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Purification, Characterization, and Structural Investigation of a New Moderately Thermophilic and Partially Calcium-Independent Extracellular α-Amylase From *Bacillus* sp. TM1

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Abstract

A new α-amylase was extracted from a recently found strain of *Bacillus* sp. and purified by ion-exchange chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band for the purified enzyme with an apparent molecular weight of 59 kDa. The optimum temperature and pH range of the enzyme were 40–60°C and 4.5–7.5, respectively, and its activation energy was 1.974 kcal/mol. The K_m value for the enzyme activity on soluble starch was 4 mg/mL, and the T_m values obtained from the circular dichroism (CD) results of thermal unfolding were 78.7 and 80.2°C in the absence and presence of the calcium, respectively. The enzyme was almost completely inhibited by the addition of Fe^{3+}, Mn^{2+}, and Zn^{2+} and was activated by EDTA, Cr^{3+}, and Al^{3+}. Moreover, it was partially inhibited by Ca^{2+}, Ba^{2+}, Ni^{2+}, and Co^{2+}. Proteolytic digestion of the enzyme using trypsin combined with results from T_m using CD and irreversible thermoinactivation suggests that this enzyme can be considered a moderate thermophile with both mild flexibility and rigidity.

Index Entries: α-Amylase; *Bacillus* sp.; characterization; proteolytic digestion; thermal stability.

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Introduction

α-Amylases (endo-1,4-α-D-glucan glucohydrolase; EC 3.2.1.1) are among the most important enzymes in present-day biotechnology. This family of enzymes has potential application in a wide number of industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industries *(1)*. For proper application, the thermal and pH stability, and other features such as metal ion dependency (e.g., Ca^{2+}), must be checked and matched. For example, thermostable α-amylases are used for the liquefaction of starch at high temperature, and thermolabile α-amylases are used for the saccharification of starch in baking *(2)*. Moreover, these enzymes have successfully replaced the chemical hydrolysis of starch in starch-processing industries *(3)*. α-Amylases from different microorganisms have a wide range of properties and action patterns on starch substrate. They have been purified from a variety of species spanning the range of thermostability from mesophiles *(4)*, moderate thermophiles *(5,6)*, to even hyperthermophiles *(7,8)*.

Extrastability of most microbial α-amylases has been correlated with the occurrence of a calcium–sodium–calcium metal triad in the main Ca^{2+}-binding site, to connect domains A and B together *(9,10)*. In addition to this function, Ca^{2+} has been reported to exhibit the roles of allosteric activation *(11)* and protein stabilization *(10–12)*, and to trigger the refolding transition *(13,14)*. It was also shown that this metal ion stimulates the secretion of α-amylase by bacterial cells *(15)* and facilitates the release of enzyme from membrane vesicles *(16)*.

The α-amylases presently used in starch liquefaction require Ca^{2+} for activity and/or stability *(17,18)*. Therefore, there is an understandable need for a novel α-amylase that does not require Ca^{2+} owing to its high value in the starch industry. The purification, characterization, and structural studies of a nearly calcium-independent α-amylase produced by *Bacillus* sp. TM1 are reported here. This α-amylase is a moderate thermophile with a broad range of pH activity, which makes it very attractive for use in different industries.

Materials and Methods

Chemicals

Thermostable α-amylase from *Bacillus licheniformis* and mesophilic α-amylase from *Bacillus amyloliquefaciens* (type II-A), 3,5-dinitrosalicylic acid (DNS), soluble potato starch, and Tris were purchased from Sigma (St. Louis, MO). ρ-Nitrophenyl α-D-maltoheptaoside-4,6-*O*-ethylidene (exopolysaccharide [EPS]) was obtained from Boehringer Mannheim (Mannheim, Germany). DEAE-Sepharose was provided by Pharmacia (Uppsala, Sweden), and all other chemicals were obtained from Merck (Darmstadt, Germany) and were of reagent grade.

