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# Purification and characterization of a thermostable phytate resistant α-amylase from *Geobacillus* sp. LH8

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ABSTRACT

A thermophilic and amylolytic bacterium (LH8) was isolated from the hot spring of Larijan in Iran at 65 °C. Identification of strain LH8 by 16S rDNA sequence analysis showed that LH8 strain belongs to the *Geobacillus* sp. with 99% sequence similarity with the 16S rDNA of *Geobacillus thermodenitrificans*. A new α-amylase (GA) was extracted from this strain and purified by ion-exchange chromatography. SDS-PAGE showed a single band with an apparent molecular mass of 52 kDa. The optimum temperature and pH were 80 °C and 5–7, respectively. In the presence of $Mn^{2+}$, $Ca^{2+}$, $K^{+}$, $Cr^{3+}$ and $Al^{3+}$, the enzyme activity was stimulated while $Mg^{2+}$, $Ba^{2+}$, $Ni^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $Cu^{2+}$ and EDTA reduced the activity. The $K_m$ and $V_{max}$ values for starch were 3 mg ml$^{-1}$ and 6.5 μmol min$^{-1}$, respectively. The gene encoding α-amylase was isolated and the amino acid sequence was deduced. Comparison of GA and other α-amylase amino acid sequences suggested that GA has conserved regions that were previously identified in α-amylase family but GA exhibited some substitutions in the sequence. Its phytate resistant is an important property of this enzyme. 5 and 10 mM phytic acid did not inhibit this enzyme. Therefore, features of phytate resistant α-amylase from *Geobacillus* sp. LH8 are discussed.

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## 1. Introduction

Enzymes involved in starch bioconversions are of major industrial interest and considerable attention has been focused on obtaining new enzymes with improved properties or new applications. Thermostable starch-hydrolyzing enzymes such as amylases, pullulanases and glucoamylases play an important role in food, chemical, and pharmaceutical industries [1–3]. α-Amylase (1,4-α-D-glucan glucanohydrolase [EC 3.2.1.1]) hydrolyzes the internal α-(1,4) glycosidic links in amylose and amylopectin to produce a less viscous solution with lower molecular mass products limited by α-(1,6) glycosidic bonds which form the branch points in the native starch molecule. α-Amylases isolated from thermophilic bacteria are thermostable and active at high temperature. *Bacillus* species such as *Geobacillus stearothermophilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquifaciens*, *Bacillus cereus*, *Bacillus globisporus* and *Bacillus alvei* are known for production of starch-hydrolyzing enzymes. More recently, Ezeji and Bahl reported that *G. thermodenitrificans* could produce a phytic acid resistant α-amylase [4]. Phytic acid which is the principal storage form of phosphorous in many plant tissues especially bran and seeds, is an inhibitor of α-amylase. This inhibitory effect is one of the main drawbacks in industrial use of α-amylase. In the present work, we have purified, characterized, cloned and sequenced a phytate resistant α-amylase from *Geobacillus* sp. LH8. The relationship between α-amylase amino acid sequence and the phytate-resistance behavior is discussed.

## 2. Materials and methods

### 2.1. Chemicals

3,5-Dinitrosalicylic acid (DNS), soluble potato starch and Tris were purchased from Sigma (St. Louis, MO). Restriction enzymes, T4 DNA ligase and alkaline phosphatase were obtained from Fermentas (Germany). Q and monoQ-Sepharose were provided by Pharmacia (Uppsala, Sweden). All other reagent chemicals were obtained from Merck (Darmstadt, Germany).

### 2.2. Microorganism and culture conditions

*Geobacillus* sp. LH8 which was previously isolated from the hot springs of Larijan-Iran was cultured in Horikoshi II medium (1% potato starch, 0.5% yeast extract, 0.5% peptone, 0.2% $KH_2PO_4$ and 0.02% $MgSO_4 \cdot 7H_2O$) [5]. pH was strictly controlled at 6.8 by HCl. 100 ml trace elements solution containing $CaCl \cdot 2H_2O$ ($10^{-3}$ M),

*Abbreviations:* GA, α-amylase from *Geobacillus* sp. LH8; BAA, α-amylase from *Bacillus amyloliquefaciens*; BLA, α-amylase from *Bacillus licheniformis*; BStA, α-amylase from *Bacillus stearothermophilus*.

