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A Novel α-Amylase from *Bacillus mojavensis* A21: Purification and Biochemical Characterization

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Abstract α-Amylase from *Bacillus mojavensis* A21 (BMA.2) was purified to homogeneity by ultrafiltration, Sephadex G-75 gel filtration and Sepharose mono Q anion exchange chromatography, with a 15.3-fold increase in specific activity and 11% recovery. The molecular weight of the BMA.2 enzyme was estimated to be 58 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. The optimum temperature and pH were 80 °C and 6.5, respectively. BMA.2 belonged to the EDTA-sensitive α-amylase, but its activity was not stimulated by the presence of Ca^{2+} ions. The major end-products of starch hydrolysis were maltohexaose, maltopentaose and maltotriose. The N-terminal amino acid sequence of the first ten amino acids of the purified α-amylase was ASVNGTLMQY. Compared to sequences of other amylases, the ten amino acid sequence contains Val at position 3, while amylases from *Bacillus licheniformis* NH1 and *Bacillus* sp. SG-1 have Leu and Thr at position 3, respectively.

Keywords α-Amylase · *B. mojavensis* A21 · Purification · Biochemical characterization

Introduction

Amylases constitute a class of industrial enzymes having approximately 30% of the world enzyme production [1]. α-Amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) were classified in family 13 of glycosyl hydrolases and hydrolyzes starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages to produce different sizes of oligosaccharides. They have diverse applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries [2]. Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence [3]. Although they can be derived

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from several sources, such as plants, animals and microorganisms, enzymes from microbial sources generally meet industrial demands. Among the various species of *Bacillus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are the two species used most frequently in the commercial production of thermostable amylase. Several amylolytic enzymes, with different molecular weight, optimum pH, temperature and specificities have been reported [4–9]. Screening of microorganisms with higher α-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications such as bread and baking industry as antistaling agents [2]. The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. Najafi et al. [10] showed that the optimum pH and temperature of the α-amylase from *Bacillus subtilis* AX20 were 6.0 and 55 °C, respectively. The end products of soluble starch were glucose (70–75%) and maltose (20–25%). Purified α-amylase from *Bacillus fllavothermus* displayed maximal activity on starch at pH 5.5–6.0 and 60 °C [11]. Hamilton et al. [12] purified the α-amylase of *Bacillus* sp. IMD 435 in a single step using an α-cyclodextrin (CD) Sepharose 6B column and this enzyme displayed maximum activity at pH 6.0 and 65 °C. Glucose and maltose were released as the main end-products on hydrolysis of both soluble starch and raw corn starch.

During a screening programme on protease-producing strains, *Bacillus mojavensis* A21 strain was isolated and the purification of three different proteolytic enzymes with molecular masses of 15.5, 20 and 29 kDa were reported [13, 14]. This strain was also found to produce thermostable α-amylases.

In this study, we describe the purification and biochemical characterization of a novel extracellular α-amylase produced by the newly isolated *B. mojavensis* A21 strain.

Materials and Methods

Microorganism

The microorganism used in this study was an alkaliphilic bacterium, which was isolated from marine water in Sfax, Tunisia by selective screening on skim milk agar plates. It was identified as *B. mojavensis* according to the methods described in Bergey's Manual of determinative Bacteriology and on the basis of the 16S rDNA sequence analysis and assigned accession number EU366229 [13]. *B. mojavensis* A21 strain was routinely maintained on Luria–Bertani (LB)-agar plates and conserved in LB medium added to 30% glycerol at −80 °C.

Medium Composition and Culture Condition

Inocula were routinely grown in LB broth medium composed of (g/L): peptone 10.0, yeast extract 5.0, NaCl 5.0 and initial pH was adjusted to 7.0 [15]. The growth medium used for α-amylase production by *B. mojavensis* A21 strain was composed of (g/L): chicken feathers, ten; yeast extract, one; $MgSO_4$, 0.1, K_2HPO_4, 1.4, KH_2PO_4, 0.7 and NaCl, 0.5. The medium was adjusted to pH 7.0. Media were autoclaved at 121 °C for 20 min. The flasks containing growth medium were inoculated with 18-h-old liquid subculture (4%, *v*/*v*).

Cultivations were conducted in 25 ml of medium in 250 ml conical flasks maintained at 37 °C. Incubations were carried out in an orbital shaking incubator (Orbital Incubator SI 50, Stuart Scientific) at 37 °C and 200 rpm for 24 h. The cultures were centrifuged at

13,000 rpm for 15 min and the cell-free supernatant were used as a crude enzyme preparation.

