Journal of Molecular Catalysis B: Enzymatic 99 (2014) 114–120

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Molecular cloning and biochemical characterization of a thermoacidophilic, organic-solvent tolerant α-amylase from a *Bacillus* strain in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 15 July 2013
Received in revised form 31 October 2013
Accepted 31 October 2013
Available online 9 November 2013

Keywords:
Gene cloning
α-Amylase
Thermoacidophilic
Organic solvent-tolerant
Bacillus sp. DR90

ABSTRACT

The α-amylase gene from *Bacillus* sp. DR90 was isolated, inserted into pET28a(+) vector and subsequently expressed in *Escherichia coli* BL21 (DE3) using 1 mM IPTG as an inducer. Recombinant enzyme containing N-terminal His-tag was sufficiently purified via nickel metal affinity chromatography with purification factor of 6.8-fold and specific activity of 4091 U/mg. The molecular mass of α-amylase was estimated to be about 76 kDa by SDS-PAGE. The recombinant enzyme was active in wide ranges of pH and temperature, exhibiting an optimal activity at pH 4 and 75 °C with $T_{1/2}$ of 125 min. Amylase activity did not enhance in the presence of calcium ions. Apart from good stability toward SDS, urea, and EDTA, the purified enzyme showed high compatibility with various solid and liquid detergents. Furthermore, results indicated the stability and stimulation of enzyme in the presence of different organic solvents. Following the incubation of amylase with imidazolium-based ionic liquids, maximum remaining activity was observed in [BMIm][Cl]-containing solution. Overall, presenting outstanding properties including high thermostability, Ca^{2+}-independency, broad pH and temperature profiles, organic-solvent tolerance as well as excellent stability and compatibility with detergents, the present recombinant α-amylase will be a suitable candidate in industrial fields, particularly in food and detergent industries.

1. Introduction

Alpha-amylases catalyze the hydrolysis of internal glycosidic bonds between glucose residues within starch molecules, which results in the release of smaller polymers [1]. Amylases are broadly found amongst a host of organisms including plants, animals, fungi and also microorganisms. However, from bio-industrial viewpoint, large-scale production of amylolytic enzymes has prevalently been performed through fungal and bacterial sources including *Bacillus* strains [2]. Occupying the biggest share of the market in enzyme, amylases present numerous applications in food, baking, textile, paper and detergent industries and likewise in medicinal and analytical chemistry. Researchers in the domains of industry and biotechnology now focus their attention on the utilization of amylolytic enzymes presenting outstanding properties such as thermostability, activity/stability within a wide pH range, resistance against heavy metals, denaturants and organic solvents, and also metal ion independency [3]. Thermostable acidophilic amylases have been extensively utilized as an alternative of starch chemical hydrolysis in industrial starch processing [4]. Although the pH range of native starch is 3.2–4.5, starch processing is commonly performed at pH around 6.5 by industrial amylases that functionally/structurally depend on calcium ions being unstable at low pH [5]. Therefore, thermostable α-amylases active at low pH values are more intended to involve in liquefaction process due to omitting pH adjustment step limiting the formation of by-products [6]. Obviously, compared to the aqueous solutions, organic solvents as anhydrous milieu for enzymatic reactions have some advantages such as the enhancement of thermal stability, the suppression of undesirable water-dependent side reactions as well as decrease in microbial contaminations. Therefore, enzymes with high resistance toward organic solvents have received a great deal of attention [7,8].

The acidophilic bacterium *Bacillus* sp. DR90, isolated from Dig Rostam hot mineral spring, Iran, was found to produce amylolytic enzyme, but in order to enhance the level of enzyme production, the isolation, cloning and expression of α-amylase encoding gene from the mentioned strain was attempted. Moreover, the

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http://dx.doi.org/10.1016/j.molcatb.2013.10.025

properties of recombinant α-amylase were biochemically characterized and discussed.

2. Materials and methods

2.1. Materials

Restriction enzymes and DNA ligation kit were purchased from Takara (Dalian, China). PCR cloning, DNA gel extraction and plasmid DNA isolation kits, T4 DNA ligase, isopropyl-b-D-thiogalactopyranoside (IPTG) and 1 kb DNA ladder were obtained from Fermentas (Maryland, USA). Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrix was procured from Qiagen (CA, USA). Carbohydrates including soluble potato starch, glycogen, α-cyclodexterin, and β-cyclodexterin were prepared from Merck (Germany). All other used chemicals were of analytical grade.

2.2. Bacterial strains, plasmid vectors and growth media

Bacillus DR90 being deposited in Iranian Biological Resource Center with acquisition number of BRC-M10742 was utilized as α-amylase gene donor strain [9]. *Escherichia coli* DH5α [F^- *end*A1 *hsd*R17 (rk^-, mk^+) *sup*E44 *thi*-1 l^-*gyr*A96 *rel*A1 Δ (*arg*F-*lac*zya)U169] and *E. coli* BL21(DE3)pLysS (Novagen, USA) were exploited as cloning and expression hosts, respectively. The plasmid vectors for cloning and expression were pTZ57R/T plasmid (Fermentas, Maryland, USA) and pET28a(+) vector (Novagen, USA) containing ampicillin and kanamycin resistance genes, respectively. The *E. coli* strains were grown aerobically in Luria-Bertani (LB) media supplemented with ampicillin (100 μg/ml) or kanamycin (50 μg/ml).

