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Cloning and Characterization of Cold-Adapted α-Amylase from Antarctic *Arthrobacter agilis*

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Abstract In this study, the gene encoding an α-amylase from a psychrophilic *Arthrobacter agilis* PAMC 27388 strain was cloned into a pET-28a(+) vector and heterologously expressed in *Escherichia coli* BL21(DE3). The recombinant α-amylase with a molecular mass of about 80 kDa was purified by using Ni^{2+}-NTA affinity chromatography. This recombinant α-amylase exhibited optimal activity at pH 3.0 and 30 °C and was highly stable at varying temperatures (30–60 °C) and within the pH range of 4.0–8.0. Furthermore, α-amylase activity was enhanced in the presence of $FeCl_3$ (1 mM) and β-mercaptoethanol (5 mM), while $CoCl_2$ (1 mM), ammonium persulfate (5 mM), SDS (10 %), Triton X-100 (10 %), and urea (1 %) inhibited the enzymatic activity. Importantly, the presence of Ca^{2+} ions and phenylmethylsulfonyl fluoride (PMSF) did not affect enzymatic activity. Thin layer chromatography (TLC) analysis showed that recombinant *A. agilis* α-amylase hydrolyzed starch, maltotetraose, and maltotriose, producing maltose as the major end product. These results make recombinant *A. agilis* α-amylase an attractive potential candidate for industrial applications in the textile, paper, detergent, and pharmaceutical industries.

Keywords *Arthrobacter agilis* · Cold-adapted α-amylase · Cloning · Expression

Introduction

Around 80 % of the Earth's biosphere is occupied by cold ecosystems. This includes the oceans, which cover 70 % of the Earth's surface, along with polar and alpine regions [1–3]. Cold-tolerant bacteria have received considerable attention as they often produce enzymes with optimum activity at moderate to low temperatures [4–6].

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Cold-adapted enzymes offer the economic benefit of being more efficient at low temperatures than their mesophilic and thermophilic homologs, thereby saving energy during enzymatic processes. Consequently, cold-adapted enzymes have been applied in diverse industries involving food, detergents, biomass conversion, molecular biology, and baking [7]. Moreover, there is a growing scientific interest in the relationship between the structure and thermal stability of these enzymes [4, 6, 8, 9].

α-Amylases (EC.3.2.1.1.1,4-α-D-glucan-glucanohydrolase) are endo-acting enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in linear amylose chains. As a result, they generate dextrins and smaller polymers composed of glucose units [10–13]. α-Amylases occupy approximately 30 % of the world's enzyme market and have a wide range of applications in starch-processing industries such as baking, brewing, distilling, textiles, detergents, and paper. Furthermore, they have biotechnological applications such as in the food, pharmaceutical, and bioremediation industries [10–12, 14]. Therefore, cold-adapted α-amylases have drawn considerable attention in industrial applications as they provide economic and ecologic benefits by reducing energy consumption and environmental impact [15, 16].

Although some cold-adapted α-amylases have been identified, these studies are limited [17]. Because of the diverse applications of α-amylases, it is important to search for new cold-adapted α-amylases with desirable properties for industrial applications from as many different sources as possible [14, 15, 18]. In this study, we successfully cloned and expressed the α-amylase coding gene from the psychrophilic strain *Arthrobacter agilis* (PAMC 27388) in *Escherichia coli*. Furthermore, the recombinant α-amylase was purified by using Ni^{2+}-NTA affinity chromatography, and its biochemical properties were characterized.

Materials and Methods

Bacterial Strains, Plasmid, and Culture Conditions

Arthrobacter agilis PAMC 27388 (Polar and Alpine Microbial Collection, Incheon, South Korea) was used as the source for the α-amylase gene in this study. It was isolated from seawater in Antarctica King George Island (South Shetland Islands), and its amylase activity was confirmed by its ability to hydrolyze starch on marine agar containing 0.5 % soluble starch. *A. agilis* was grown in marine broth at 20 °C. *Escherichia coli* DH5α (Invitrogen, Waltham, MA, USA) and *E. coli* BL21(DE3) (Invitrogen) were used as cloning and expression host strains, respectively, and were cultivated in Luria-Bertani (LB) broth at 37 °C. The plasmid pET-28a(+) containing a kanamycin resistance gene was purchased from Invitrogen for gene cloning and protein expression.