α-Amylase Activity Assay

α-Amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture containing 0.5 ml of appropriately diluted enzyme and 0.5 ml of 1.0% (*w*/*v*) potato starch (Sigma) in 100 mM acetate buffer (pH6.5) was incubated at 80 °C for 10 min. The amount of liberated reducing sugars was determined by the dinitrosalicylic (DNS) acid method [16]. 3 ml of DNS reagent was added to the reaction volume, boiled for 10 min and 20 ml of distilled water was added and then the absorbance at 550 nm was measured. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups per minute. D-Glucose was used to construct a standard curve.

Amylase activities represent the mean value of at least two determinations carried out in duplicate. The difference between values did not exceed 5%.

Purification of α-Amylase from *B. Mojavensis* A21

The culture broth was centrifuged at 13,000×*g* for 10 min at 4 °C, and then the cell-free supernatant (2,180 U) was applied to a stirred ultrafiltration cell (Millipore 8400) using 10-kDa MW cut-off membrane (PBGC membrane, Millipore).

The concentrated crude enzyme was subjected to gel filtration on a Sephadex G-75 column (2.5 cm×90 cm) pre-equilibrated with buffer A (25 mM acetate buffer, pH6.5 containing 0.05% Triton X-100). Fractions of 5 ml were eluted at a flow rate of 30 ml/h with the same buffer. Protein contents and α-amylase activity were determined.

Fractions (63–77) showing α-amylase activities were pooled and applied to a Sepharose mono Q column (2×25 cm) previously equilibrated with buffer B (25 mM acetate buffer, pH 6.5) and the unadsorbed protein fractions were eluted with the same buffer at a flow rate of 80 ml/h. Bound proteins were eluted with a linear gradient of sodium chloride (0–0.5 M) in the same buffer. Fractions showing α-amylase activities were collected and stored at −20 °C for further analysis. All the purification steps were conducted at temperatures below 4 °C.

Protein concentration was determined by the method of Bradford [17] using bovine serum albumin as standard, and during the course of enzyme purification by measuring the absorbance at 280 nm.

SDS-PAGE Electrophoresis and Zymogram

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the α-amylase as described by Laemmli [18]. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit as markers (Amersham Biosciences) consisting of: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa).

α-Amylase activity staining was performed by layering the gel on a thin starch gel containing agarose (2%, *w*/*v*) and soluble potato starch (1%, *w*/*v*) and incubating the sandwich for 1 h at 50 °C. Upon staining the agarose gel with iodine solution at room temperature, protein bands with amylolytic activity became visible as white bands against a dark blue background.

N-Terminal Amino Acid Sequencing

The purified enzyme was transferred from SDS-PAGE to a polyvinylidene difluoride membrane (PVDF). The PVDF band corresponding to the α-amylase was excised and the N-terminal amino acid sequence was determined by the Edman degradation method on an ABI Procise 494 protein sequencer (Applied Biosystems).

Biochemical Properties of the Purified α-Amylase

Effect of pH on Activity and Stability

The effect of pH on α-amylase activity was performed in the pH range of 2.0–12.0 at 80°C. For the measurement of pH stability, the enzyme was incubated at 40°C for 1 h in different buffers and the residual activity was determined under the enzyme assay conditions. The following buffer systems were used: 100 mM glycine-HCl buffer, pH2.0–4.0; 100 mM acetate buffer, pH4.0–6.0; 100 mM Tris-HCl buffer, pH7.0–8.0; 100 mM glycine–NaOH buffer, pH9.0–11.0; 100 mM Na_2HPO_4-NaOH buffer, pH12.0.

Effect of Temperature on Activity and Stability

The effect of temperature on α-amylase activity was studied from 30°C to 90°C. Thermal inactivation of the purified enzyme was examined by incubating the enzyme preparation for 1 h at different temperatures ranging from 40 to 80°C in the absence and presence of $CaCl_2$ (5 mM) at 70°C.

Aliquots were withdrawn at desired time intervals and the remaining activity was measured under enzyme assay conditions. The non-heated enzyme was taken as 100%.

Effect of Metal Ions, Denaturing Reagents and Enzyme Inhibitors

The influence of various metal ions (5 mM) on enzyme activity was investigated using $CaCl_2$, $ZnCl_2$, $FeCl_2$, $HgCl_2$, $BaCl_2$, $MnCl_2$, $MgCl_2$, $CoCl_2$, NaCl and KCl. Activity in the absence of ions was taken as 100%.

The effect of some surfactants such as Triton X-100, Tween 20 (5%, *v*/*v*) and SDS (1%, *w*/*v*) on enzyme stability was studied by pre-incubating the enzyme for 1 h at 40°C. The residual activity was measured at pH6.5 and 80°C. The activity of the enzyme without any additive was taken as 100%.