Microorganism and Culture Conditions

The bacterial strain used, *Bacillus* sp. TM1, was isolated from the wastewater of the flour mill in Iran by our group; stored on cultured medium containing 30% (v/v) glycerol at –15°C; and grown in a complex fermentation medium containing 1% (w/v) potato starch, 1% (w/v) soybean peptone, 1% (w/v) meat extract, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $CaCl_2 \cdot 2H_2O$, and 0.5% (w/v) $MgSO_4 \cdot 7H_2O$ for the production of α-amylase. Culture was grown at 37°C, in an orbital incubator, with stirring at 180 rpm for 18 h.

Determination of Enzyme Activity and Protein Concentration

α-Amylase activity was determined at room temperature using potato starch as a substrate in 20 m*M* Tris-HCl, pH 7.4, containing 10 m*M* $CaCl_2$. The concentration of reducing sugars obtained from the catalyzed reaction was measured by the DNS method according to Bernfeld *(19)*. α-Amylase was also assayed using blocked EPS as substrate in the aforementioned conditions. The released ρ-nitrophenol was measured by monitoring the absorption at 405 nm *(20)*. Protein concentration was determined by the Lowry method *(21)*.

Purification of α-Amylase

Proteins were precipitated by the addition of ammonium sulfate (at 80% saturation) to crude culture. The precipitate was centrifuged at 8000*g* for 10 min at 4°C; dissolved in 20 m*M* Tris, pH 7.4; and dialyzed overnight against the same buffer. The partially purified enzyme was applied onto a DEAE-Sepharose column using an AKTA Fast Performance Liquid Chromotography (FPLC) system (Amersham Biosciences, Uppsala, Sweden). The active fractions were pooled and concentrated by ultrafitration (Amicon, Beverly, MA).

Gel Electrophoresis of Protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide gel was carried out, and protein bands were detected by Coomassie brilliant blue R250 *(22)*.

Measurement of Melting Temperature by Circular Dichroism Spectropolarimeter

Melting temperature (T_m) of the samples was determined from the thermal denaturation curves using a JASCO J-715 circular dichroism (CD) spectropolarimeter (Tokyo, Japan). The CD signal at 222 nm as a function of temperature was recorded with a rate of 1°C/min. T_m was determined from the peaks on the first temperature derivatives of normalized melting profiles.

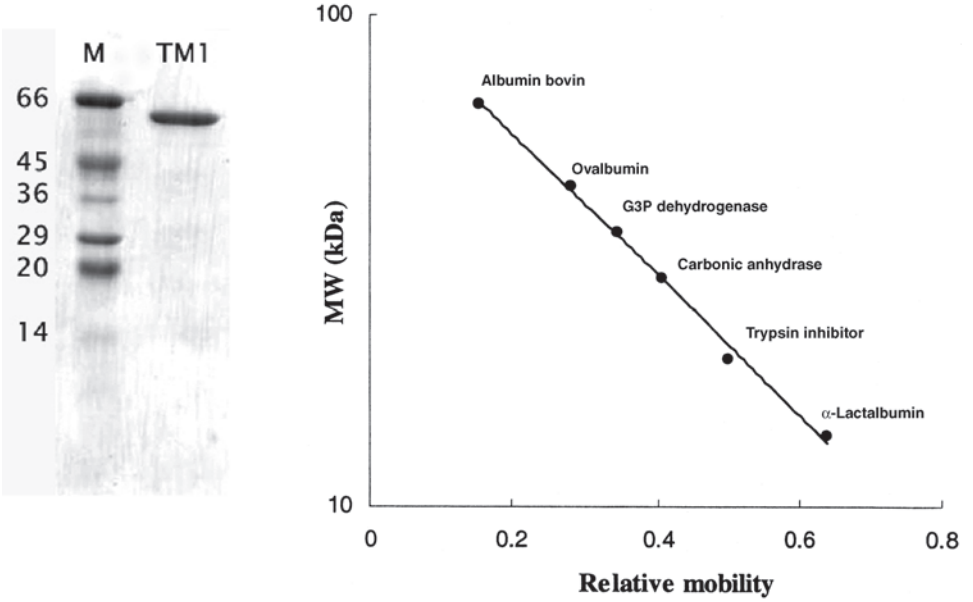

Fig. 1. SDS-PAGE of purified α-amylase from *Bacillus* sp. TM1. Lane M, molecular size markers; lane TM1, purified enzyme.

