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ORIGINAL ARTICLE

Cloning, Expression, and Purification of Hyperthermophile α-Amylase from *Pyrococcus woesei*

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Abstract
Objectives: In an attempt α-amylase gene from *Pyrococcus woesei* was amplified and cloned into a pTYB2 vector to generate the recombinant plasmid pTY-α-amylase.
Methods: *Escherichia coli* BL21 used as a host and protein expression was applied using IPTG. SDS-PAGE assay demonstrated the 100 kDa protein. Amylolytic activity of proteins produced by transformed *E. coli* cells was detected by zymography, and the rate of active α-amylase with and without the intein tag in both soluble conditions and as inclusion bodies solubilized by 4M urea were measured.
Results: Amylolytic activity of ∼185,000 U/L of bacterial culture was observed from the soluble form of the protein using this system.
Conclusion: These results indicate that this expression system was appropriate for the production of thermostable α-amylase.

1. Introduction

Pyrococcus woesei is known as an ultra-thermophilic marine archaebacterium that is sulfur-reducing and capable of growing at between 100°C and 103°C. Its cells have a roughly spherical, elongated, and constricted appearance, similar to *Thermococcus celer*, and frequently occur as diploforms. Cells grown on solid supports have dense tufts of flagellae or pili attached to one pole.

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The α-amylase is an enzyme that catalyzes the hydrolysis of α-l,4 glucosidic linkages in polysaccharides of three or more α-1,4-linked D-glucose units to yield glucose and maltose [1]. The α-amylase isolated from the ubiquitous mesophilic soil bacterium *Bacillus licheniformis* [2–4] operates optimally at 90°C and pH 6, and requires the addition of calcium (Ca^{2+}) to maintain thermostability [5]. The ideal conditions for starch hydrolysis are pH ∼4.5 and a temperature of 105°C. Given that this enzyme is unstable under these conditions, the pH must be increased to 5.7–6.0 and Ca^{2+} added [6]. These pH adjustments increase the energetic costs associated with the process. Furthermore, the Ca^{2+} requirement creates the need for ion-exchange refining to remove added Ca^{2+} that has a potent inhibitory effect on xylose isomerase, which is used in processing starch hydrolysate to fructose syrup [7]. Given that the majority of industrial processing of starch gelatinization is completed near 100°C, thermostable α-amylases are used. Hyperthermophilic archaeon *Pyrococcus woesei* producing α-amylase is a good source of such an enzyme, which displays a temperature optimum of 100°C and retains ∼60% of maximal activity in pH ranges of 4.5 to 7.0. Moreover, this enzyme precludes the necessity to add Ca^{2+} salt to the substrate [7]. In view of the industrial importance of *P. woesei* α-amylase, we cloned, expressed, and purified *P. woesei* α-amylase in *Escherichia coli*.

2. Materials and methods

P. woesei (DSM 3773, Braunschweig, Germany) was cultivated in artificial seawater supplemented with 0.25% soluble starch, 2.5% tryptone, 2% yeast extract, and 0.1% elemental sulfur. The pH of the medium was adjusted to 7.0 with 1M NaOH. Extraction of *P. woesei* chromosomal DNA and genomic library construction were performed as described previously [8]. *E. coli* BL21 DE3 plysS cells (Stratagene, La Jolla, CA, USA) transformed with the ligation mixture were plated on Luria-Bertani (LB) agar–ampicillin (100 mg/mL) plates. After 16–20 h of incubation at 37°C, colonies were transferred to a new set of LB-ampicillin plates.

2.1. Construction of expression plasmid

To express the α-amylase protein with its fusion intein protein, the plasmid pTYB2 (New England BioLabs, Beverly, MA, USA) was used. *P. woesei* chromosomal DNA was used as a source of the α-amylase gene and polymerase chain reaction (PCR) amplification was done with the following primers:

5′-GCTAGCTTGGAGCTTGAAGAGGGAG-3′ and 5′-GAGACCACAATAACTCCATACGGAG-3′ containing recognition sites for restriction endonucleases *Nhe*I and *Xho*I (Fermentas; Vilnius, Lithuania).