The effects of enzyme inhibitors on α-amylase activity were studied using phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol and ethylene-diaminetetraacetic acid (EDTA) at a final concentration of 5 mM. The purified enzyme was pre-incubated with inhibitors at 20°C for 30 min and then the remaining enzyme activity was measured under enzyme assay conditions using potato starch as a substrate. The activity of the enzyme assayed in the absence of inhibitor was taken as 100%.

Chromatographic Analysis of the Starch Hydrolysis Products

The purified enzyme was examined for its ability to hydrolyze various carbohydrates such as starch (potato, wheat and corn), amylose, amylopectin and maltooligosaccharides at a final concentration of 1% (*w*/*v*). Enzyme activity measured in the presence of potato starch was taken as 100%. Potato starch and oligosaccharides (G7, G6, G5, G4 and G3), at a final

concentration of 1% (*w*/*v*), were hydrolysed by BMA.2 enzyme. The reaction mixture, containing 0.2 U of amylase enzyme, was incubated at 60°C for 6 h. Starch hydrolysis by *B. mojavensis* A21 crude enzyme and purified BLA.NH1 enzyme [9] were used under the same conditions. Hydrolysis products were subjected to thin-layer chromatography (TLC) with silica gel 60 (20×20 cm, Merck, Germany) in a solvent system composed of chloroform/acetic acid/water (60:70:10, *v*/*v*/*v*). The spots were visualized by spraying TLC plates with H_2SO_4/ethanol (5:95, *v*/*v*) followed by heating at 120°C for 10 min [9].

Statistical Analysis

Statistical analyses were performed with Statgraphics ver. 5.1, professional edition (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at $p<0.05$.

Results and Discussion

Purification of BMA.2 α-Amylase from *B. mojavensis* A21

Among various carbon source tested, chicken feather was found to be the most suitable substrate for the production of amylolytic activity. The level of amylase production in chicken feathers medium (10 g/l) was 4.78 U/ml. Zymogram analysis of the crude enzyme revealed the presence of two clear zones, suggesting the presence of at least two amylases (BMA.1 and BMA.2) with different molecular weights (Fig. 1). α-Amylase (BMA.2) from *B. mojavensis* A21 was purified by a three-step procedure as described in Materials and Methods section. In the first step, the cell-free supernatant (456 ml, 2,180 U) was applied to a stirred ultrafiltration cell using 10-kDa MW cut-off membrane. The concentrated enzymes were subjected to gel filtration on a Sephadex G-75 column. The elution profiles of α-amylase activity and proteins from Sephadex G-75 are shown in Fig. 2. This procedure yielded two peaks of α-amylase activity designated P1 and P2. Fractions containing α-amylase activity from each peak were pooled separately. Zymogram analysis of α-amylase activity of the peak P1 indicated the presence of at least two amylolytic activities, similar to the crude enzyme (Fig. 1). However, the pool 2 shows only one band of amylolytic activity, suggesting the presence of at least one

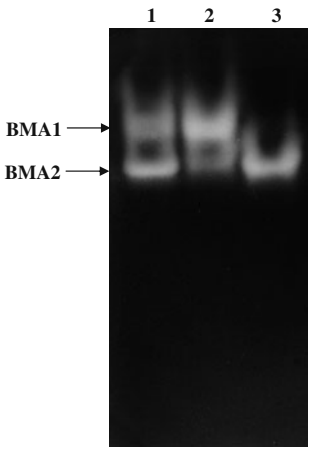

Fig. 1 Zymogram analysis. *Lane 1*, crude enzyme; *lane 2*, pool 1; *lane 3*, pool 2

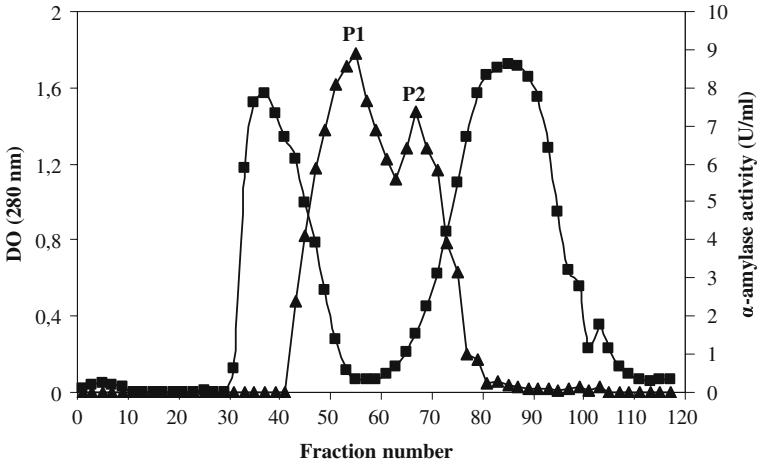

Fig. 2 Purification profile of α-amylase from *B. mojavensis* A21 by gel filtration on Sephadex G-75 column. The concentrated enzyme preparation was applied to a 2.5 cm×90 cm column, equilibrated and eluted with 25 mM acetate buffer (pH6.5) at a flow rate of 30 ml h^{-1}