Cloning of the α-Amylase Gene

Genomic DNA of *A. agilis* was isolated by using a genomic DNA purification kit (Invitrogen) according to the manufacturer's protocol, and partial genomic DNA sequences were analyzed by a MiSeq sequencing system (Illumina, San Diego, CA, USA). To amplify the DNA fragments of the putative α-amylase gene from *A. agilis*, the forward primer (5′-AAA CAT ATG GTG ACC ACC ACT ACT-3′) and reverse primer (5′-AAA AAG CTT TCA CCG GAG CCG TC-3′) were designed based on genome sequences. Polymerase chain reaction (PCR)

was performed by using *Pfu* DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) as follows: denaturation at 95 °C for 5 min, followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 40 s, extension at 72 °C for 80 s, and a final elongation at 72 °C for 10 min. After the amplified fragment and pET-28a(+) vector were digested by *NdeI* (Promega, Madison, WI, USA) and *HindIII* (Promega), the two cleaved fragments were ligated to obtain the pET28a/amyl plasmid. The plasmid (pET28a/amyl) was transformed into *E. coli* DH5α and then confirmed by colony PCR, double digestion, and DNA sequencing. Afterwards, pET28a/amyl was transformed into *E. coli* BL21(DE3) competent cells for heterologous expression.

The open reading frame of α-amylase from *A. agilis* was analyzed by the NCBI ORF Finder tool (http://www.ncbi.him.nih.gov/gorf/gorf/gorf.html) and InterProtool (https://www.ebi.ac.uk/interpro/). Amino acid sequences were aligned by using CLUSTAL OMEGA from EMBL-EBI online software (http://www.ebi.ac.uk/Tools/msa/clustalo/). Then, the aligned sequences were investigated by using MEGA 3.1 software (http://www.megasoftware.net/) to search for conserved regions. The NCBI accession number of the α-amylase gene from *A. agilis* is KX516725.

Expression of Recombinant α-Amylase

The *E. coli* BL21(DE3) cells harboring pET28a/amyl were cultivated overnight at 37 °C in LB broth containing 50 μg/mL kanamycin. The culture (1 % *v*/*v*) was inoculated into 100 mL of fresh LB broth containing 30 μg/mL kanamycin and grown at 37 °C until the OD_{600} reached 0.5–0.6. Next, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 1 mM to induce the heterologous expression of α-amylase, and cells were further incubated for 4 h at 30 °C. After incubation, the induced cells were harvested by centrifugation (8000×*g* for 20 min at 4 °C). The cell pellet obtained was resuspended in PBS, and membranes were disrupted by sonication. After centrifugation at 20,000×*g* for 10 min at 4 °C, SDS-PAGE analysis was performed by using the supernatant and pellet of cell lysates to detect the recombinant protein.

Purification of Recombinant α-Amylase

The insoluble fraction of the cell lysate was resuspended in solubilization buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl at pH 8.0) with lysozyme and incubated for 20 min to solubilize the inclusion bodies. After centrifugation at 24,000×*g* for 15 min at 4 °C, the supernatant containing soluble recombinant protein was collected. The recombinant α-amylase was purified by using Ni^{2+}-NTA affinity chromatography. The supernatant was loaded onto a Ni^{2+}-NTA affinity resin column (Thermo Scientific, Waltham, MA, USA) and equilibrated with binding buffer (8 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, 0.005 M imidazole at pH 8.0). Once the column was washed by using washing buffer (8 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, 0.02 M imidazole at pH 8.0), the recombinant protein was eluted with elution buffer (8 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, 0.4 M imidazole at pH 8.0). The eluted protein was dialyzed to remove urea slowly, which allowed for refolding of the recombinant α-amylase. The purified recombinant protein was detected by using SDS-PAGE as previously described [19]. The molecular mass of α-amylase was quantified on an 8 % polyacrylamide gel by using defined protein markers (Bioprince, Chuncheon, South Korea).