The reaction was performed using 10 ng DNA, 2 μL (10 μM) of each primer, 5 μL (10mM) dNTPs, 5 μL 10× PCR buffer [100mM Tris-HCl (pH 8.9), 500mM KCl, 20mM $MgCl_2$, 1% Triton X-100] and High-fidelity PCR Enzyme Mix (Fermentas). After 2 minutes of preliminary heating at 95°C in a thermal cycler, each of the 30 cycles was conducted at 95°C for 0.5 minutes, 56°C for 1 minute, and 72°C for 1 minute, with a final step of 5 minutes at 72°C. The amplified gene was cloned into pTZ57R/T (InsTAclone PCR Cloning Kit; Fermentas), and then subcloned into pTYB2 and transformed into *E. coli* BL21 (DE3) pLysS cells.

2.2. Purification of α-amylase

Isolation and purification of α-amylase was undertaken as described previously [9]. Briefly, transformed *E. coli* with pTY-amylase was induced with 1mM isopropyl- β-D-thiogalactopyranoside (IPTG) and incubated for 20 h at 20oC. The size of the purified expression product according to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis corresponded to the predicted molecular mass of the recombinant protein (100 kDa; Figure 1). The transformed *E. coli* cells obtained from 0.5 L culture induced with IPTG were sonicated in 50 mL 0.05M phosphate buffer (pH 7.2) containing 1 mL of 50 μmol NaCl and 1 μmol dithiothreitol. The acquired suspension was centrifuged (4°C) at 10,000*g* for 15 minutes. The *E. coli* proteins were denatured at 75°C for 30 minutes and then precipitated to obtain recombinant α-amylase. The supernatant containing thermostable α-amylase was then used for further purification. Urea (4M) was used to renature inclusion bodies, and the precipitated enzyme was extracted (2 h, 20°C) from the pellet.

2.3. Enzyme assay

A 1% starch solution in 0.05M acetate buffer (pH 5.5) was used for determining α-amylase activity. Before reaction initiation, the starch (2.5 mL) was preincubated at 80°C, and then the reaction initiated by adding 0.5 mL diluted enzyme solution. After different time periods, cooling on ice was used to stop the reaction. The reducing sugars formed during the reaction were assayed according to the dinitrosalycilic acid method of Bradford [10]. To determine the effect of pH on enzyme activity, 0.05M citrate-phosphate buffers in the pH range of 4.0–8.0 adjusted at 80°C were used. The specific activity of α-amylase (U/mg of protein) was determined as described by Bradford [10].

2.4. Detection of amylolytic activity by electrophoresis

A 12% polyacrylamide gel at room temperature was used to determine amylolytic activity, as described previously [11]. The samples (10 μg of the protein suspension) were dissolved in 0.01M phosphate buffer