Determination of Thermal Stability

The thermal stability of the enzyme was measured by incubating the enzyme at 70 and 80°C for a series of time intervals, cooling on ice, and finally determining the residual activity under standard assay conditions.

Proteolysis of Enzymes

Each enzyme was incubated with trypsin (7:1) for 3 h at 37°C. At the end of the incubation time, samples were removed from the reaction mixture and placed in Laemmli solvent immediately for SDS-PAGE.

Results

Isolation, Purification, and Determination of Molecular Weight of α-Amylase

Precipitation is a valuable method for protein purification, especially at an initial step for an isolation process. Important reagents for precipitation are inorganic salts ($[NH_4]_2SO_4$), organic solvents (acetone or alcohols), and nonionic hydrophilic polymers (polyethyleneglycol). Proteins were precipitated when the crude enzyme was saturated to 80% with ammonium sulfate, and the α-amylase from *Bacillus* sp. TM1 was purified using ion-exchange chromatography with a DEAE-Sepharose column. An FPLC chromatogram showed a small and a large protein peak. The large peak was symmetric and had α-amylase activity. The molecular weight of the enzyme was about 59 kDa (Fig. 1).

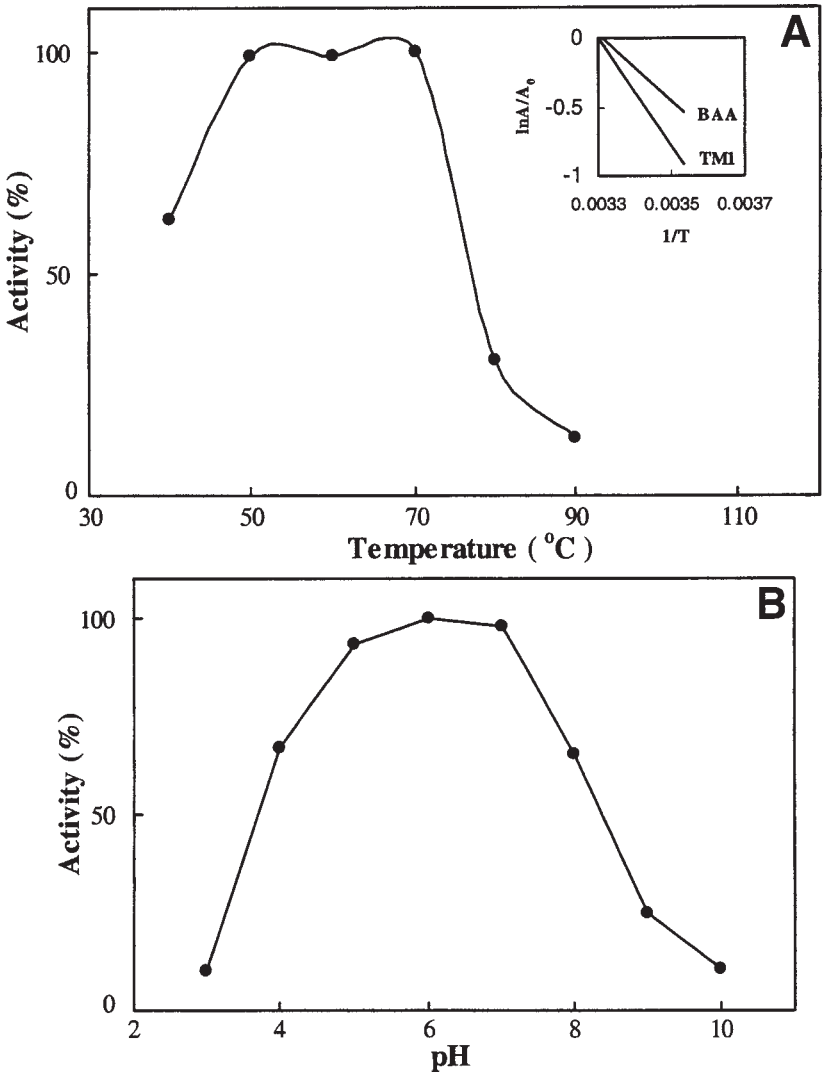

Fig. 2. **(A)** Effect of temperature on enzyme activity in 10 m*M* Tris, pH 7.4. The inset is Arrhenius plots corresponding to BAA and TM1 α-amylase. Activation energies for BAA and TM1 α-amylase were 1.199 and 1.974 kcal/mol, respectively. **(B)** Effect of pH on enzyme activity at 25°C. A mixture of acetate, phosphate, Tris, and glycine buffers at a concentration of 10 m*M* was used.

Effect of Temperature and pH

The influence of temperature and pH on the activity of enzyme is shown in Fig. 2. The optimum temperature for activity was from 50 to 70°C, and the activity reduced to 60% at 40°C and a sharp decrease in activity was observed above 70°C (Fig. 2A). The activation energy for *Bacillus* sp. TM1 α-amylase and *B. amyloliquefaciens* α-amylase (BAA) was calculated to be between 10 and 40°C based on the Arrhenius plot (Fig. 2A, inset).

