INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18F–032/2018/20–7–1657–1662
DOI: 10.17957/IJAB/15.0702
http://www.fspublishers.org

Full Length Article

Cloning and Characterization of α-Amylase from *Thermotoga neapolitana* in *Escherichia coli*

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Abstract

The enzyme 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1), which randomly hydrolyzes the 1,4-α-D -glucosidic linkages between adjacent glucose residues in linear amylose chains, is one of the most crucial starch-converting enzymes in present-day biotechnology. The intracellular α-amylase gene from the hyperthermophilic bacterium *Thermotoga neapolitana* was isolated, then inserted into pET-28a (+) vector and expressed in *Escherichia coli* BL21 (DE3) using IPTG as an inducer. In this study, we mainly investigated the expression, purification and enzymatic properties of recombinant α-amylase in *E. coli*. The recombinant α-amylase assayed maximum activity at 75°C and pH 5.5. The molecular mass of the purified enzyme was supposed about 62 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant enzyme named Amy1680, with excellent heat resistance and Ca^{2+}-independent activity, has a beneficial development prospect which is worthy of further more exploration.

Keywords: α-amylase; *Thermotoga neapolitana*; Cloning; pET-28a; Enzymology; Characterization

Introduction

The enzyme 1,4-α-D-Glucan-glucanohydrolase (EC 3.2.1.1) are widely distributed in animal, plant, and microorganism (Vihinen and Mantsala, 1989), and it is a random way to decompose starch, glycogen, polymeric or polysaccharide molecular into glucose units by hydrolyzing on α-1,4-glycosidic linkages (Cipolla *et al.*, 2012). It is significant that α-amylase is one of the most crucial starch converting enzymes in present-day biotechnology with applications ranging from baking, fermentation, distilling, pharmaceutical industries to clinical, textile, analytical chemistries, detergent and paper industries (Gupta *et al.*, 2003; Horváthová *et al.*, 2006; Fang *et al.*, 2016). Since the advent of α-amylase, researchers have quickly discovered its value as it has been occupying a more significant market share over the years (Sajedi *et al.*, 2005; Saxena *et al.*, 2007).

The double-enzymatic method is commonly used for industrial processing of raw starch slurry. The initial pH of the starch slurry is 4.5, and it is required to add acid and alkali to regulate pH during the liquefaction (pH 6.5) and saccharification (pH 4.5). In the processing, a large number of acid and alkali, not only make the process more complicated and increase the cost, but increase the environmental burden at the same time. If the α-amylases used for the liquefaction have robust acid stability, the pH adjustment step is not required. Therefore, acid-stable α-amylase in the industrial application can save acid-base reagent consumption and simplify the processing technology.

α-Amylases can be divided into high-temperature α-amylase and medium temperature α-amylase, according to the thermal stability of α-amylase (Sodhi *et al*., 2005). It is highly desirable that α-amylases remain relatively active at the high temperatures (100~110°C) of starch gelatinization (Arikan, 2008). Therefore, there has been a need to continually search for more not only acid-stable but also thermostable α-amylases (Emtenani *et al.*, 2015).

It was reported that acid-stable α-amylases could be obtained from bacteria, fungi, yeasts, and archaea (Gupta *et al.*, 2003). Thermophilic microorganisms compared with others has a significant advantage that its original growth environment temperature is higher than others (Mjec *et al.*, 2002). Moreover, high-temperature resistance of these microorganisms as the source of α-amylases is higher than non-thermophilic microorganisms (Niehaus *et al.*, 1999). Industries need high-temperature α-amylases to reduce starch viscosity, equipment wear and cost (Gupta *et al.*, 2003).

Bacillus species such as *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are well-known for the production of α-amylases (Burhan *et al*., 2003; Khajeh *et al*., 2006; Hmidet *et al*., 2009). α-Amylases isolated from thermophilic bacterias are acid stable and insensitive at high temperature. But there was a marine hyperthermophilic bacterium isolated from geothermally heated biotopes, called *Thermotoga neapolitana* (Conners *et al*., 2006), and its heat resistance has been a hot topic automatically. The researchers hope to use its heat resistance to develop heat-resistant enzyme preparation, so as to save the production cost of starch processing. In recent years, the relationship of the thermotogales between growth and utilization is gradually clear.