2.3. Cloning and plasmid construction

The genomic DNA of *Bacillus* DR90 was isolated according to Sambrook et al. [10]. To amplify the open reading frame of α-amylase (ORF) gene, a set of oligonucleotide primer was designed based on the alignment of *Bacillus* alpha-amylase gene sequences deposited in the GenBank database. Forward primer (5′-CGCGGATCCGCGATGTTTGCAAAACGATTCAAAACC-3′) and reverse primer (5′-CCCAAGCTTGGGTCAATGGGGAAGAGAACCGC-3′) contained *Bam*HI and *Hind*III restriction sites, respectively, which were introduced into both ends of the target gene by the polymerase chain reaction (PCR) before insertion into the TA cloning vector. The amplification of alpha-amylase encoding gene was performed using chromosomal DNA as template. The PCR fragment was electrophoretically analyzed and purified from agarose gel. The target gene was ligated with PTZ vector and transformed into *E. coli* DH5α competent cells. Positive colonies selected on LB-ampicillin plates were cultivated for recombinant plasmid extraction. Cloning vector carrying the amylase encoding gene was used for nucleotide sequencing. The recombinant PTZ plasmid and the expression vector were treated with aforementioned restriction enzymes and subsequently purified from agarose gel. The target gene was cloned into expression carrier through compatible sticky ends. Competent cells of *E. coli* BL21(DE3) were transformed with recombinant pET28a(+) vector and plated onto LB plates supplemented with 50 μg/ml kanamycin. The enzyme encoding gene was inserted in-frame to the C-terminus of 6xHis tag as an N-terminal fusion partner, facilitating the protein purification.

2.4. Expression of recombinant amylase

Single transformants of *E. coli* BL21(DE3) were cultured in LB broth containing 50 μg/ml kanamycin at 37 °C overnight with shaking at 200 rpm. An aliquot of overnight culture was inoculated into fresh LB broth and incubated at 37 °C to mid-exponential phase (OD_{600} 0.5). The expression of recombinant α-amylase under the control of T7 promoter was performed in the presence of IPTG at final concentration of 1 mM following incubation at 25 °C. The induced cells were collected at 2, 4, 6, and 8 h-post induction.

2.5. Purification and biochemical characterization of recombinant enzyme

2.5.1. Purification

The induced cells were centrifuged at 12,000 × *g* for 15 min and pellets were resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and 1 mM PMSF, pH 8.0). Following the cell disruption by sonication (6 cycles of intermittent sonication at 25 W for 30 s), the cell lysates were cleared by centrifugation at 10,000 × *g* for 20 min at 4 °C. The supernatant, containing recombinant proteins with an N-terminal His tag, was applied onto Ni-NTA affinity column, which facilitated the isolation of proteins with His fusion tag. After washing the column, the bound target protein was eluted with buffer B (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8.0) and through-flow fractions were collected. The purification was carried out at 4 °C.

2.5.2. Polyacrylamide gel electrophoresis and zymography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the homogeneity of purification and the molecular mass of recombinant enzyme [11]. Followed by Coomassie brilliant blue R-250 staining, the molecular mass of protein band was determined on 12% gel using standard protein markers. Non-denaturing-PAGE containing 0.5% starch was conducted in order to demonstrate the amylase activity of purified protein [12]. Non-heated samples were run on 10% native gel. Subsequently, SDS was removed from gel by 2.5% Triton X-100 and the gel was stained with Lugol's solution. White areas in a dark blue background represented amylase activity.

2.5.3. Measuring protein concentration and amylase activity

Purified protein samples were determined for protein concentration according to the method of Bradford [13] by measuring the absorbance at 280 nm using bovine serum albumin as the standard. Using dinitrosalicylic acid (DNSA), Bernfeld [14] method was performed to assay the amylase activity. An aliquot of purified enzyme was mixed with 1% soluble starch as substrate in 50 mM sodium acetate buffer (pH 4) and incubated for 10 min at 42 °C. Upon the addition of DNSA, sample was boiled for 15 min and the amylase activity was subsequently evaluated by measuring the reducing sugars released following starch hydrolysis at 540 nm. One unit of amylase activity was defined as the amount of enzyme needed to liberate 1.0 μmol of reducing sugar/min using maltose as a standard under the assay condition.

2.5.4. Effect of pH and temperature on amylase activity and stability

Different buffer systems, including 50 mM glycine–HCl buffer pH 2–3, sodium acetate buffer pH 3.5–5.5, sodium phosphate buffer pH 6–7.5, Tris–HCl buffer pH 8–9.5, and Na_2HPO_4–NaOH buffer pH 10 were prepared to study the effect of pH on enzyme activity. A reaction mixture containing enzyme and 1% starch in each buffer was incubated at 42 °C for 10 min and the amylase activity was determined using DNSA. The stability of recombinant amylase at different pH values was investigated by pre-incubating the enzyme in each buffer for 60 min. The remaining activity was subsequently assessed by 10-min incubation of enzyme with starch under standard conditions as previously described.