amylase activity (BMA.2). Active fractions from peak P2 were collected and then loaded on Sepharose mono Q column pre-equilibrated with buffer B. The column was washed with the same buffer, and then bound proteins were eluted with a linear gradient 0–0.5 M NaCl in the same buffer. Enzyme activity was eluted with unabsorbed fractions (data not shown). The results of the purification procedure are summarized in Table 1. After the final purification step, the enzyme was purified 15.3-fold with a recovery of 11%. The specific activity of the purified BMA.2 α-amylase (648.15 U/mg) was higher than that of α-amylase from *B. licheniformis* NH1 (178.5 U/mg) [9].

The purified enzyme was analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 3, the enzyme gave a single band on SDS-PAGE with a molecular weight of approximately 58 kDa, corresponding to that determined by gel filtration. Gel staining for purified α-amylase activity by the iodine assay showed a unique clear band of starch hydrolysis, indicating the homogeneity of the purified α-amylase (Fig. 3). The molecular weight of BMA.2 enzyme correlates with several previous reports showing molecular masses of α-amylases from *Bacillus* strains to be approximately 58 kDa [9, 19].

The N-terminal amino acid sequence of the ten amino acids of the purified α-amylase was found to be ASVNGTLMQY. The N-terminal amino acid sequence of *B. mojavensis* A21 α-amylase (BMA.2) showed uniformity indicating that it was isolated in a pure form. The N-terminal amino acid sequence alignment analysis of BMA.2 enzyme showed 90%

Table 1 Summary of the purification of *B. mojavensis* A21 α-amylase (BMA.2).

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	2,180	51.47	42.36	100	1
Ultrafiltration	2,134	33.36	63.96	97.86	1.5
Gel filtration Sephadex G-75	558.8	6.79	82.3	25.6	1.94
Mono Q	246.3	0.38	648.15	11.3	15.3

All operations were carried out at 4°C

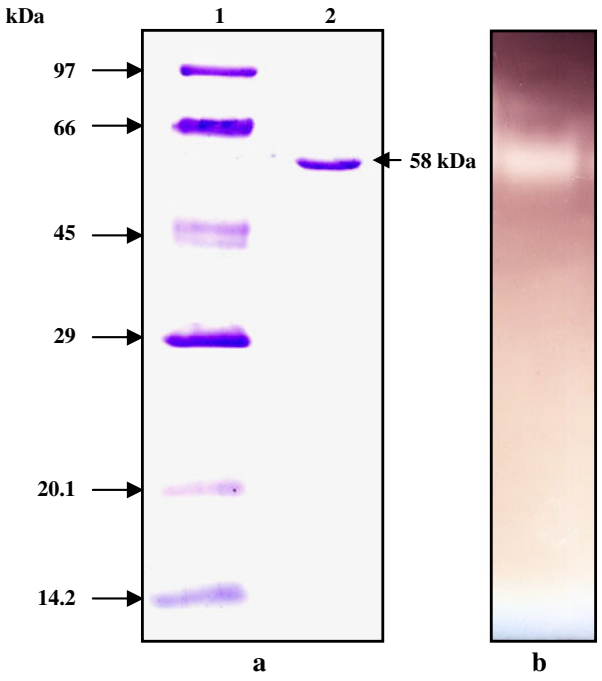

Fig. 3 SDS-PAGE (**a**) and zymogram analysis (**b**) of the purified α-amylase from *B. mojavensis* A21 (BMA.2). *Lane 1*, molecular weight markers, *lane 2*, purified BMA.2 enzyme

identity with α-amylase of *B. licheniformis* NH1 [9], and cytoplasmic α-amylase of *Bacillus* sp. SG-1 [20]. BMA.2 enzyme has Val residue at position 3 while amylases from *B. licheniformis* NH1 [9] and *Bacillus* sp. SG-1 [20] had Leu and Thr at position 3, respectively. The enzyme showed 80% identity with α-amylase of *B. licheniformis* NCIB 8061 [21] and *B. amyloliquefaciens* [22]. These results suggest that BMA.2 enzyme of *B. mojavensis* A21 is a novel enzyme.