The role of industrial α-amylases in the action of pH is about 6.5 and requires the addition of calcium ions (Ca^{2+}) to enhance the activity and stability (Asoodeh *et al*., 2010). However, Ca^{2+} has to be given up in some industrial production. Therefore, it is necessary to study Ca^{2+}-independent acid-stable and heat insensitive α-amylases, and much research on the characteristics of Ca^{2+}-independent α-amylases has been published. At present, acid-stable and high-temperature α-amylases do not need Ca^{2+} to increase the stability, which can reduce the addition of metal ions. It is needless to say that Ca^{2+}-independent, heat insensitive and acid- stable α-amylases will be a hot commodity.

In this study, we report the Ca^{2+}-independent α-amylase cloning of the *T. neapolitana* gene. After that, we mainly investigated the expression, purification and enzymatic properties of recombinant α-amylase in *E. coli*.

Materials and Methods

Materials

Both *T. neapolitana* (DSM 4359) and *E. coli* strain (BL21(DE3)) were the preservation of bacteria in the laboratory. *E. coli* strain was used for cloning and expression with plasmid pET-28a (+) kept by the laboratory. Taq DNA Polymerase and other PCR reagents, restriction endonuclease NheI and BamH I, T4 DNA ligase kit and protein molecular weight standards were all purchased from TaKaRa. Bacterial genome DNA extraction kit and a small amount of high-purity plasmid preparation kit were purchased from BioTeke, while DNA gel extraction kit was from Shanghai Sangon. In addition, the tryptone and yeast extract from OXOID, and Amy1680 in kanamycin was purchased from the Beijing Ding States. All other chemicals were of analytical grade.

Luria-Bertani (LB) broth was used as the medium for the genic engineering strain which was used as the heterologous expression host. LB broth containing bacto-tryptone, sodium chloride, yeast extract and dH_2O (1:1:0.5:100, w/w/w/v) was sterilized at 121°C for 20 min (Emtenani *et al.*, 2015). *E. coli* transformants were cultured in LB broth containing 50 μg·mL^{-1} kanamycin at 37°C with stirring at 200 r/min for 16 h.

Preparation of Recombinant α-amylases Gene from *T. neapolitana*

T. neapolitana genomic DNA was made ready according to "Molecular Cloning A Laboratory Manual" (Deininger, 2001), designated Amy1680. Encoding genes were amplified by polymerase chain reaction (PCR). The template used by PCR is *T. neapolitana* genomic DNA with the following combination of Taq polymerase and primers. PCR reaction conditions are as follows: 3 min per denaturation at 94°C; 45 s denaturation at 94°C, 35 s annealing at 55°C and 90 s elongation at 72°C (30 cycles); 10 min elongation at 72°C. Gene fragment was amplified and cloned into the expression vector pET-28a (+). Two primers were designed by software Primer 5, which were the forward primer (5'-CTAGCTAGCATGTTTTGCCAAGAAGGAGGAGC-3') and the reverse primer (5'-CGCGGATCCTCATTTTCGCACCTCCTCACTTTTG-3'). Two primers containing BamH I and NheI restriction sites were used for the overexpression of DNA protein in *E. coli*. The obtained recombinant plasmid was named pET-Amy1680. The nucleotide sequence of the gene amplified by the PCR is determined by the Sangon biotech bioassay.

Expression and Purification of the Recombinant Amy1680 α-amylase

The recombinant *E. coli BL21(DE3)* strain carrying pET-Amy1680 was grown in LB broth containing 50 μg·mL^{-1} kanamycin at 37°C. The pET-Amy1680 genes were induced with IPTG to a final concentration of 0.8 m*M*, which the optical density at 520 nm was 0.7. After 7 h induction with IPTG at 30°C, the cells were harvested by centrifugation at 9500 r/min for 15 min at 4°C, followed by resuspended in 20 m*M* pH 5.5 sodium acetate buffer. The target proteins were released from the cells by sonication (intermittent for 5 s, working for 4 s, 400 W, 100 cycles). The lysates of cells were separated by centrifugation at 9500 r/min at 4°C for 15 min. To get rid of the host proteins which were thermolabile, the supernatant fraction was incubated at 75°C for 30 min. The denatured protein was eliminated by centrifugation at 9500 r/min for 15 min at 4°C, and the supernatant was the crude α-amylase solution. The resulting clear supernatant was dialyzed against 20 m*M* Tris-HCl (pH 7.4) at 4°C for overnight and freeze-dried into a powder.