Protein Quantification and Activity Assay of α-Amylase

Concentration of the purified protein was determined by using a Bradford assay [20]. The absorbance was measured at 595 nm, and bovine serum albumin was used as a calibration standard. α-Amylase activity was assayed with the dinitrosalicylic acid (DNS) method reported by Miller et al. [21]. The reaction was performed by incubating purified enzyme in 50 mM Tris-HCl (pH 7.0) containing 2 % soluble starch as a substrate at 25 °C for 10 min. After adding DNS reagent, the sample was boiled for 5 min, and the absorbance was read at 575 nm by using a UV-vis spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). One unit of α-amylase activity was defined as the amount of enzyme required to liberate 1.0 mg of reducing sugar per minute under the assay conditions. Each assay was performed in triplicate.

Effects of pH and Temperature on α-Amylase Activity and Stability

The effect of pH on recombinant α-amylase activity was determined at pH values from 2.0 to 10.0 by using different 50 mM buffers (glycine-HCl for pH 2.0–3.0, sodium acetate for pH 4.0–5.0, potassium phosphate for pH 6.0–8.0, Tris-HCl for pH 9.0, and carbonate for pH 10.0). To measure the optimum pH for α-amylase activity, the enzyme was incubated in the aforementioned buffers containing 2 % soluble starch as a substrate at 25 °C for 10 min, and its activity was measured. In order to study pH stability, α-amylase was pre-incubated in the mentioned buffers at 25 °C for 1 or 24 h. After incubation, the pH of the buffer was adjusted to pH 7.0. Residual activity was assayed under standard α-amylase assay conditions (25 °C for 10 min, pH 7.0) and calculated by assuming enzyme activity at the beginning of the reaction as 100 %.

To determine the optimal temperature for α-amylase activity, the enzyme was incubated at different temperatures (10–70 °C) for 10 min in 50 mM Tris-HCl buffer pH 7.0 containing 2 % soluble starch. Thermal stability was studied by pre-incubating the enzyme at the aforementioned temperatures for 1 or 24 h. Afterwards, the residual activity was measured as described above. All experiments were carried out in triplicate.

Effects of Metal Ions and Chemical Reagents on α-Amylase Activity

Purified α-amylase was incubated in reaction mixtures containing various metal ions (1 mM Ca^{2+} [$CaCl_2$], Co^{2+} [$CoCl_2$], Mg^{2+} [$MgCl_2$], Fe^{3+} [$FeCl_3$], Zn^{2+} [$ZnCl_2$], Na^+ [NaCl], or K^+ [KCl]) and different chemical compounds (10 % SDS or Triton X-100, 5 mM ammonium persulfate, 5 mM β-mercaptoethanol, 5 mM ethylenediaminetetraacetic acid [EDTA], 1 % urea, or 1 mM phenylmethylsulfonyl fluoride (PMSF)) to evaluate the effect of each of these on the enzyme activity. Enzyme activity was assayed as described previously and expressed as a percentage of relative activity assuming the reaction without any additives to be 100 %. The assay was performed in triplicate.

Analysis of End Products by Thin Layer Chromatography

Thin layer chromatography (TLC) analysis was performed to identify the end products of hydrolysis by recombinant α-amylase as described by Zhang et al. [22]. Purified enzyme was incubated with 2 % maltotriose, maltotetraose, or soluble starch in 50 mM Tris-HCl pH 7.0 buffer at 25 °C for 24 h. Reaction samples were spotted on a TLC silica gel 60F plate (Merck,

Darmstadt, Germany) and dried. The plate was put in a TLC chamber saturated by *n*-butanol/acetic acid/water solution (4:8:1, *v*/*v*) as the mobile phase. After development, the plate was dried, and the end products were visualized by spraying with 10 % (*v*/*v*) sulfuric acid in methanol and heating at 110 °C for 10 min.