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$FeSO_4{\cdot}7H_2O$ ($5 \times 10^{-4}$ M), $MnCl_2{\cdot}4H_2O$ ($7 \times 10^{-3}$ M), $ZnSO_4{\cdot}7H_2O$ ($8.7 \times 10^{-5}$ M), $H_3BO_3$ ($4 \times 10^{-3}$ M), $CuSO_4{\cdot}5H_2O$ ($5 \times 10^{-5}$ M), $Na_2MoO_4{\cdot}2H_2O$ ($5.2 \times 10^{-5}$ M), $Co(NO_3)_2{\cdot}6H_2O$ ($7.9 \times 10^{-5}$ M) and 0.25 ml of concentrated $H_2SO_4$ was prepared. In order to increase the production of α-amylase 300 μl of the prepared solution was added to 1 L of culture medium. Cultures were incubated at 65 °C in an orbital incubator. Growth medium was gently stirred at 185 rpm to maintain homogeneity.

### 2.3. DNA isolation and phylogenetic analysis through 16S rDNA sequence analysis

To determine the phylogenic relationship, 16S rDNA sequencing studies were carried out on the bacterium [6]. Preparation of LH8 genomic DNA was performed according to Sambrook et al. [12]. The 16S rRNA gene was amplified using universal forward (5′-AGT TTG ATC CTG GCT CAG-3′) and reverse (5′-GGC/T TAC CTT GTT ACG ACT T-3′) primers [7,8]. These primers are complementary to phylogenetically conserved regions of the 16S rDNA in *Bacillus* sp. A DNA thermal cycler (Eppendorf) was programmed as follows: (1) an initial denaturing temperature of 94 °C for 5 min, (2) a run of 30 cycles with each cycle consisting of 45 s at 94 °C, 45 s at 48 °C and 90 s at 72 °C and (3) 5 min final extension at 72 °C. The amplified products were purified with DNA extraction kit (Fermentas), and then DNA sequencing was performed on both strands by SEQ-LAB (Germany). The phylogenic relationship of the new isolate was determined through comparing the sequencing data with annotated sequences of related *Bacillus* and *Geobacillus* (GenBank database of the National Center for Biotechnology Information (Bethesda, MD), www.ncbi.nlm.nih.gov/GenBank/). A phylogenetic tree was constructed by the ClustalW software. The LH8 16S rDNA sequence has been deposited in GenBank under accession number of DQ192572.

### 2.4. Enzyme purification

#### 2.4.1. Ammonium sulphate precipitation and dialysis

All steps were carried out at 4 °C. The crude culture was precipitated with ammonium sulphate (at 80% saturation) by slow continuous stirring at 4 °C for 5 h. The saturated solution was centrifuged and the pellet was dissolved in minimum amount of 20 mM Tris–HCl (pH 8.5) containing 2 mM $CaCl_2$ and dialyzed against 20 mM Tris buffer (pH 7.4). The dialyzed crude enzyme was filtered through a sterile filter (Schleicher and Schuell, Dessel, Germany).

#### 2.4.2. Ion-exchange chromatography

Filtered crude was applied onto Q-Sepharose column at a flow rate of 1 ml $min^{-1}$, which was previously equilibrated with 20 mM Tris buffer, pH 7.2. The enzyme was eluted with a linear gradient of sodium chloride (0–0.5 M) in the same buffer. Protein was monitored by measuring the absorbance at 280 nm and 250 μl aliquots from each fraction was assayed for amylase activity. The active fractions were pooled and concentrated by ultrafiltration with a molecular weight cut-off of 30 kDa (Amicon, Bevery, MA, USA). Then, the concentrated α-amylase solution was applied to the monoQ-Sepharose column, connected to FPLC (Amersham Biosciences, Uppsala, Sweden) and eluted with Tris buffer at a flow rate 1 ml $min^{-1}$. Fractions containing α-amylase activity were pooled and dialyzed against 20 mM Tris buffer, pH 7.4. To determine molecular mass of the purified enzyme, the protein was subjected to SDS-PAGE and the gel stained with coomassie brilliant blue R250 [9,10].

