halophilic α -amylases.

Materials and Methods

Strains, Vectors, and Chemicals

The donor strain of α -amylase gene *amySL3*, *Alkalibacterium* sp. SL3, was isolated from the sediment of a soda lake [14]. The strain has been deposited at China General Microbiological Culture Collection Center as CGMCC 1.13866. *E. coli* Top10 and vector pMD19-T from TaKaRa (Japan) were used for gene cloning. *E. coli* BL21(DE3) and vector pET-28a (+) from Novagen (USA) were used for gene expression. Kits for genomic DNA extraction, DNA purification, and plasmid isolation were purchased from Omega (USA). T4 DNA ligase, restriction endonucleases, DNA polymerase, and dNTPs were purchased from Thermo Fisher Scientific (USA).

The nickel-nitrilotriacetic acid (NTA) agarose from Qiagen (Germany) was used to purify His₆-tagged protein. The soluble starch substrate was purchased from Sigma (USA). All other chemicals were of analytical grade and commercially available.

Cloning of the Full-Length α -Amylase Gene *amySL3*

Genomic DNA of *Alkalibacterium* sp. SL3 was extracted and purified using the Omega kits following the manufacturer's instructions. Two degenerate primers, *amy*-F and *amy*-R, were designed according to the conserved motifs of nine *Alkalibacterium* α -amylases (Table 1). The gene fragment of *amySL3* was obtained using the purified genomic DNA as template and the degenerate primer set *amy*-F/-R designed above. The optimized PCR conditions were: 4 min at 95°C; 12 cycles of 94°C for 30 sec, 60°C (decreasing by 0.5°C after each cycle) for 30 sec, and 72°C for 30 sec; followed by 28 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 5 min. The PCR products were excised, purified, and ligated into vector pMD19-T. The recombinant plasmid was then transformed into *E. coli* Top10, and the objective gene fragment was sequenced by Invitrogen (USA). Because the gene fragment of *amySL3* shared 94% nucleotide sequence identity with the α -amylase gene from *Alkalibacterium pelagium* DSM19183 (NZ_FNZU01000004), primers *amySL3*-F and *amySL3*-R were then designed accordingly to amplify the full-length gene of *amySL3*. The PCR product was obtained and sequenced as described above.

Sequence and Structure Analysis

DNA and protein sequence similarities were assessed using the BLASTn and BLASTp programs (<http://www.ncbi.nlm.nih.gov/BLAST/>), respectively. The signal peptide sequence was predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). To obtain the predicted tertiary structure of AmySL3, the protein sequence was directly submitted to I-TASSER [16], one of the best protein structure prediction servers. The top template identified by I-TASSER was 1HVX, the α -amylase from *Bacillus stearothermophilus* [17]; its sequence identity with AmySL3 was 50% with 99% coverage. The alignment z-score by MUSTER [18] on the I-TASSER server was 23.1, which is significantly higher than the cutoff of 6.1. The prediction was classified as "Easy" by I-TASSER, and the final model was therefore considered to be reliable.

Table 1. Primers used in this study.

Primers	Sequences (5' → 3') ^a	Size (bp)
<i>amy</i> -F	GAGGAGACGAAGAARTGGATHCAYTGG	27
<i>amy</i> -R	CTCTACGAAAGTNACNGCYTGYTCNGC	27
<i>amySL3</i> -F	ATGAATGGAACAATGATGCAGTACTTTG	28
<i>amySL3</i> -R	TTATTCTGTTTTCTGACCCATACGG	26
<i>AmySL3</i> -m-F	TTCG AGCT CATGAATGGAACAATGATGCAGTAC	33
<i>AmySL3</i> -m-R	GTG CTCGAG TTTATTCTGTTTTCTGACCCATACG	35

^aRestriction sites are bold.

Expression and Purification of rAmySL3 in *E. coli*

An expression primer set (Table 1) was used to amplify the full-length *amySL3*, which was then cloned into the *SacI-XhoI* site of pET-28a (+). The recombinant plasmid, pET-*amySL3*, was transformed into *E. coli* BL21 (DE3) competent cells. Expression, purification, and SDS-PAGE analysis of the recombinant α -amylase rAmySL3 were performed following the protocol described by Wang *et al.* [14]. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was used to identify the purified protein at ProTech (China).