Results and Discussion

Cloning of the α-Amylase Gene from *A. agilis*

Genomic DNA of *A. agilis* PAMC 27388 was partially sequenced and annotated. Among the annotated genes, there was an open reading frame with homology to other amylase genes. This open reading frame was composed of 2163 nucleotides, which encoded a polypeptide of 720 amino acid residues. The molecular mass and theoretical pI of this putative α-amylase were predicted to be 79.61 kDa and 9.11, respectively, by using the ExPASy server (http://web.expasy.org/protparam/). Protein sequence analysis by using the InterPro tool (https://www.ebi.ac.uk/interpro/) indicated that this putative α-amylase has an alpha-1,4-glucan-maltose-1-phosphate maltosyltransferase domain (IPR021828) (aa 20–206) and a GH13 family domain (aa 199–574) of glycoside hydrolase (IPR015902). Figure 1 shows the alignment of this putative α-amylase protein from *A. agilis* PAMC 27388 with other α-amylase proteins. The amino acid sequence of this putative α-amylase shared the highest identity of 97 % with alpha-1,4-glucan-maltose-1-phosphate maltosyltransferase from *Arthrobacter* sp. L77. Also, the putative α-amylase shared 89, 86, 82, and 79 % sequence identity with alpha-1,4-glucan-maltose-1-phosphate maltosyltransferase from *Arthrobacter* sp. Leaf234, *Arthrobacter* sp. H41, *Arthrobacter* sp. RIT-PI-e, and and *Arthrobacter* sp. Soil782, respectively. According to the amino acid sequence alignment, the primary structure of this putative α-amylase from *A. agilis* PAMC 27388 contained highly conserved regions (regions I–VIII), a catalytic triad (Asp 400, Glu 429, and Asp 486), and six acceptor binding sites (Thr 432, Arg 433, Pro 434, Tyr 451, Trp 454, and Arg 455). The sequence of this *A. agilis* α-amylase gene was submitted to the GenBank nucleotide sequence database under the accession number KX516725. After PCR amplifying the *A. agilis* α-amylase gene, the PCR product was ligated into a pET28a(+) vector and successfully transformed into *E. coli* DH5α for cloning and sequencing. Afterwards, the constructed pET28a/amyl was transformed into *E. coli* BL21(DE3) for α-amylase gene expression.

Fig. 1 Conserved regions (I–VIII) of *A. agilis* α-amylase sequence (*shaded*) obtained by aligning amino acid sequences from *Arthrobacter agilis* PAMC 27388 (KX516725), *Arthrobacter* sp. L77 (WP_052274089.1), *Arthrobacter* sp. Leaf234 (WP_055765122.1), *Arthrobacter* sp. H41 (WP_049822566.1), *Arthrobacter* sp. RIT-PI-e (WP_049830883.1), and *Arthrobacter* sp. Soil782 (WP_056546396.1). The catalytic triad is *boxed*, and acceptor binding sites are indicated by *dots*. The residue numbers of amino acids in *A. agilis* α-amylase were shown on the top of sequences

Expression and Purification of Recombinant α-Amylase Protein

Recombinant α-amylase was expressed by adding 1 mM IPTG to the culture medium. SDS-PAGE (12 % polyacrylamide gel) analysis showed that the majority of the recombinant protein was observed at 80 kDa in the insoluble fraction of the cell lysate (Fig. 2a). This suggested that the heterologous protein accumulated as inclusion bodies in *E. coli* BL21(DE3).