Effect of pH on α-Amylase Activity and Stability

The effect of pH on α-amylase activity was studied by using potato starch as a substrate at various pH values at 80°C. The pH-activity profile of BMA.2 α-amylase is shown in Fig. 4a. The purified enzyme was highly active between pH4.0 and 9.0 with an optimum around pH6.5. The relative activities at pH5.0 and 9.0 were about 84% and 63%, respectively, of that measured at pH6.5. However, the activity decreased rapidly to 11% at pH10.0. It is worth noting that BMA.2 enzyme is more active at higher pH than previously reported α-amylase from *B. subtilis* AX20, which exhibited optimal activity at pH6.0 and relative activity of 80% and 67% at pH5.0 and 8.0, respectively [10].

The BMA.2 α-amylase was very stable at pH6.0 and 7.0 and retained more than 80% activity after 1 h incubation at 40°C (Fig. 4b). However, the activity decreased significantly above pH7.0 and was 25% and 11% of the initial activity at pH10.0 and 11.0, respectively.

Effect of Temperature on α-Amylase Activity and Stability

The optimum temperature of BMA.2 α-amylase activity was determined by assaying enzyme activity at different temperatures (Fig. 5a). BMA.2 enzyme was active at

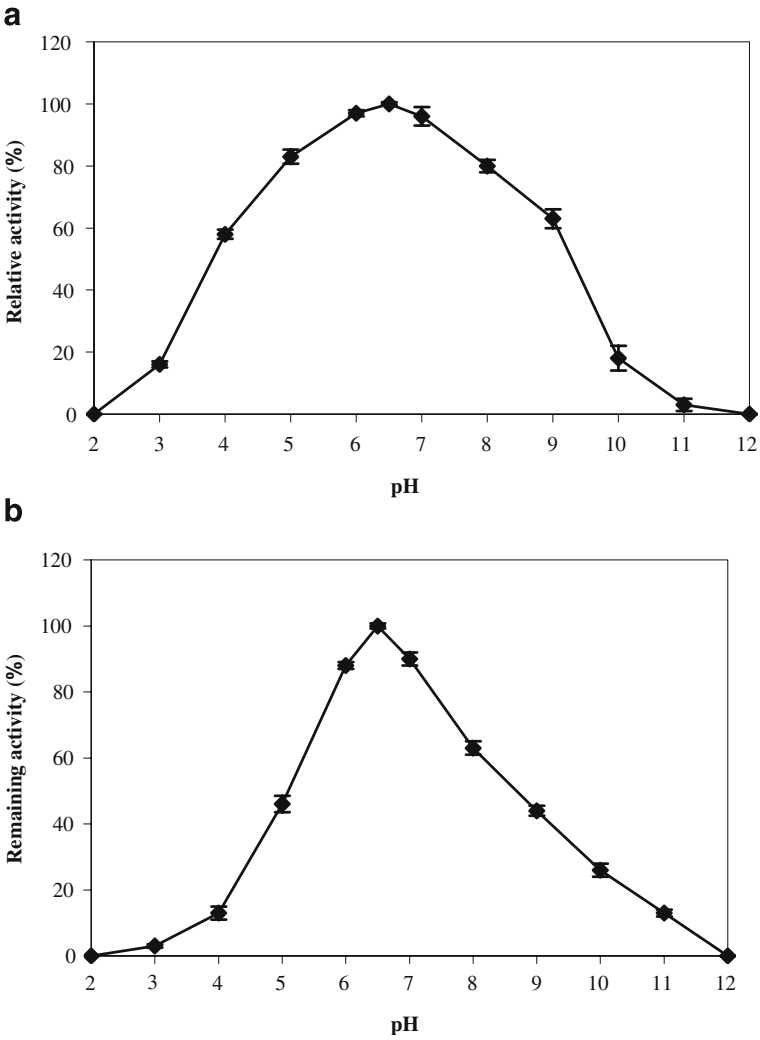

Fig. 4 Effect of pH on the activity (**a**) and stability (**b**) of the purified BMA.2 α-amylase. The pH profile was determined in different buffers by varying pH values at 80°C. The maximum activity obtained at pH6.5 was considered as 100%. The pH stability of the α-amylase was determined by incubating the enzyme in different buffers for 1 h at 40°C and the residual activity was measured at pH6.5 and 80°C. Buffer solutions used for pH activity and stability are presented in "Materials and Methods". *Error bars* represent the standard deviation

temperatures from 30 to 90°C and had optimum activity at 80°C. The relative activities at 70 and 90°C were about 94% and 61%, respectively. BMA.2 enzyme is less active at higher temperatures than α-amylase from *B. licheniformis* NH1, which exhibited optimal activity at 90°C [9]. However, BMA.2 enzyme was more active than α-amylase from *B. subtilis* AX20 with optimum activity at 60°C and decreased sharply at higher temperatures with relative activity of 60% and 35% at 40 and 70°C, respectively [10].