Enzyme Assay

The α -amylase activity was determined using the dinitrosalicylic acid (DNS) method [19]. The standard reaction system contained 0.1 ml of appropriately diluted purified rAmySL3 and 0.9 ml of McIlvaine buffer (0.2 M Na_2HPO_4 , 0.1 M citric acid, pH 7.5) containing 1% (w/v) soluble starch and 4 M NaCl. After incubation at 45°C for 10 min, reactions were terminated by the addition of 1.5 ml of DNS reagent and a 5 min-boiling water bath. The amount of reducing sugar was determined spectrophotometrically at 540 nm. Maltose was used to generate a standard curve. One unit (U) of α -amylase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to maltose per minute. All reactions were performed in triplicate.

Biochemical Characterization

The substrate specificity of rAmySL3 was determined in McIlvaine buffer (pH 7.5) containing 1% of substrate under standard conditions (45°C, 10 min). The substrates used were soluble starch, amylose, amylopectin, pullulan, glycogen, corn starch, potato starch, and tapioca starch.

The optimal pH of rAmySL3 was determined at 37°C in buffers with pH ranging from 4.0 to 9.0. The pH stability was estimated by measuring the rAmySL3 activity under standard conditions (pH 7.5, 45°C and 10 min) after 1 h-incubation at pH 4.0 to 10.0 and 37°C without substrate. The initial rAmySL3 activity was set to 100%. The buffers used were McIlvaine buffer (pH 4.0–8.0), 0.1 M Tris-HCl (pH 8.0–9.0), and 0.1 M glycine-NaOH (pH 9.0–10.0). The optimal temperature of rAmySL3 was determined in McIlvaine buffer (pH 7.5) over the temperature range of 5°C to 70°C for 10 min. The thermostability was determined by measuring the residual activity under standard conditions after pre-incubation of the enzyme in McIlvaine buffer (pH 7.5) at 35°C, 40°C, 45°C, and 50°C for various durations.

For kinetic analysis, the enzymatic activities of rAmySL3 were assayed in McIlvaine buffer (pH 7.5) containing 1–10 mg/ml soluble starch at 45°C for 5 min. The K_m , V_{max} , and k_{cat} values were determined using the non-linear regression program GraFit (Erithacus, Horley, UK).

The effect of metal ions, surfactants, and organic solvents on rAmySL3 activity and stability was also determined. The rAmySL3 thermostability in the presence of 4 M NaCl was assayed at 45 and 50°C as described above. The salt-stability was

determined in the presence of 4 or 5 M NaCl at 37°C. The effect of Na^+ , Ca^{2+} , ethylenediaminetetraacetic acid (EDTA), Triton X-100, Tween-20, Tween-80, methanol, ethanol, butanol, isobutanol, isoamyl alcohol, acetone, glycerol, *n*-hexane, and chloroform on rAmySL3 activity was determined at 45°C in McIlvaine buffer (pH 7.5) containing 0–5.0 M NaCl, 0–20 mM CaCl_2 , 0–100 mM EDTA, 5 or 10% (v/v) surfactant, or 20 or 50% (v/v) organic solvent. To determine the interacted effect of EDTA and calcium ion on rAmySL3 activity, the purified enzyme was treated with 100 mM EDTA for 30 min at 4°C, followed by three-time dialysis in Tris-HCl buffer (pH 7.5). The EDTA-treated enzyme was then subjected to amylase activity assay in the presence of 0, 0.1, 0.5, 1, 2, or 5 mM CaCl_2 . The effect of SDS on rAmySL3 activity was also tested at the concentrations of 0.1% and 1% with 2 M NaCl (SDS will salt out when its concentration is higher than 1% in the presence of 2 M NaCl). The enzyme activity without any addition was defined as 100%.

Hydrolysis Product Analysis

Purified rAmySL3 was mixed with 1% (w/v) of each substrate (soluble starch, amylose, amylopectin, corn starch, potato starch, and tapioca starch) in McIlvaine buffer (pH 7.5) and incubated at 45°C for 24 h. Thin-layer chromatography (TLC) with the silica gel G-60 and a solvent system of butanol, ethanol, and water (5:3:2, v/v/v) was used to analyze the hydrolysis products. Carbohydrate products were visualized by spraying with a mixture of 1% (v/v) aniline, 1% (w/v) diphenylamine, and 5% (v/v) phosphoric acid in acetone. Glucose (G), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7) were used as standards.