To purify α-amylase, Ni^{2+}-NTA affinity chromatography was carried out as the α-amylase gene was inserted near an N-terminal six-histidine tag. A single band of purified protein was observed, and the molecular mass was estimated to be 80 kDa by SDS-PAGE (Fig. 2b). For further characterization of recombinant α-amylase, the purified protein under denaturing conditions was refolded into an active state by dialyzing to remove the denaturants. The specific activity of the purified recombinant *A. agilis* α-amylase was 27.96 U/mg protein.

Effects of pH and Temperature on α-Amylase Activity and Stability

The effect of pH and temperature on recombinant α-amylase activity is illustrated in Fig. 3. The α-amylase exhibited optimal activity of 60 U/mg at pH 3.0 and retained high specific activity of 50 U/mg at pH 2.0. Enzyme activity declined sharply at pH values above pH 3.0, retaining only about 48, 45, 30 28, 24, 34, and 27 U/mg between pH 4.0 and 10.0, respectively (Fig. 3a). Recombinant α-amylase displayed the greatest activity at 30 °C (Fig. 3b). Interestingly, the enzyme was active at a wide range of temperatures from 30 to 60 °C with specific activities of 32, 30, 29, and 30 U/mg, respectively, showing that the purified amylase belongs to the cold-adapted enzyme class [9, 23].

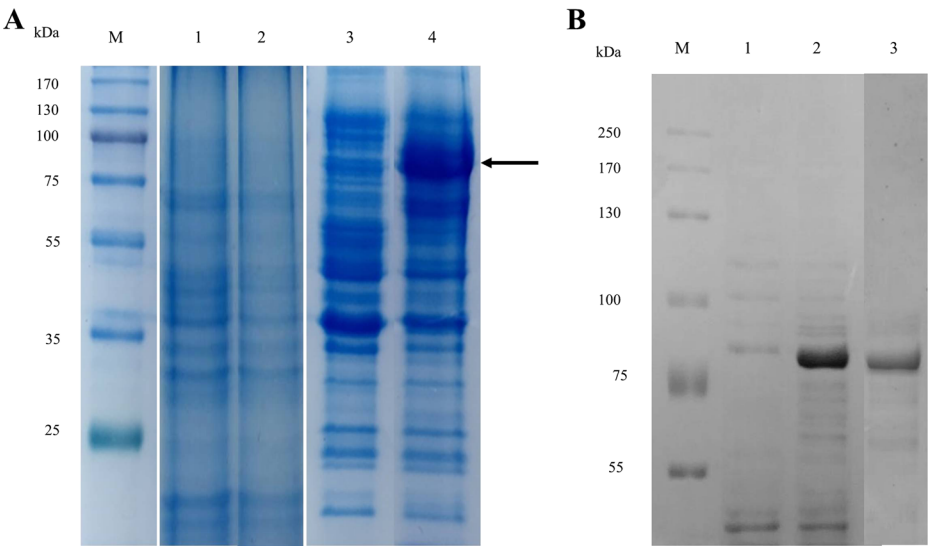

Fig. 2 Recombinant α-amylase expression in *E. coli*. **a** SDS-PAGE analysis of recombinant α-amylase expressed in *E. coli* after induction with 1 mM IPTG. ACCU pre-stained protein marker (Bioprince, Chuncheon, South Korea) (*M*), supernatant of cell lysate from *E. coli* BL21(DE3) harboring pET-28a(+) (*1*), supernatant of cell lysate from *E. coli* BL21(DE3) harboring pET28a/amyl after induction (*2*), pellet of cell lysate with pET-28a(+) (*3*), and pellet of total lysate with pET28a/amyl after induction (*4*). **b** SDS-PAGE analysis of the purified α-amylase. Standard protein marker (*M*), cell pellet before induction (*1*), solubilized sample after induction with 1 mM IPTG (*2*), and purified α-amylase (*3*)

According to the pH stability curve shown in Fig. 4a, α-amylase was highly stable between pH 6.0 and 8.0 after a 24-h pre-incubation. The enzyme retained more than 60 % of its activity between pH 4.0 and 9.0 after incubation for 24 h, indicating that recombinant *A. agilis* α-amylase has a broad range of pH stability. Furthermore, after 24 h of pre-incubation at different temperatures, the enzyme was highly stable in temperatures ranging from 10 to 60 °C (Fig. 4b).