For investigation of thermal stability, the purified enzyme was incubated for 1 h in the absence of $CaCl_2$ at temperatures ranging from 40 to 80°C (Fig. 5b). No loss of the activity was observed after a 1-h incubation at 40°C. At 50°C, the enzyme retained 80% of its

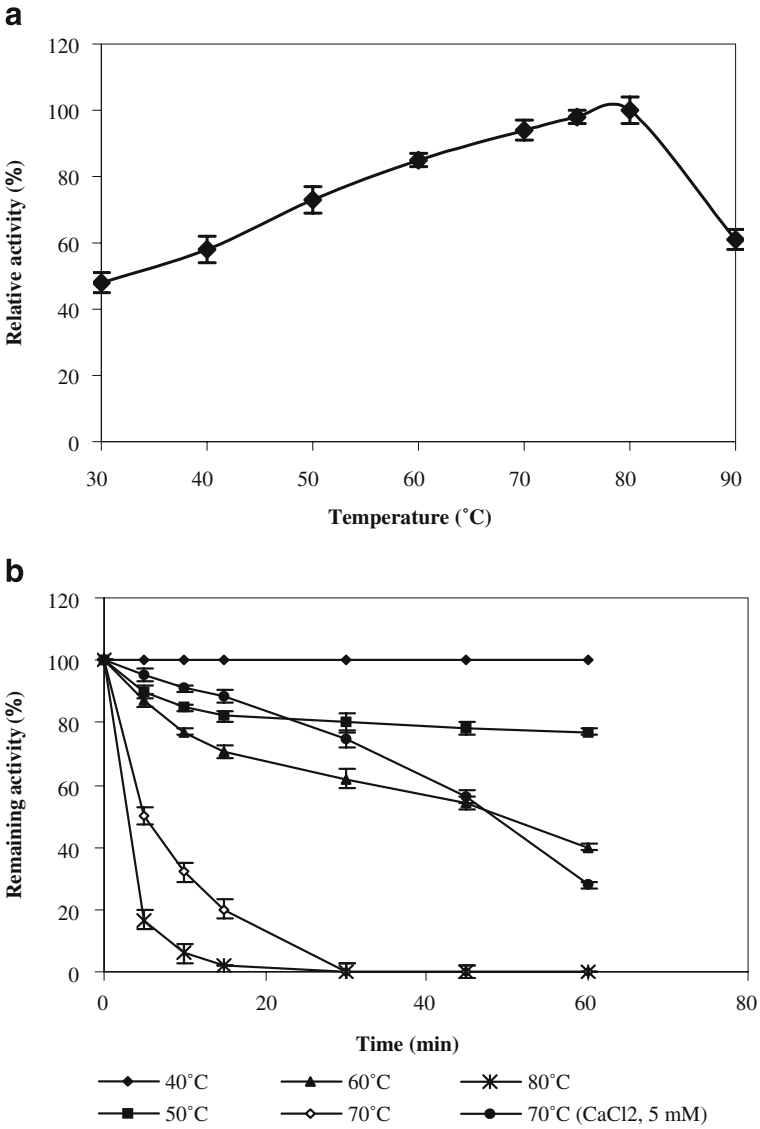

Fig. 5 Effect of temperature on activity (**a**) and stability (**b**) of the purified BMA.2 enzyme. The temperature profile was determined by assaying enzyme activity at various temperatures values at pH6.5. The activity of the enzyme at 80°C was taken as 100%. The temperature stability was determined by pre-incubating the purified enzyme at different temperatures in the absence and presence of $CaCl_2$ (5 mM). The residual enzyme activity was measured at 80°C and pH6.5. *Error bars* represent the standard deviation

initial activity. However, the activity was rapidly lost at 70 and 80°C and no activity was detected after a 30-min incubation. In contrast, the α-amylase from *B. subtilis* 65 was rather unstable at higher temperatures and was unstable when it was kept at more than 60°C for 5 min [23]. The α-amylase from *B. subtilis* AX20 showed stability at 50°C only for 45 min [10].

Several works have reported the enhancement of enzyme thermostability by Ca^{2+} metal ion [9, 19]. To determine whether Ca^{2+} enhanced thermal stability of the BMA.2 α-amylase, the

Table 2 Effect of various metal ions (5 mM) on α-amylase activity.