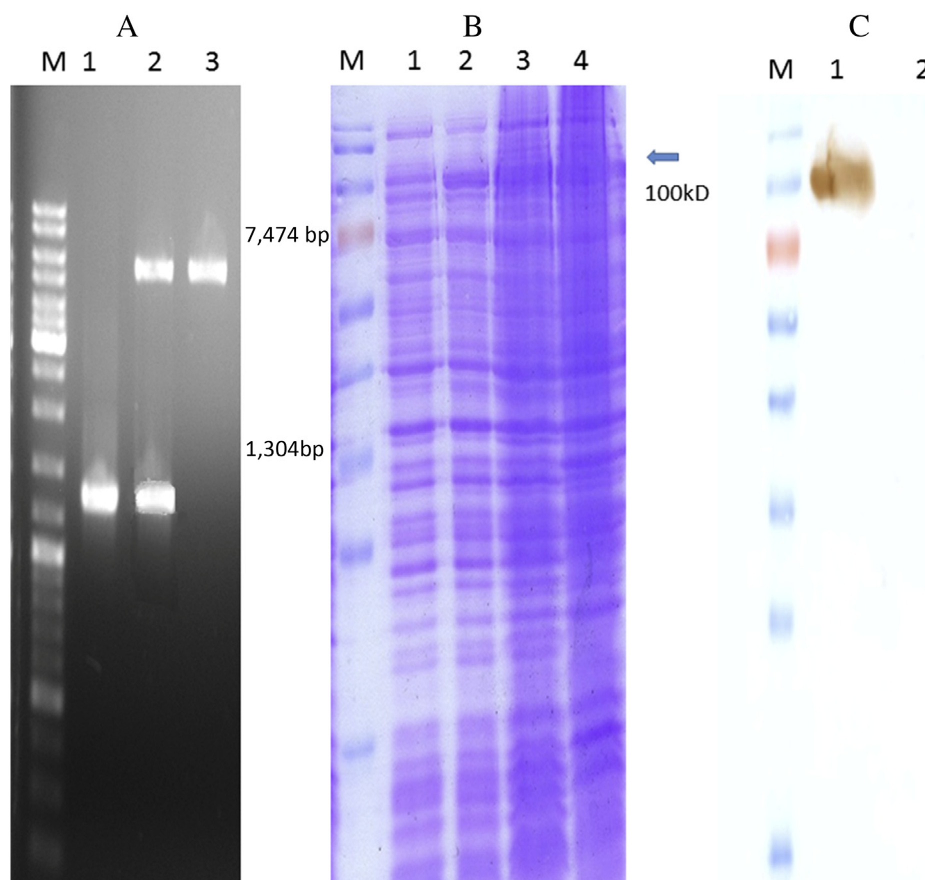

Figure 1. (A) Digestion of pTY-α-amylase with *Nhe*I and *Xho*I restriction enzymes. Lane 1: pTYB2 digested with *Nhe*I and *Xho*I; lane2: pTY-α-amylase digested with *Nhe*I and *Xho*I; lane 3: PCR product of α-amylase. (B) SDS-PAGE analysis of α-amylase expression. Lane 1: before IPTG induction; lane 2: after IPTG induction; lane 3: 16 hours after IPTG induction; lane 4: 20 hours after IPTG induction. (C) Western blot analysis of α-amylase with anti-intein polyclonal antibody. Lane1: α-amylase; lane 2: lysate of untransformed bacteria. IPTG = isopropyl- β-d-thiogalactopyranoside; M = molecular marker; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

(pH 7.2) and detected by electrophoresis. The gel was incubated for 15 minutes at 40°C in 0.05M acetate buffer (pH 5.5), then submerged in the same buffer including 1% of soluble starch at 80°C for 15 minutes, and stained with iodine solution (0.01 mol/L I_2 in 0.1M KI). Proteins displaying amylolytic activity emerged as bands on a dark brown background.

3. Results

3.1. Construction of pTY-α-amylase

The PCR amplified product of the α-amylase gene, added between *Nhe*I and *Xho*I restriction sites at the 5′ and 3′ termini of the α-amylase gene, respectively, was electrophoresed and the 1304-bp fragment gel purified, T/A cloned in pTZ57R/T, and subcloned into pTY2 to generate pTY-α-amylase expression plasmid. The expression plasmid was first transformed into *E. coli* TOP 10 cells for vector propagation, and then transformed into *E. coli* BL21 cells. Presence of the insert in the plasmid preparation from the positive transformants was confirmed by restriction enzyme digestion (Figure 1).

3.2. Expression and purification of α-amylase

SDS-PAGE analysis of proteins from the culture of *E. coli* cells carrying pTY-α-amylase and following induction with 1mM IPTG for 20 hours at 20°C showed a clear band at a position corresponding to 100 kDa. Further confirmation was undertaken with western blot analysis using an anti-intein antibody. Amylolytic activity of proteins produced by transformed *E. coli* cells was detected by zymography using 12% SDS-PAGE gels (Figure 2). Enzymatic activity of the fusion construct of α-amylase and intein was observed in the soluble fraction, as well as in inclusion bodies solubilized by 4M urea. These results showed that heating was unable to cleave the intein fusion from α-amylase, however, the enzyme fused with intein showed activity. Heat treatment of the crude extract effectively removed proteins and can be used as an efficient step for enzyme