Taken together, these results suggest that recombinant *A. agilis* α-amylases with high activity at low pH values are desirable in industrial processes such as starch liquefaction, because most current commercial α-amylases are unstable at low pH values [24, 25]. Furthermore, although most bacterial α-amylases, such as those from *Bacillus* species, exhibit optimal activity from 50 to 90 °C [26–28], recombinant α-amylase from Antarctic *A. agilis* showed increased activity and stability at low and moderate temperatures. These special properties of α-amylase at low temperatures make the enzyme highly attractive for mechanistic studies as well as for industrial applications [26, 29].

Effects of Metal Ions and Chemical Reagents on α-Amylase Activity

The effects of different metal ions and chemicals on recombinant α-amylase are shown in Table 1. α-Amylase was stimulated by Fe^{3+}, Na^{+}, and K^{+} ions to achieve 252, 115, and 110 % relative activities, respectively. In contrast, the enzyme was strongly inhibited in the presence of Co^{2+} and Zn^{2+} ions. Interestingly, Ca^{2+} ions did not affect the activity of recombinant *A. agilis* α-amylase, in contrast to most α-amylases that require calcium ion for catalytic activity and for maintaining structural integrity, such as α-amylase from *Pseudoalteromonas arctica* GS230, *Nocardiopsis* sp. 7326, and *Aeromonas veronii* NS07 [30–32]. Recently, some Ca^{2+}-independent α-amylases such as α-amylase from *Talaromyces pinophilus* 1–95, *Bacillus* sp. KR-8104, *Bacillus thermooleovorans* NP54, and *Streptomyces* strain A3 have been reported as candidate substitutes for current commercial Ca^{2+}-dependent α-amylases [33–36]. In the liquefaction step of starch processing, $CaCl_2$ is usually added for α-amylase stability, and the added Ca^{2+} ions need to be eliminated because they inhibit glucose isomerase, which isomerizes glucose to fructose. Thus, the use of a Ca^{2+}-independent enzyme is desirable, as it would eliminate the need for $CaCl_2$ [33, 37, 38].

Among the numerous chemical reagents tested, the addition of ammonium persulfate, EDTA, SDS, Triton X-100, and urea decreased enzyme activity. The inhibitory effect of

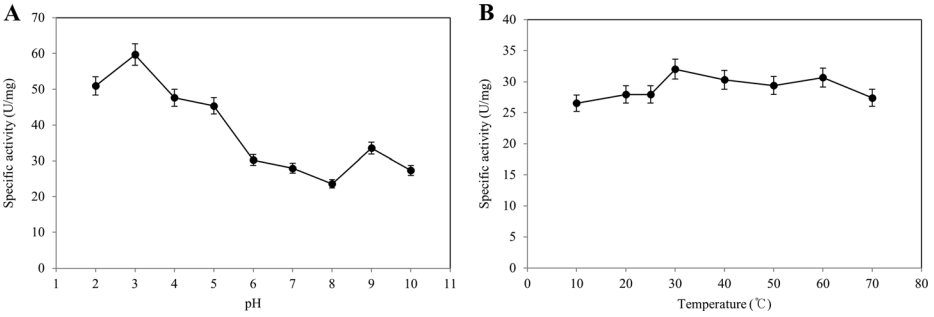

Fig. 3 Effect of pH and temperature on the activity of recombinant α-amylase. **a** Enzyme activity was assayed at different pH values (2.0–10.0) at 25 °C for 10 min. **b** Enzyme activity was measured at temperatures ranging from 10 to 70 °C for 10 min. All experiments were performed in triplicate