Results

Sequence and Structure Analysis of *amySL3*

A full-length α -amylase gene (*amySL3*; GenBank Accession No. MH763825) of 1,455 bp was successfully cloned from *Alkalibacterium* sp. SL3. *amySL3* encodes a polypeptide of 484 amino acid residues with a calculated mass of 56.6 kDa and a theoretical isoelectric point of 4.56. No signal peptide was predicted by the SignalP. The deduced amino acid sequence of *amySL3* has the highest identities with putative α -amylases from *Alkalibacterium*: 98% with *A. pelagium* (WP_091479515.1), 97% with *A. thalassium* (WP_091267104.1), 93% with *A. olivapovlenticus* (WP_106194330.1), 83% with *A. putridalgalicola* (WP_091487088.1), 83% with *Alkalibacterium* sp. AK22 (WP_034301409.1), and 82% with *A. gilvum* (WP_091633519.1), but shares low sequence identities (33–53%) with functionally characterized α -amylases from *Bacillus amyloliquefaciens* [20] and other halophilic α -amylases (Table 2). Deduced AmySL3 belongs to GH family 13, containing four highly conserved regions [2] and three

Table 2. Enzymatic characteristics and amino acid compositions of AmySL3 and other halophilic or halotolerant counterparts.

Enzyme	Microbial source	Characteristic	T _{opt} (°C)	pH _{opt}	NaCl _{opt} (M)	Specific activity (U mg ⁻¹) ^a	K _m (mg ml ⁻¹)	V _{max} (μmol mg ⁻¹ min ⁻¹)	k _{cat} (s ⁻¹)	Asp+Glu (%)	Lys+Arg (%)	Hydrophobic amino acids (%) ^b	Reference
AmySL3	<i>Alkalibacterium</i> sp. SL3	Halophilic	45	7.5	5.0	313.9	3.6	527.5	497.7	20.7	8.9	30.0	This study
AmyHh	<i>Haloarcula</i> <i>hispanica</i>	Halophilic	50	7.5	4.0	-	-	-	-	16.5	6.1	32.9	[25]
AmyHj	<i>Haloarcula</i> <i>japonica</i>	Halophilic	45	6.5	2.6	24	-	-	-	18.3	7.5	35.1	[39]
AmyN	<i>Natronococcus</i> sp. Ah-36	Halophilic	55	8.7	2.5	160	25	-	-	24.2	5.4	32.8	[38]
AmyEc	<i>Escherichia coli</i> JM109	Halophilic	55	7.0	2.0	1090	4.3	909	825	17.0	8.1	35.4	[40]
AmyKv	<i>Kocuria</i> <i>varians</i>	Halophilic	-	-	2.0	273	-	-	-	16.9	3.8	33.7	[21]
AmyHt	<i>Haloterrigena</i> <i>turkmenica</i>	Halophilic	55	8.5	2.0	79.8	-	-	-	18.2	6.1	31.0	[33]
AmyHm	<i>Halomonas</i> <i>meridiana</i>	Halophilic	37	7.0	1.7	-	-	-	-	12.5	5.5	34.8	[41]
AmyZp	<i>Zunongwangia</i> <i>profunda</i>	Halotolerant	35	7.0	1.5	275.8	2.7	287.7	316.5	15.5	9.8	28.6	[27]
AmyP	<i>Pseudoalteromonas</i> sp. M175	Halotolerant	25	8.0	1.0	337.9	2.5	0.125	-	13.3	8.4	34.4	[29]
AmyHo	<i>Halothermothrix</i> <i>oreni</i>	Halotolerant	65	7.5	0.9	22.3	-	-	-	14.5	10.8	31.8	[42]
AmyPh	<i>Alteromonas</i> <i>haloplanctis</i> A23	Halotolerant	25	7.0	0.5	-	-	-	-	10.5	6.1	31.9	[43]

^aThe specific activities of amylase AmyHj and AmyHt were determined using the method of iodine-starch; the specific activities of amylase AmyN and AmyKv were determined by the method of Somogyi-Nelson; the specific activities of amylase AmySL3, AmyEc, AmyZp, AmyP and AmyHo were determined by the method of DNS.

^bIncluding Ala, Ile, Leu, Phe, Trp, and Val.

-, not determined

Fig. 1. Multiple sequence alignment of AmySL3 and three other GH13 α-amylases.

