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Molecular cloning and biochemical characterization of a novel cold-adapted alpha-amylase with multiple extremozyme characteristics

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ABSTRACT

A gene coding for an alpha-amylase (Amy-E) from *Exiguobacterium* sp. SH3 was successfully expressed in *Escherichia coli*. The enzyme was purified as a functional His-tagged protein of about 53 kDa with maximum activity at 30 °C and pH 6.5. Amy-E was also able to function well at low temperatures, retaining 41% of its maximum activity in assays conducted at 0 °C. The activation and inactivation energies of the enzyme were found to be 4.46 and 11.76 kcal mol^{-1}, respectively. The substrate specificity of Amy-E was in the following order: soluble starch (100%), maltodextrin (87.6%); amylopectin (62.2%), wheat flour (53%), and rice flour (48.7). The K_m and V_{max} of soluble starch hydrolysis were found to be 2.29 mg ml^{-1} and 1405 U. Amy-E not only was halotolerant but also its activity was stimulated at high salt concentrations in the range of 1–5 M. The enzyme activity was also stimulated by non-ionic surfactants (Triton X-100 and Tween 20) at 20% and 50% concentrations. It was remarkably stable against sodium dodecyl sulfate (SDS), alcohols, and acetone. Amy-E as an extremozyme, seems quite promising for applications that are conducted at low temperatures and low water activity conditions.

1. Introduction

Extremophiles are organisms adapted to proliferate under exotic conditions such as extreme cold, hot, acid, alkaline, and saline. Therefore, the organisms have evolved enzymes called extremozyme which are fitted to work under such conditions. The enzymes have gained particular attention due to their ability to function under abnormal conditions and their potential application in industries [1,2]. For instance, cold-adapted enzymes produced by psychrophiles and psychrotrophs feature the unique ability of high catalytic activity at cold and ambient temperatures. The industrial exploitation of cold-adapted enzymes may result in huge saving of energy and reducing the production costs [2]. Cold-adapted amylases may have great potential for industrial applications such as dough fermentation, waste treatment, brewing, detergents, food, textile, and biofuel production [3,4].

Amylolytic enzymes belong to families 13, 57, 119, and 126 of glycoside hydrolases. The enzymes are industrially important (GH) accounting for about 25–33% of the world market of commercial enzymes [5]. Among the amylolytic enzymes are α-amylases (EC 3.2.1.1) that mostly belong to the family GH13 and are capable of hydrolyzing α-1,4-glucosidic bonds in starch, amylose, amylopectin, glycogen, and related α-glucans. α-amylases share common structural features including 4–7 conserved sequence regions (CSRs) and the (β/α)8-barrel catalytic domain. However, α-amylases that belong to the family GH57 contain a (β/α)7-barrel fold for their catalytic domain [6,7]. Despite the similarities, amino acid sequence and structural differences among α-amylases obtained from various organisms are high enough to endow interesting biochemical diversity to the enzymes. This diversity in biochemical characteristics has spurred research for novel amylases. Several species of *Exiguobacterium* genus have been isolated from diverse geographical regions ranging from as cold as Arctic to as hot as Yellowstone hot spring [8]. Therefore, bacteria of the genus with huge ecogeographical distribution seem interesting in order to get some insight into implications of adaptation that are well reflected by enzymes produced by the organisms. The α-amylase of psychrophilic *Alteromonas haloplanctis* has been extensively studied [9–11]. Some marine and polar microorganisms such as *Pseudoalteromonas haloplanktis* [12], *Pseudoalteromonas arctica*

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GS230 [13] and *Nocardiopsis* sp. 7326 [14] have also been studied as cold-adapted amylase producers. However, so far, very few genes of cold-active amylases have been isolated and characterized. In this work, a gene coding for α-amylase was isolated from psychrotrophic *Exiguobacterium* sp. SH3, cloned and expressed in *E. coli*. The recombinant α-amylase was purified and characterized from biochemical and thermodynamic viewpoints.

2. Materials and methods

2.1. Bacterial strains and media

Exiguobacterium sp. SH3 has been isolated from soil [15], and was routinely grown on LB medium at 30 °C. *Escherichia coli* DH5α was used as a host for gene cloning and *E. coli* BL21 (DE3) pLysS was used for expression of the recombinant α-amylase gene. *Escherichia coli* strains were grown on LB medium and kanamycin was added when required at 0.05 mg ml^{-1} concentration.

2.2. Gene cloning

Our previous studies had shown that *Exiguobacterium* sp. SH3 was able to produced amylase and pullulanase enzymes with interesting psychrophilic characteristics [15,16]. In order to identify the genes responsible for amylolytic activity of this bacterium, a partial genomic library was prepared and sequenced (BaseClear, Leiden, NL). According to the genomic library, an amylase-coding gene, named *amy-E*, was isolated from *Exiguobacterium* sp. SH3 genome by PCR using amyE-f (5′-ATAAAGCTTAGGAGGTTACAATATGAGACGAGGCGTGATGCTTC-3′) and amyE-r (5′-ATACTCGAGGTGTGTTTCCTTCGACCGGTTAAG-3′) primers.