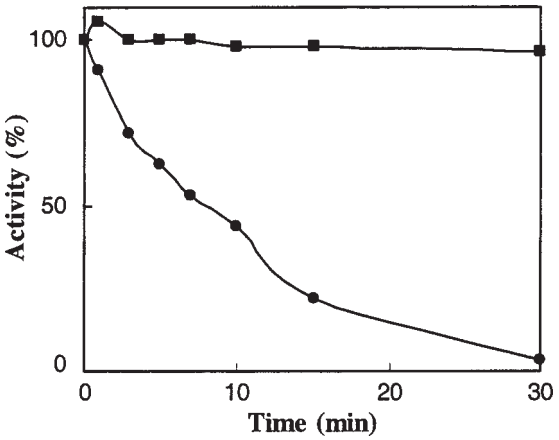

Fig. 3. Irreversible thermoinactivation of TM1 α-amylase at (■) 70°C and (●) 80°C in 10 m*M* Tris, pH 7.4, containing 10 m*M* $CaCl_2$.

This enzyme was active in a broad pH range (4.5–7.5), with maximum activity at pH 6.0 (Fig. 2B).

Catalytic Properties

The α-amylase revealed a Michaelis-Menten type of kinetics when hydrolyzing soluble starch. As calculated from Lineweaver-Burke plots, the K_m and V_{max} for soluble starch at 37°C were 4 mg/mL and 4.6 μmol/min, respectively.

Thermal Denaturation of Enzyme

The irreversible thermoinactivation of the enzyme was recorded in 20 m*M* Tris, pH 7.4, containing 10 m*M* $CaCl_2$ at 70 and 80°C. *Bacillus* sp. TM1 α-amylase retained its full activity after 30 min of incubation at 70°C, but as shown in Fig. 3, it lost 50% of its original activity after 10 min of incubation at 80°C. Under the same condition, a complete inactivation of BAA was witnessed. The CD results from thermal unfolding of the enzyme were recorded in the far-ultraviolet region (Fig. 4). The T_m values of the strain TM1 α-amylase and BAA are presented in Fig. 4.

Effect of Metal Ions on α-Amylase Activity

The α-amylase activity was measured at pH 7.4 in the presence of various metal ions (10 m*M*) and EDTA (10 m*M*) (Table 1). The purified enzyme was dialyzed extensively at 4°C against 20 m*M* Tris buffer (pH 7.4) prior to the experiment. As shown in Table 1, the addition of Ca^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}, Fe^{3+}, Ni^{2+}, and Co^{2+} decreased α-amylase activity, whereas the addition of 10 m*M* Cr^{3+}, Al^{3+}, and EDTA increased the activity. Furthermore, K^+, Na^+, and Mg^{2+} had no effect on α-amylase activity.