Metal ions	Relative activity (%)
None	100
Ca^{2+}	90±2.3
Fe^{2+}	96±2.45
Mg^{2+}	118±2.2
Ba^{2+}	100
Hg^{2+}	0
Zn^{2+}	16±1.56
K^{+}	100
Na^{+}	86±2.0
Mn^{2+}	94±1.8
Co^{2+}	18±1.0

The activity of the α-amylase was determined by incubating the enzyme in the presence of various metal ions (5 mM) for 10 min at 80°C and pH6.5

stability was examined by incubating the enzyme at 70°C in the presence of 5 mM Ca^{2+}. The enzyme showed a half-life value of 48 min, while in the absence of calcium the half-life of the enzyme was only 5 min (Fig. 5b). As was explained in previous works, the improvement in amylase thermostability against thermal inactivation in the presence of Ca^{2+} is due to the formation of a calcium–sodium–calcium metal triad in the main Ca^{2+} binding site, bridging domains A and B of the enzyme [24].

Effect of Metal Ions, Enzyme Inhibitors and Chemical Reagents

The effect of various metal ions on enzyme activity was studied at 80°C and pH 6.5 (Table 2). Although Ba^{2+} and K^{+} did not affect the enzyme activity, Ca^{2+}, Fe^{2+}, Ba^{2+} and Na^{+} had little effect on enzyme activity. However, Hg^{2+} and Zn^{2+} completely inhibited enzyme activity.

Among all inhibitors tested, the chelating agent EDTA inactivated the enzyme, indicating that BMA.2 is a metalloenzyme (Table 3). This finding is in line with earlier reports showing that calcium cation is essential for enzyme folding [25, 26]. The effect of EDTA on α-amylase from alkaliphilic *Bacillus* species varies considerably, some being unaffected at EDTA concentration as high as 100 mM [27], while others are completely inhibited in the presence of low EDTA concentration. α-Amylase of *Bacillus* sp. IMD 370 [28] was completely inhibited by 1 mM EDTA.

The stability of BMA.2 α-amylase was also studied by incubating the enzyme in the presence of surfactants (SDS, Tween 20, Triton X-100) for 1 h at 40°C. As shown in Table 3, the enzyme was highly stable in the presence of the non-ionic surfactants like Tween 20 and Triton X-100 and the strong anionic surfactant SDS. BMA.2 enzyme

Table 3 Effect of inhibitors and chemical reagents on α-amylase stability.

Chemical reagents	Concentration	Remaining activity (%)
None	–	100
Tween 20	5% (*v*/*v*)	85±2.8
Triton X-100	5% (*v*/*v*)	83±0.5
SDS	1% (*w*/*v*)	71±3.2
PMSF[a]	5 mM	100
β-mercaptoethanol[a]	5 mM	87±3.35
EDTA[a]	5 mM	12±1.0

[a] Enzyme activity measured in the absence of any inhibitor was taken as 100%. The remaining α-amylase activity was measured after pre-incubation of enzyme with each inhibitor at room temperature for 30 min

Table 4 Substrate specificity of the purified BMA.2 α-amylase.

Substrate (1%, *w/v*)	Relative activity (%)
Potato starch	100
Wheat starch	78±2.2
Corn starch	64±1.77
Amylose	117±0.64
Amylopectin	80±0.03
Maltoheptaose	95±0.2
Maltohexaose	ND
Maltopentaose	ND
Maltotetraose	ND

Substrates were incubated with purified enzyme for 10 min at 80°C in 100 mM acetate buffer (pH6.5). Enzyme activity measured in the presence of potato starch was taken as 100%

ND not detected

retained 85, 83 and 71% of its initial activity in the presence of Tween 20 (5%), Triton X-100 (5%) and SDS (1%) after 1-h incubation at 40°C, respectively.

Substrate Specificity and Chromatographic Analysis of the Starch Hydrolysis Products

The purified enzyme was examined for its ability to hydrolyze various carbohydrates such as starch (potato, wheat and corn), amylose, amylopectin and maltooligosaccharides (Table 4) at a final concentration of 1% (*w/v*). BMA.2 enzyme hydrolyzed starch from potato, wheat and corn, amylopectin and amylose at a relative rate of 100%, 78%, 64%,