### 2.5. Activity determination and specific activity staining of gels

α-Amylase activity was assessed at 65 °C using potato starch (1%) as a substrate in 20 mM Tris–HCl, pH 7.4 containing 10 mM $CaCl_2$. The activity was determined using 3,5-dinitrosalicylic acid (DNS) method according to Bernfeld which is based on the reducing sugar concentration [11]. One unit of α-amylase activity is defined as the amount of enzyme that produces reducing sugar equivalent to 1 μmol maltose (as the standard) per minute under the assay conditions. Protein concentration was determined by the Lowry method [10]. SDS gel electrophoresis was performed using 10% acrylamide gel according to Laemmli [9]. To reveal activity after SDS-PAGE, the gel was rinsed with deionized water and shaken in Triton X-100 2.5% (v/v) for 45 min and it was then washed in 0.2 M sodium acetate buffer (pH 5.5) at 40 °C for 45 min. The rinsed gel was transferred to fresh buffer containing 1% soluble starch and the incubation temperature was shifted to 65 °C for duration of 30 min. Upon applying the Lugol's solution at ambient temperature, protein bands with amylolytic activity became visible as white bands against a dark blue background.

### 2.6. Thermal stability

Thermal stability of *Geobacillus* sp. LH8 α-amylase was measured by incubating the enzyme at 75, 85 and 90 °C for different time periods (5, 10, 30 and 60 min) and subsequently cooled on ice for 30 min. The residual activity of GA was measured according to Bernfeld method [11]. Thermal stability was also determined in the presence of calcium chloride and EDTA. It should be noted that a stability control reaction was set up without using any of these additives.

### 2.7. Cloning of α-amylase gene

Genomic DNA was prepared as described by DNA extraction procedures [12]. The encoding gene of α-amylase was selectively amplified using specific primers. The sequences of these primers are represented here: forward primer: 5′-GCG AGC TCG TGC TAA CGT TTC ACC-3′ and reverse primer: 5′-GACTCGAGT CAA GGC CAT GCC ACC-3′. These primers were designed based on the α-amylase gene of *G. stearothermophilus*. The PCR reaction was programmed and carried out as follows (1) an initial temperature of 93 °C for 5 min (2) a run of 30 cycles, each consisting of 45 s at 93 °C, 45 s at 66 °C and 90 s at 72 °C and (3) 5 min at 72 °C to allow for the extension of any incomplete products. Amplified products were separated on 1% agarose gel and ~1.8 kb length fragment were purified from the gel using the QIAquick gel extraction kit (Qiagen). Two different *Escherichia coli* strains were used: DH5α and BL21. pET24d was used as the cloning vector. Preparation of competent cells of *E. coli* and other experimental procedures used to construct the designed plasmid were carried out through standard protocols [12]. Transformation of recombinant plasmids into *E. coli* cells was performed by $CaCl_2$ method and electroporation [13–15]. Expression vector pET24d (+) was digested with restriction endonucleases SacI and XhoI. DNA fragments were ligated using the T4 DNA ligase. Competent cells of DH5α were transformed and grown overnight at 37 °C in LB broth containing kanamycin. Plasmid DNA was extracted from the cells, transformed into BL21 (expression vector) and then plated on LB-kanamycin medium. Transformation was carried out using electroporation [16]. Recombinant plasmids containing the α-amylase gene were selected based on restriction analysis and sent for DNA sequencing, performed by MWG Biotech using an automatic DNA sequencer (LiCor) [17].

### 2.8. Sequence analysis and homology modeling

Analysis and translation of the nucleotide sequences were performed with the tools available at the ExPASy Molecular Biology Server (www.expasy.ch). Pair wise and multiple amino acid sequence alignments were carried out using the BLAST and

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