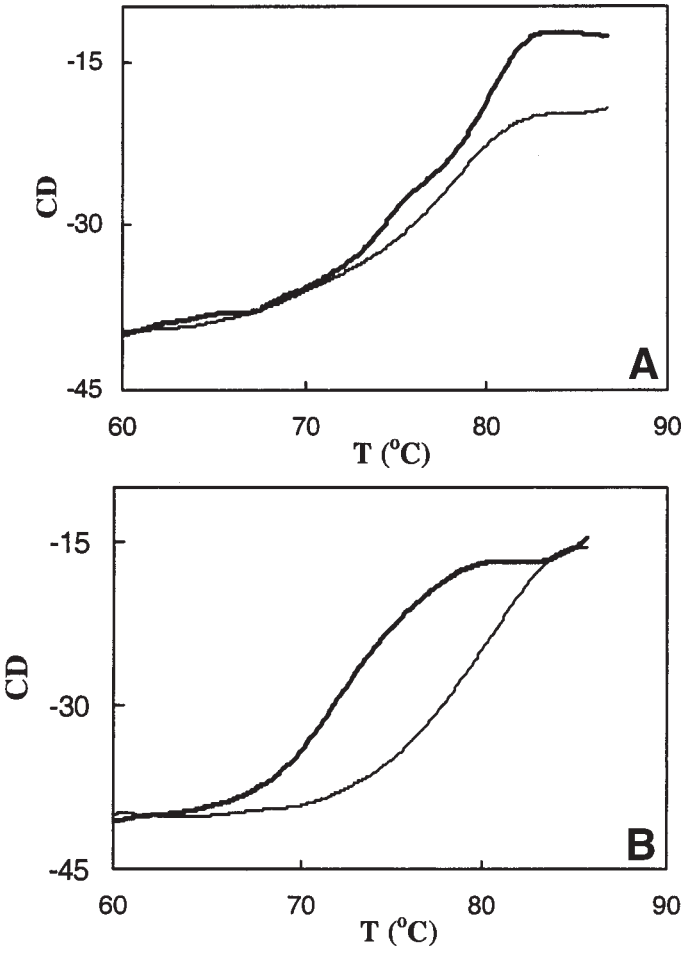

Fig. 4. CD at 222 nm exhibited by **(A)** TM1 α-amylase and **(B)** BAA at various temperatures in Tris buffer in (thick line) absence and (thin line) presence of 10 m*M* $CaCl_2$. In the absence and presence of Ca^{2+}, the T_m values of TM1 α-amylase were 78.7 and 80.2°C and for BAA were 71.7 and 80°C, respectively.

Proteolytic Digestion of α-Amylase

Proteolysis was achieved by incubating *Bacillus* sp. TM1 α-amylase with trypsin in Tris buffer, pH 7.4. BAA and *B. licheniformis* α-amylase (BLA) were used as a control in this experiment. SDS-PAGE analysis of the proteolysis revealed that the digestion of *Bacillus* sp. TM1 α-amylase was more extensive than BLA and less extensive than BAA (Fig. 5).

Discussion

α-Amylase purified from *Bacillus* sp. TM1 appeared as a single polypeptide with a molecular weight of about 59 kDa. It is larger than *B. licheniformis* and *B. amyloliquefaciens* α-amylases (56 kDa).

Table 1
Effect of Various Metal Ions (10 m*M*)
and EDTA (10 m*M*)
on α-Amylase From *Bacillus* sp. TM1[a]

Metal ions	Relative activity
Control (no addition)	1.00
Na^{+}	0.91
K^{+}	1.22
Ca^{2+}	0.79
Mg^{2+}	1.10
Mn^{2+}	0.09
Zn^{2+}	0.13
Ba^{2+}	0.92
Fe^{3+}	0.01
Ni^{2+}	0.75
Co^{2+}	0.57
Cr^{3+}	1.60
Al^{3+}	1.30
EDTA	2.13

[a]All the metal ions were added as chloride salts.