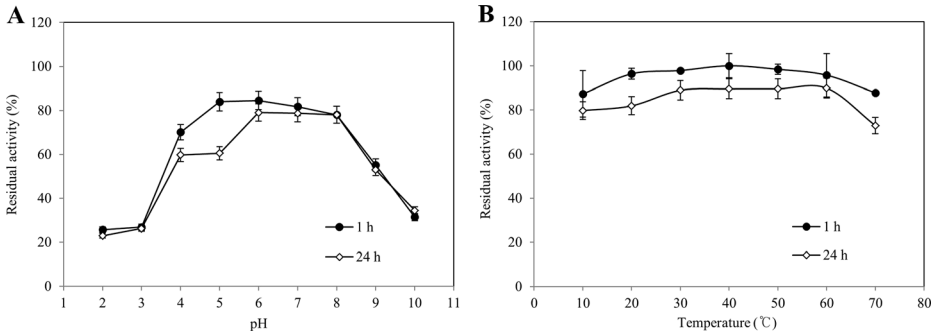

Fig. 4 Effect of pH and temperature on the stability of recombinant α-amylase. **a** The residual activity of α-amylase was determined after incubating the enzyme at different pH values (2.0–10.0) at 25 °C for 1 or 24 h. **b** The residual activity was measured after incubation of the enzyme solution in Tris-HCl buffer (pH 7.0) at 10–70 °C for 1 or 24 h. The enzyme activity at the beginning of the reaction was considered as 100 %, and all experiments were performed in triplicate

EDTA indicated that metal ions, including iron, are essential for catalytic activity. The effect of EDTA on α-amylase activity varies depending on the enzyme origin. Some α-amylases are hardly or completely unaffected by EDTA, while others, such as α-amylase from *A. agilis* PAMC 27388, are rendered considerably or totally inactive by EDTA [39]. Specially, urea significantly inhibited enzyme activity to 36 % relative activity. Surprisingly, recombinant α-amylase activity was enhanced in the presence of β-mercaptoethanol and PMSF. The enhancement by PMSF suggested the absence of a serine in the active site, and this absence of serine was also observed in this recombinant α-amylase.

Table 1 Effect of various metal ions and chemical reagents on recombinant α-amylase activity[a]

Reagent	Concentration	Relative enzyme activity (%)
Tris-HCl (control)	None	100.00 ± 8
Ca ($CaCl_2$)	1 mM	92.93 ± 9
Co ($CoCl_2$)	1 mM	36.84 ± 3
Mg ($MgCl_2$)	1 mM	77.63 ± 4
Fe ($FeCl_3$)	1 mM	252.26 ± 4
Zn ($ZnCl_2$)	1 mM	64.24 ± 5
Na (NaCl)	1 mM	114.60 ± 7
K (KCl)	1 mM	110.13 ± 12
Ammonium persulfate	5 mM	45.89 ± 8
β-Mercaptoethanol	5 mM	191.20 ± 13
EDTA	5 mM	58.64 ± 7
SDS	10 %	61.19 ± 6
Triton X-100	10 %	55.45 ± 8
Urea	1 %	35.56 ± 4
PMSF	1 mM	112.05 ± 10