Identical and similar amino acids are highlighted in solid black and grey, respectively. The four conserved regions (I, II, III, and IV) are boxed. The three conserved catalytic residues (Asp²³³, Glu²⁵³, and Asp³³⁰) are marked with triangles. Na⁺ or Ca²⁺ binding sites are marked with asterisks. The sequence name, microbial source, and GenBank accession numbers of each α-amylase are shown as follows: AmySL3, *Alkalibacterium* sp. SL3 (this study); AmyBH, *Bacillus halmapalus* (WP_078382693); AmyB707, *Bacillus* sp. 707 (P19571); and AmyBA, *Bacillus amyloliquefaciens* (WP_013352208).

putative catalytic residues, Asp²³³, Glu²⁵³, and Asp³³⁰, which form the catalytic triad (Fig. 1).

The amino acid composition of AmySL3 was analyzed and compared with those of ten halophilic or halotolerant α-amylases (Table 2). AmySL3 is distinguished by high Asp+Glu content (20.7%) and low hydrophobic amino acid content (30.0%). Using 1HVX [17] as the template, homology-modeled AmySL3 has the typical (β/α)₈ barrel structure of GH family 13 proteins (Figs. 2A and 2B). Surface electrostatic potential analysis indicated that most acid amino acid residues are distributed on the surface of AmySL3 (Figs. 2C and 2D).

Expression and Purification of rAmySL3

After induction with 1 mM IPTG at 30°C for 12 h, significant α-amylase activity was detected in the cell lysate. The purified rAmySL3 migrated as a single band on SDS-PAGE (Fig. 3), but had a molecular mass slightly higher than the calculated value (56.6 kDa). Similar migration has been reported in the halophilic α-amylase from moderately halophilic bacterium *Kocuria varians* [21]. Three internal peptides obtained by LC-ESI-MS/MS,

Fig. 2. The tertiary structure and corresponding electrostatic potentials of the homology-modeled AmySL3.

(A) and (B) show top and bottom views of the model, respectively. (C) and (D) show surface electrostatic potentials for (A) and (B), respectively. The negative and positive electrostatic potentials are indicated by red and blue, respectively.

Fig. 3. SDS-PAGE analysis of the purified rAmySL3. Lanes: M, the molecular-weight protein marker; 1, the cell lysate of an uninduced transformant harboring pET-amySL3; 2, the cell lysate of an induced transformant harboring pET-amySL3; and 3, the purified rAmySL3 after Ni-affinity chromatography.

SVGQDDVGYGIYDLYDLGEFDQK, PLAYGFILLSYYGYPCVFYSDYYGYK, and EMSVGELHANEVYVDLMNNR, matched the deduced amino acid sequence of AmySL3. The specific activity of purified rAmySL3 towards soluble starch was determined to be 313.9 U/mg.

Enzyme Characterization

The pH and temperature properties of rAmySL3 were determined using soluble starch as the substrate. rAmySL3 showed the highest activity at pH 7.5, and more than 60% of the maximum activity remained between pH 6.5 and 8.0 (Fig. 4A). The enzyme was stable between pH 6.5 and 10.0, retaining more than 50% of its initial activity after incubation at 37°C for 1 h (Fig. 4B). rAmySL3 was apparently optimal at 45°C when assayed at pH 7.5, and it retained more than 50% of its maximum activity when assayed at 25–55°C (Fig. 4C). Without NaCl, the enzyme was stable at 35°C and 40°C for more than 1 h, but lost 50% activity at 45°C and 50°C after 20 min and 7 min (Fig. 4D), respectively.

Fig. 4. Enzymatic properties of the purified rAmySL3. (A) Effect of pH on rAmySL3 activity. Activities at various pH values were assayed at 37°C for 10 min in the presence of 4 M NaCl. (B) pH stability of rAmySL3. Residual activities after incubation of the purified enzyme at various pH values for 1 h at 37°C were assayed at pH 7.5, 45°C, and 4 M NaCl for 10 min. (C) Effect of temperature on rAmySL3 activity in McIlvaine buffer (pH 7.5, 4 M NaCl). (D) Thermostability of rAmySL3. Residual activities were assayed at pH 7.5, 45°C and 4 M NaCl for 10 min after pre-incubation at 35°C, 40°C, 45°C, and 50°C without NaCl for different periods of time. Thermostability of rAmySL3 at 45°C and 50°C in the presence of 4 M NaCl was also assayed. Each value in the panel represents the mean $\pm$ SD ($n = 3$).