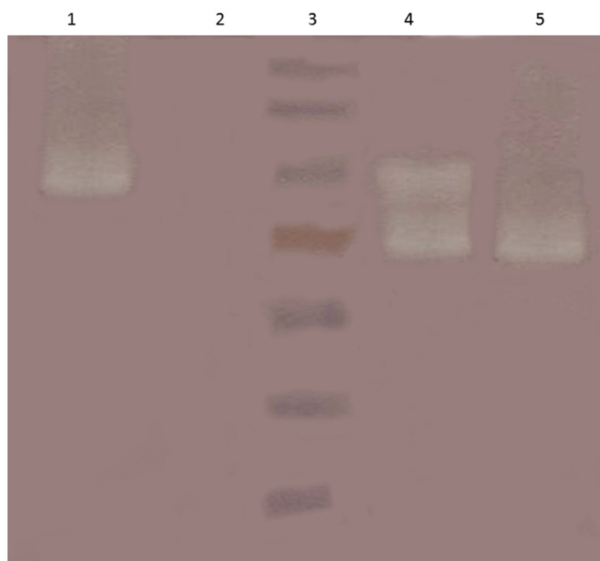

Figure 2. Zymography of the amylolytic activity of α-amylase expressed in *Escherichia coli* BL21 cells transformed with pTYB2α-amyl. Lane 1: lysate of *E. coli* BL21 cells transformed with pTYB2α-amyl before separation of intein fusion by heat; lane2: lysate of untransformed bacteria; lane 3: supernatant obtained from centrifugation of heated lysate of *E. coli* BL21 cells transformed with pTYB2α-amyl; lane 4: purified thermostable α-amylase produced by *Bacillus licheniformis*. M = molecular marker.

purification. Most of the α-amylase activity was observed in the cell-free extract, and only 35% of the total enzyme activity was observed in inclusion bodies. The intein fused with the recombinant protein can be partially separated from its fusion partner by heat treatment at ∼85°C in the presence of thiol compounds. Interestingly, the recombinant protein fused with intein also showed enzyme activity (Figure 2).

3.3. Enzyme activity

Amylolytic activity (∼185,000 ± 8,000 U/L bacterial culture) was observed from the soluble form of the protein, while the solubilized inclusion bodies exhibited similar activity levels. Our findings suggested that the pTYB2 plasmid is an appropriate system to express and produce thermostable α-amylase. No reduction in enzyme activity was observed following mutation of two amino acids in the C- and N-terminal regions of the enzyme.

4. Discussion

α-Amylases are enzymes that hydrolyze starch by cleaving α-l,4-glucosidic bonds. They are among the most important commercial enzymes, having broad applications in starch processing, textiles, brewing and alcohol manufacturing, and other industries. Several α-amylases have been characterized and their genes cloned from microorganisms, plants, and animals. With the exception of two enzymes obtained from eubacteria and archaea [12], α-amylases are considered members of the same α-amylase family, sharing a comparable structure, similar catalytic site, and identical catalytic mechanism [13]. Because starch is soluble beginning at ≥100°C, the only α-amylases used in industry are those that are active at temperatures ≤110°C [14]. The majority of thermostable α-amylases used in industry are purified from *B. licheniformis*, with optimal temperatures of 90°C and requiring the addition of Ca^{2+} for thermostability [5]. Hyperthermophilic archaea are increasingly attractive to applied research, given that their enzymes show extreme thermostability [15,16]. Recently, numerous amylolytic enzymes have been obtained from *Pyrococcus furiosus* [17–20], *P. woesei* [9,18], and *Thermococcus profundus* [21]. Only the *P. furio*sus and *P. woesei* intracellular α-amylase genes were cloned and expressed in *E. coli* [22] and their sequences shared minimal homology with other α-amylase sequences. Starch hydrolysis at high temperatures requires an α-amylase with improved thermostable characteristics that does not require Ca^{2+}. Expression of the enzyme in *E. coli* transformed with pTY2-α-amyl led to high levels of soluble enzyme [9]. In this study, we cloned the α-amylase gene of *P. woesei* in *E. coli* with two changes in amino acids in comparison of previous work [9].

The activity of the soluble enzyme obtained here (185,000 ± 8,000 U/L of growth medium) was higher that previously reported for native α-amylase from *P. woesei* and the yield achieved during expression of the recombinant enzyme from *P. furious* in *E. coli* (10 g, 1,000 U/L, and 38,000 U/L of induced culture) [18,19]. The amount of soluble enzyme obtained was similar to that reported by Grzybowska et al (195,000 ± 11,000 U/L of the growth medium) [9]. Our result emphasized that the pTYB2-α-amyl plasmid can be used as an expression system for the production of catalytically active recombinant fusion proteins of thermostable α-amylase and intein in *E. coli*. In regard to other systems used for *P. woesei* α-amylase expression, the inclusion bodies described here displayed 85% of total enzyme activity [9]. The soluble form of the active enzyme suggested that this expression system is adequate for the production of thermostable α-amylase, which can be separated from the fusion protein in the presence of thiol compounds by heat treatment at ∼85°C.

In summary, this study emphasized that the pTYB2α-amyl plasmid can be used as an expression system for the production of catalytically active, recombinant, thermostable α-amylase in *E. coli,* with no impact to enzyme solubility by two amino acid mutations in the C- and N-terminal regions.

Conflicts of interest

The authors declare no conflicts of interest.

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