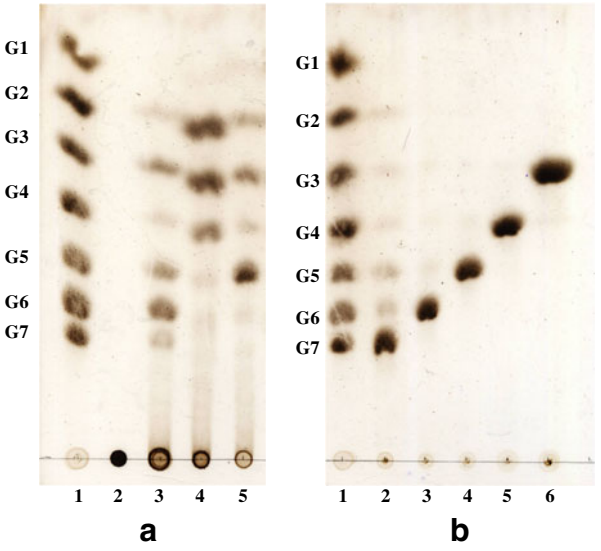

Fig. 6 Thin-layered chromatography analysis of the main hydrolysis products of soluble starch (**a**) and maltooligosaccharides (**b**) by the purified BMA.2 enzyme. The reaction mixture contained 0.2 U of amylase enzyme and 1% substrate or maltooligosaccharides in 0.1 M acetate buffer (pH6.5) was incubated at 60°C for 6 h. **a** *1* Standard maltooligosaccharides (G1–G7), *2* non-hydrolysed starch, *3* starch hydrolysed by the purified BMA.2 enzyme, *4* starch hydrolysed by *B. mojavensis* A21 crude enzyme, *5* starch hydrolysed by the purified BLA.NH1 enzyme [9]. **b** *1* Standard maltooligosaccharides (G1–G7), BMA.2 was reacted with maltoheptaose (*lane 2*), maltohexaose (*lane 3*), maltopentaose (*lane 4*), maltotetraose (*lane 5*) and maltotriose (*lane 6*) for 6 h at 60°C. *G1* glucose, *G2* maltose, *G3* maltotriose, *G4* maltotetraose, *G5* maltopentaose, *G6* maltohexaose, *G7* maltoheptaose

80% and 117%, respectively. No activity was detected on maltoheptaose, maltohexaose and maltopentaose as substrates.

Figure 6a compares the products of hydrolysis of potato starch by BMA.2 enzyme, crude enzyme from A21 strain and α-amylase from *B. licheniformis* NH1 strain. The main hydrolysis products of potato starch by BMA.2 enzyme were maltohexaose, maltopentaose and maltotriose. These findings differ from previous findings reported on hydrolysis products from *B. licheniformis* NH1 α-amylase which were maltopentaose, maltose and maltotriose [9]. They equally differ from the main hydrolysis products produced by the crude enzyme of *B. mojavensis* A21 strain, containing both BMA.1 and BMA.2 enzymes, were maltotetraose, maltotriose and maltose. Morgan and Priest [29] showed that G1, G2, G3 and G5 are the end products of starch hydrolysis by the α-amylase of *B. licheniformis* NCIB 6346. Amylase of *Bacillus* sp. KSM-1378 strain produced G5 to G7 and on further incubation, G3 and G5 increased, while G6 to G8 decreased [30]. *B. halodurans* LBK 34 amylase produced maltohexaose (G6) as predominant initial product of starch hydrolysis, while lower maltooligosaccharides were formed in the order G4>G2>G5>G3>G1 [7].

In an attempt to gain more information on the action of BMA.2 enzyme, intermediate saccharides of starch hydrolysate were used as substrates and end product profiles were examined by TLC. As shown in Fig. 6b, the enzyme did not hydrolyse maltotriose, maltotetraose and maltopentaose and was less active on maltohexaose. The hydrolysis of maltoheptaose was a mixture of maltooligosaccharides formed of G6, G5, G4, G3 and G2. Therefore, BMA.2 enzyme could be classified as liquefying-type amylase, since large maltooligosaccharides were generated from starch while G1, G2 and G3 are the main products of starch hydrolysis by saccharifying-type enzymes.

Conclusion

In the present study, a novel α-amylase from *B. mojavensis* A21 (BMA.2) was purified and characterized. BMA.2 enzyme was purified to homogeneity by ultrafiltration, Sephadex G-75 gel filtration and Sepharose mono Q anion exchange chromatography. After the final purification step, the enzyme was purified 15.3-fold with a specific activity of 648 U/mg and 11% recovery. The purified enzyme was homogenous on SDS-PAGE and its molecular weight was estimated to be 58 kDa. The optimum temperature was 80 °C and its thermostability is enhanced in the presence of Ca^{2+}. The enzyme is active and stable over a wide range of pH from 5.0 to 9.0. BMA.2 enzyme produced maltohexaose, maltopentaose and maltotriose as major end products of starch hydrolysis as in the case of a liquefying type α-amylase. Cloning and characterization of the α-amylase gene of BMA.2 enzyme will help to elucidate the structure of this enzyme. Experimentation in this direction is now in progress.

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