The genomic DNA of *Exiguobacterium* sp. SH3 was isolated according to Sambrook and Russell [17], and used as template in a PCR containing 1 μg of the genomic DNA, 10 pmol of forward and reverse primers, and 1 unit of *pfu* DNA polymerase. The PCR reaction was conducted in a Touchgene Gradient thermocycler (Techne, Barloworld Scientific Ltd.) using the following conditions: 94 °C, 2 min; 25 cycles of 94 °C, 45 s; 54.7 °C, 40 s; 72 °C, 2 min and finally 72 °C, 10 min. The PCR product was purified using a high pure PCR product purification kit (Roch Indianapolis, USA), digested with *HindIII* and *XhoI*, and inserted in pET26b(+). The resulting plasmid named as pEamy was validated by restriction digestion and sequencing.

2.3. Expression and purification of Amy-E

Escherichia coli BL21(DE3) transformed with pEamy was grown in LB medium containing 0.05 mg ml^{-1} kanamycin at 30 °C to an optical density of 0.8 read at 600 nm. The cells were then induced by 0.1 mM isopropyl-D-thiogalactopyranoside (IPTG) and let grow at 25 °C for 16 h. At the end, the cells were harvested (12000 × *g*, 10 min, 4 °C), washed with lysis buffer (50 mM Tris–HCl, 50 mM ethylenediamine tetraacetic acid (EDTA), 50 mM Triton X-100, pH 8, 1 mg ml^{-1} lysozyme), and sonicated (Hielscher Ultrasonics GmbH, Teltow, Germany) in the same buffer on ice for 8 cycles (45 s on, 45 s off). The cell lysate was centrifuged (12000 × *g*, 10 min, 4 °C), and the resulting supernatant along with the crude culture supernatant were analyzed using the amylase assay and the sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In order to purify Amy-E, the cell lysate was applied by gravity flow on a Ni-NTA agarose column (Qiagen) that was previously equilibrated by 5 volumes of binding buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20% Tween 80, 10 mM imidazole, pH 8). To remove unbound proteins, the column was then washed enough with washing buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20% Tween 80, 20 mM imidazole, pH 8) until no further protein was detected at 280 nm in the flow-through. Finally, the Amy-E was eluted from the column by 3 volume of elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 20% Tween 80, 250 mM imidazole, pH 8). For this purpose, about 3 ml of the elution buffer was passed through the column by gravity flow and the flow-through was collected in fractions of 500 μl. Each fraction was analyzed for amylase activity and purity by SDS-PAGE.

2.4. SDS-PAGE and activity staining

The expression and purity of Amy-E were analyzed by SDS-PAGE using 2% stacking and 10% separating gels containing 0.1% SDS. A 10–170 kDa protein marker (Fermentas, Canada) was used for molecular weight estimation. In order to analyze the activity of the purified protein, zymogram staining was conducted using an SDS-free polyacrylamide gel containing 1% starch [18]. After electrophoresis, the gel was washed first in Triton X-100 (2.5%) for 30 min and then two times in citrate buffer (pH 6.5) each for 15 min. Subsequently, the gel was washed with distilled water and stained by Gram's iodine solution to visualize the zone of amylase activity.

2.5. Enzyme activity assay

The amylase activity was assayed principally according to Bernfeld [19], using the 3,5-dinitrosalicylic acid (DNS). The standard reaction mixture was composed of 150 μl of the purified enzyme and 150 μl of 200 mM sodium phosphate buffer (pH 7.4) containing 1% soluble starch. After incubation of the reaction mixture at 30 °C for 60 min, the reaction was stopped by the addition of 900 μl of DNS reagent. In this method, reducing-end sugars react with DNS to produce a characteristic color which can be measured using a spectrophotometer. The absorbance at 570 nm of each reaction was read and converted to sugar concentration using a standard curve. One unit of amylase activity was defined as the amount of enzyme that released 1 μM glucose equivalent of reducing sugars per minute under the defined assay conditions.

2.6. Effect of temperature and pH

The effect of temperature on the amylase activity was investigated in the range of 0–80 °C with 10 °C increments under standard conditions. To study the effect of temperature on the enzyme inactivation, Amy-E was held at different temperatures including 30, 40, 50, and 60 °C between 0 to 30 min with 5-min intervals. Subsequently, the residual activity was measured under standard conditions. The effect of pH on the amylase activity was analyzed in the range of 4–9 with 0.5 increments. For this purpose, enzyme assays were conducted in either citrate–phosphate buffer (pH 4–7) or Tris–HCl (pH 7.5–9) at 30 °C.

2.7. Effect of metal ions, additives and chemicals

The effect of cations (Na^+, K^+, Ca^{2+}, Li^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}), EDTA, SDS, and urea on the activity of Amy-E was analyzed at 5 and 10 mM concentrations. The effect of glucose, sucrose, maltose, mannitol, trehalose, sorbitol, *myo*-inositol, iodoacetate, ammonium sulfate, and guanidine hydrochloride on the activity was studied at 5, 10, and 200 mM concentrations. The effect of Triton X-100, Tween 20, glycerol, methanol, ethanol, butanol, dimethyl sulfoxide (DMSO), and acetone at 20% and 50% (v/v) concentrations was studied on the activity of the enzyme. The effect of high salt concentrations on the activity of Amy-E was analyzed by conducting enzyme assays in the presence of various concentrations of NaCl ranging from 0 to 5 M. Reactions were conducted in citrate–phosphate buffer (pH 6.5) at 30 °C. All the materials were purchased from Sigma–Aldrich (St Louis, MO, USA).