Fig. 5. Effect of CaCl_2 and EDTA on the rAmySL3 activity. (A) The effect of 0–20 mM CaCl_2 on the activity of rAmySL3. (B) The effect of 0–5 mM CaCl_2 on the activity of EDTA-treated rAmySL3. (C) The effect of 0–100 mM EDTA on the activity of rAmySL3. Activities were assayed at pH 7.5, 45°C and 4 M NaCl for 10 min. Each value in the panel represents the mean $\pm$ SD ($n = 3$).

Using soluble starch as the substrate, the K_m , V_{max} and k_{cat} values were 3.6 ± 0.2 mg/ml, 527.5 ± 12.3 μmol mg/min, and 497.7 ± 11.6 s^{-1} , respectively.

The effect of CaCl_2 and EDTA on rAmySL3 activity was investigated. As shown in Fig. 5A, low concentrations (<2 mM) of CaCl_2 had no effect on the activity of rAmySL3, while high concentrations (>10 mM) of CaCl_2 obviously inhibited the activity. EDTA-treated enzyme retained high activity when CaCl_2 was absent (Fig. 5B). EDTA alone

Fig. 6. Effect of NaCl on the rAmySL3 activity and stability. (A) Effect of different concentrations of NaCl on the activity of rAmySL3. (B) Effect of 4 M and 5 M NaCl on the stability of rAmySL3. Residual activities were assayed at pH 7.5, 45°C and 4 M NaCl for 10 min. Each value in the panel represents the mean $\pm$ SD ($n = 3$).

inhibited the rAmySL3 activity (Fig. 5C), causing an activity reduction of 14% at 5 mM and 64% at 100 mM, respectively.

Extremely Halophilic and Salt-Dependent Stability

The effect of NaCl on rAmySL3 activity and stability was also investigated. As shown in Fig. 6A, rAmySL3 lost α -amylase activity without NaCl, but showed increased activity along with the increased concentration of NaCl up to 5.0 M. Moreover, rAmySL3 was highly stable at high concentrations of NaCl, retaining more than 90% activity after a 1 h-incubation at 37 °C and pH 7.5 with 4 M or 5 M NaCl (Fig. 6B). The thermostability of rAmySL3 was improved in the presence of 4 M NaCl. After incubation at pH 7.5 and 45 or 50°C for 1 h, the enzyme retained 87% and 71% of its initial activity (Fig. 4D), respectively. Under the same conditions, it lost 74–100% activity without NaCl.

Table 3. Effects of various surfactants and organic solvents on the activity of the purified rAmySL3.

Chemicals	Concentration (%)	Relative activity (%) ^a	Chemicals	Concentration (%)	Relative activity (%)
Control	0	100.0 ± 0.0	Butanol	20	88.6 ± 2.5
				50	5.4 ± 0.0
Tween-20	5	103.7 ± 2.9	Isobutanol	20	90.7 ± 0.9
	10	115.2 ± 4.1		50	9.5 ± 1.3
Tween-80	5	95.9 ± 1.5	Isoamyl alcohol	20	89.8 ± 0.6
	10	117.3 ± 1.9		50	38.0 ± 1.1
Triton X-100	5	102.3 ± 0.0	Acetone	20	6.6 ± 0.9
	10	81.5 ± 1.3		50	ND
SDS	0.1	40.2 ± 0.8	Glycerol	20	85.8 ± 0.2
	1	11.0 ± 0.6		50	73.8 ± 2.9
Methanol	20	ND	n-Hexane	20	90.0 ± 0.5
	50	ND		50	95.9 ± 0.3
Ethanol	20	3.3 ± 0.3	Chloroform	20	85.1 ± 0.4
	50	3.8 ± 0.3		50	83.2 ± 0.6

^aThe AmySL3 activity was assayed in the reaction systems containing 0.1 ml of appropriately diluted purified rAmySL3, 0.9 ml McIlvaine buffer (pH 7.5), 1 M NaCl, and different concentrations of chemical reagent at 45°C. The specific activity (307.6 U mg⁻¹) without any reagent was defined as 100%. The data are shown as means ± SD (*n* = 3). ND, not detected.