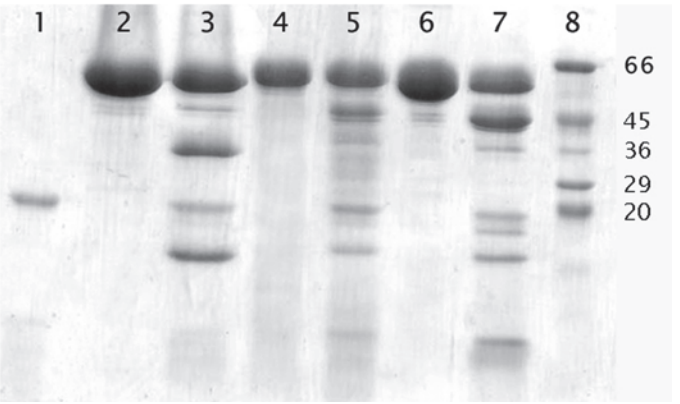

Fig. 5. Extensive proteolysis of BLA, TM1, and BAA by trypsin. Lane 1: trypsin; lanes 2, 4, and 6: controls for BLA, TM1 α-amylase, and BAA, respectively; lanes 3, 5, and 7: digestion of BLA, TM1 α-amylase, and BAA, respectively; lane 8: molecular size markers.

Although it is difficult to compare the kinetic values of amylases obtained by other groups in view of the different starch substrates and their assay conditions, the K_m value of *Bacillus* sp. TM1 α-amylase (4 mg/mL) is within the range of the majority of other amylases (0.35–4.7 mg/mL) *(23)*.

The pH profile of this enzyme shows a broad pH range of activity at room temperature (4.5–7.5). According to earlier reports, α-amylases produced by several bacterial sources have a variety of pH optima. The maximum activity

of most of them is in the range of 6.0–7.0 *(24–26)* or 5.0–7.0 *(2,27,28)*. Some of these enzymes have low pH optima, such as α-amylase produced by *Bacillus subtilis* X-23 *(29)* and *Lactobacillus manihotivorans* LMG 18010^T *(23)*.

The presence or absence of 10 m*M* Ca^{2+} had no effect on thermal denaturation of *Bacillus* sp. TM1 α-amylase. The T_m of the enzyme slightly increased in the presence of the calcium by 1.5°C, whereas the T_m of BAA in the presence of calcium increased by about 10°C. Thus, it is shown that this α-amylase is almost Ca independent. Ca^{2+} ion usually increases thermal stability in α-amylases, as mentioned in earlier reports *(24,30,31)*.

Our studies indicate that the extracellular α-amylase secreted by *Bacillus* sp. TM1 is more stable than the α-amylase from mesophilic microorganisms such as *B. amyloliquefaciens*, but less stable than the same enzyme from *B. licheniformis*. A thermostable substitute for the mesophilic α-amylase used in the cake-baking industry can be advantageous, because the enzyme activity of thermophile ceases as the temperature approaches and passes through the temperature range of gelatinization (70–80°C). The broad range of pH activity and moderate thermostability make this enzyme a useful additive to liquid detergents.

Bacillus sp. TM1 α-amylase is almost calcium independent and thermostable. These features will be extremely useful in starch liquefaction because the α-amylases presently used in the starch industry are calcium dependent. The use of this type of enzyme in starch hydrolysis eliminates the need for calcium in starch liquefaction and a subsequent extra step for its removal by ion exchange.

A clear correlation between rigidity and sites of proteolysis in globular proteins has been established *(32–34)*. Although TM1 α-amylase seems more flexible than BLA, it is more rigid than BAA. The digestion pattern of *Bacillus* sp. TM1 α-amylase is similar to that of BAA treated with trypsin. However, there is more undigested *Bacillus* sp. TM1 α-amylase compared to BAA.

In conclusion, because thermophilic enzymes lack enough activity at lower temperature and because mesophilic enzymes have lower stability, their application is limited. Therefore, moderately thermophilic enzymes such as TM1 α-amylase with intermediate stability could be a suitable candidate for industrial applications. Furthermore, owing to its lower sensitivity toward calcium ion, this enzyme is economically valuable in starch technology.

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