Values represent the mean ± SD of three independent replicates

[a] Enzyme activities were measured at pH 7.0 and 25 °C for 10 min

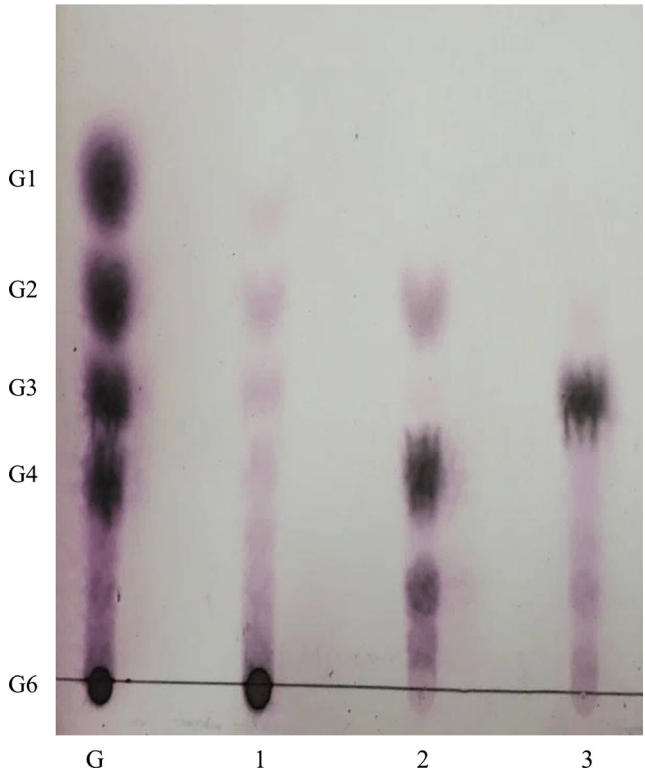

Fig. 5 TLC analysis of hydrolysis products of starch (*1*), maltotetraose (*2*), and maltotriose (*3*) by recombinant α-amylase after enzymatic reaction for 24 h. Glucose (*G1*), maltose (*G2*), maltotriose (*G3*), maltotetraose (*G4*), and starch (*G6*)

Analysis of Hydrolysis Products

The end products of maltotriose, maltotetraose, and starch hydrolysis were analyzed by TLC. After enzymatic reaction for 24 h, various products were identified as maltose (G2), maltotriose (G3), and maltotetraose (G4) (Fig. 5). Among the three products, maltose was the major end product, indicating that the purified recombinant α-amylase was an endo-acting maltose-forming α-amylase [40, 41]. Commonly, most α-amylases produce maltose as the major product, small oligosaccharides, and a minor amount of glucose [10, 11]. Besides maltose, recombinant α-amylase produced larger molecules than the maltotetraose from maltotriose and maltotetraose, suggesting that the recombinant α-amylase is an α-1,4-glucan-maltose-1-phosphate maltosyltransferase that cleaves an α-1,4 glycosidic bond of the donor molecule and transfers part of the donor to an acceptor by forming a new α-1,4 glycosidic bond [42]. Similar results have been reported by other studies [43, 44].

Conclusions

Cold-adapted α-amylases have garnered considerable interest in biotechnological and industrial applications because they can catalyze reactions at low and moderate temperatures more

effectively than mesophilic and thermophilic α-amylases [5, 15, 16, 45]. However, there is little information available on cold-adapted α-amylases from microorganisms isolated from the deep sea or the Antarctic [18, 29].

In the present study, a cold-adapted α-amylase gene from the psychrophilic *A. agilis* was successfully cloned and heterologously expressed in *E. coli* BL21(DE3). According to other studies related to cold-adapted α-amylases, α-amylase from *P. arctica* GS230 showed optimum activity at 30 °C. In addition, a cold-adapted α-amylase from *Nocardiopsis* sp. 7326 displayed peak activity at 35 °C, and this activity declined at temperatures above 45 °C [30, 32]. On the other hand, the α-amylase purified in this study showed optimal activity at 30 °C and retained its high activity in a broad temperature range from 30 to 60 °C. Moreover, the enzyme was stable in a wide temperature range (30–60 °C) for 24 h. It indicates that this enzyme can be used at higher temperatures unlike other cold-adapted α-amylases [23, 46]. Regarding pH profiles, this enzyme displayed optimal activity at pH 3.0 and was highly stable within pH values of 4.0–8.0 for 24 h. These results indicated that the recombinant *A. agilis* α-amylase might be applicable in various industrial processes and biotechnological applications, with desirable properties including wide optimal ranges of pH and temperature [15, 16, 23, 25].

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