Tolerance to Various Surfactants and Organic Solvents

The tolerance of rAmySL3 against various surfactants and organic solvents was determined (Table 3). Of the four surfactants tested, Tween-20 and Tween-80 had no obvious effects on rAmySL3 activity at the concentration of 5% (v/v), but enhanced the activity by more than 15% at the concentration of 10% (v/v). The enzyme was also highly resistant to Triton X-100, retaining more than 80% of its initial activity at the concentrations of 5 and 10% (v/v). However, rAmySL3 was highly sensitive towards SDS,

retaining approximately 40% and 11% of its initial activity at the concentrations of 0.1% and 1% (m/v), respectively. Of the tested organic solvents, rAmySL3 activity was greatly inhibited by methanol, ethanol, and acetone and high concentration (50%) of butanol, isobutanol, and isoamyl alcohol. Moreover, rAmySL3 was highly tolerant to hydrophobic solvents, retaining more than 70% activity in the presence of n-hexane and chloroform at concentrations up to 50%.

Fig. 7. TLC analysis of the hydrolysis products generated by rAmySL3.

(A) Hydrolysis products generated from soluble starch at pH 7.5, 45°C and 4 M NaCl. Samples were taken at 30 min (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), and 24 h (lane 6), respectively. (B) Hydrolysis products generated from potato starch (lane 1), corn starch (lane 2), tapioca starch (lane 3), amylopectin (lane 4), and amylose (lane 5) after 24 h incubation. Lane M, the standard of malto-oligosaccharides. G, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; and G7, maltoheptaose.

Substrate Specificity and Hydrolysis Product Analysis

rAmySL3 had high specific activities towards soluble starch (313.9 U/mg), tapioca starch (291.4 U/mg), amylopectin (213.7 U/mg), potato starch (191.6 U/mg), and corn starch (120.3 U/mg). It had weak activity towards amylose (38.6 U/mg) and glycogen (37.8 U/mg), and no activity against pullulan. It suggests that AmySL3 is specific on the cleavage of α -1,4-glucosidic bonds.

The hydrolysis products of various substrates by rAmySL3 were determined by TLC. The major hydrolysis products of soluble starch were maltose and maltotriose, plus a small proportion of glucose and maltotetraose (Fig. 7A). With potato starch, corn starch, and tapioca starch as the substrate, the major hydrolysis products were composed of maltobiose, maltotriose, and maltotetraose; while the products from amylopectin and amylose were mainly maltose and maltotriose (Fig. 7B).

Discussion

A soda lake represents a unique habitat which is characterized by both high alkalinity and high salinity, and harbors a rich diversity of novel enzyme-producing microorganisms [22]. *Alkalibacterium* sp. SL3 was isolated from the sediment of a typical soda lake with high salinity and alkalinity [23]. In our previous study, a cold-adapted and highly salt-tolerant esterase and a salt-stable alkaline xylanase have been obtained from this strain [14, 15]. In this study, a new α -amylase gene, *amySL3*, was cloned from strain SL3. Although deduced AmySL3 has high similarity to those putative amylases from *Alkalibacterium*, none of them has been functionally characterized. The low identities (<53%) of AmySL3 with functionally characterized α -amylase and halophilic or halotolerant counterparts (Table 2) suggest that the AmySL3 from *Alkalibacterium* sp. SL3 is a new α -amylase with great research and application potential.

One characteristic feature of halophilic enzymes is that they require high salt concentrations for activity and stability [13]. rAmySL3 under study was adaptable to high concentrations of NaCl up to 5 M (Fig. 6A), and remained stable in the presence of 5 M NaCl (Fig. 6B). The enzyme lost activity without NaCl, and its thermostability was greatly enhanced by 4 M NaCl (Fig. 4D). These results suggested that rAmySL3 is an extremely halophilic α -amylase. Extremely halophilic α -amylases are mainly from halophilic Archaea. For example, the halophilic α -amylase from *Haloarcula* sp. strain S-1 exhibited maximal activity at 4.3 M NaCl [24]; the halophilic α -amylase AmyH

from *Haloarcula hispanica* exhibited maximal activity at 4–5 M NaCl [25]; and the polyextremophilic α -amylase from *Halorubrum xinjiangense* exhibited maximal activity at 4 M NaCl [26]. In contrast, halophilic or halotolerant α -amylases from bacteria have an optimal salinity of lower than 3 M [13]. To the best of our knowledge, rAmySL3 is the first bacterial α -amylase with an optimal salinity of up to 5 M NaCl (nearly saturated).

To date, only the α -amylases from marine bacteria *Pseudoalteromonas haloplanktis* [27], *Zunongwangia profunda* [28], and *Pseudoalteromonas* sp. M175 [29] were found to be both cold-active and halotolerant. rAmySL3 showed its maximum activity at 45°C, which is higher than the optimal temperature of three cold-active and halotolerant α -amylases, but lower than that of most other reported halophilic or halotolerant α -amylases (Table 2). Although rAmySL3 is not a cold-active enzyme, it retained more than 60% and 33% relative activity at 30°C and 20°C, respectively. In addition, the specific activity of rAmySL3 towards soluble starch is 313.9 U/mg, which is lower than the halophilic amylase AmyEc from *Escherichia coli* JM109 and the halotolerant α -amylase AmyP from *Pseudoalteromonas* sp. M175, but higher than most other halophilic or halotolerant α -amylases (Table 2). These results suggest that rAmySL3 has application potential in industries where low temperature and high salinity are required [30].

Calcium ion functions as an activator for most halophilic α -amylases, while high concentrations of EDTA inhibit α -amylase activity significantly [21, 25, 27]. In the case of rAmySL3, calcium ion had no activating effect on rAmySL3 activity, but was inhibitory at high concentrations (Fig. 5A). In addition, EDTA-treated rAmySL3 remained highly active and showed Ca^{2+} -independence (Fig. 5B). Usually, EDTA greatly inhibits the activity of Ca^{2+} -dependent α -amylases but has no or minor effect on the Ca^{2+} -independent α -amylases. Although rAmySL3 did not require Ca^{2+} for catalysis, its activity was inhibited by EDTA (Fig. 5C). Another study has reported that some α -amylases do not require Ca^{2+} for activity but are inhibited by EDTA [31]. One possible explanation is that EDTA itself might be an inhibitor of the α -amylase, rather than inhibiting the enzyme by chelating the metal ions.

Halophilic α -amylases might be highly tolerant to organic solvents because they function under low water conditions [32]. rAmySL3 remained highly active in the presence of hydrophobic solvents, while it lost activity in hydrophilic solvents (Table 3). Polar solvents may strip off essential water molecules from the active site of the enzyme and result in enzyme inactivation. The same phenomenon has

been found in the organic solvent-tolerant α -amylases from halophilic Archaea *Haloarcula* sp. strain S-1 [24] and *Haloterrigena turkmenica* [33]. Although rAmySL3 was inhibited by anionic surfactant SDS, it was more tolerant to nonionic surfactants (Tween-20, Tween-80, and Triton X-100) at higher concentrations than other reported halophilic α -amylases [33–36].

High content of acidic amino acid is usually found in halophilic proteins. Acidic amino acids on the surface facilitate the binding and maintenance of essential water molecules, making the proteins soluble, stable, and functional under high salinity conditions [32, 37]. rAmySL3 contains 20.7% acidic amino acids, which is lower than that of α -amylase from *Natronococcus* sp. Ah-36 [38], but higher than those of other halophilic or halotolerant counterparts (Table 2). In addition, most of the acidic amino acids display on the enzyme surface, which accounts for a negative electrostatic potential (Fig. 2). Moreover, rAmySL3 has a hydrophobic amino acid content of 30.0%, which is higher than that of α -amylase AmyZ from *Z. profunda* [27], but lower than those of other nine halophilic or halotolerant α -amylases (Table 2). These factors might allow rAmySL3 to form a solvation shell that keeps the protein surface hydrated and thus highly active and tolerant to high concentrations of salt.

In this study, a new α -amylase gene (*amySL3*) was cloned from a halophilic isolate *Alkalibacterium* sp. SL3 and expressed in *E. coli*. Biochemical characterizations demonstrated that rAmySL3 is an extremely halophilic, low-temperature-active, calcium-independent α -amylase with great tolerance to surfactants and organic solvents. These enzymatic properties give rAmySL3 broad potential for various applications, such as hypersaline waste treatment, processing seafood and saline food, and so on. Moreover, the polyextremophilic rAmySL3 represents a good candidate for basic research into the structure-function relationship.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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