A HiTrap SP HP column was used to purify α-amylase at 1.0 mL·min^{-1}. The concentration of the sample α-amylase solution was prepared with 20 m*M* sodium phosphate buffer at 100 mg/mL. Then the elution flowed in 0 to 1.0 *M* sodium chloride gradient buffer in 15 column volumes. The resulting eluent was dialyzed against 20 m*M* Tris-HCl (pH 7.4) at 4°C for overnight. The molecular mass of the Amy1680 protein was estimated

by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of Recombinant Amy1680 Activity

The enzyme activity was assayed according to Bernfeld method with modification (Bernfeld, 1955). Recombinant Amy1680 activity was measured in 20 m*M* sodium acetate buffer (pH 5.5) with 3,5-dintrosalicylic acid (DNSA) at 75°C. The reaction mixture (1 mL) was comprised of 0.1 mL Amy1680 solution, 0.4 mL sodium solution and 0.5 mL 1% (w/v) soluble starch solution. The reaction mixture was incubated for 10 min at 75°C. After that 0.375 mL DNSA was added and the sample was boiled for 5 min. The enzyme activity was calculated by measuring reducing sugars released at 520 nm. All samples were analyzed in triplicate. The amylase activity for one unit (U) was defined as the amount of protein needed to liberate 1.0 μmol of reducing sugar per min under the assay condition (Wang *et al.,* 2016).

pH and Temperature Studies

The optimal temperature of Amy1680 was determined at the temperature range of 50–90°C. Thermal stability was investigated by measuring the residual activity of the Amy1680, which was pre-incubated at 75, 85 and 95°C for 60 min. The residual activity which was after cooling on the ice was measured every 20 min according to section 2.4.

Different buffer systems, including glycine-HCl buffer (pH 2-3.5), sodium acetate buffer (pH 4-5.5), sodium phosphate buffer (pH 6-7.5), Tris-HCl buffer (pH 8-9.5), and Na_2HPO_4-NaOH buffer (pH 10-11) were prepared to study the effect of pH on Amy1680 activity. The enzyme activity was measured according to section 2.4.

Effect of Various Metal Ions

The amylase activity was investigated in the presence of various metal ions (1 and 5 m*M*) including K^+, Co^+, Ca^{2+}, Na^+, Ni^+, Zn^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Al^{3+} by pre-incubation of the enzyme for 30 min with metal salts, followed by measuring the residual activity under optimum conditions. The remaining activity of each sample was calculated from the activity of the untreated sample as 100% (Asoodeh *et al.*, 2014).

SDS–PAGE Analysis

The molecular weight was assessed by SDS–PAGE. Samples were prepared at the same protein concentration, and 10 μL of sample was added to each lane. Electrophoresis lasted 2 h with a voltage of 120 V and a current of 50 mA. After the electrophoresis, the gel was dealt with Coomassie Blue R 250 dye in a solution containing isopropanol, acetic acid and dH_2O (5:2:15, v/v/v) for 1 h. Then, the gel immersed in a solution composed of methanol, acetic acid and dH_2O (2:1:17, v/v/v) for 2 h. Remove the gel, take a photo with the gel imager.

Hydrolytic Patterns of Amy1680

Purified Amy1680 was incubated at 75°C with 20 mM sodium acetate buffer (pH 5.5) and 1% (w/v) soluble starch solution, after 5 h the reaction was stopped at 100°C. The reaction products were separated and determined by thin-layer chromatography (TLC) and developed as described below. The resulting reaction products were dispersed on TLC in a solvent system made from 1-butanol/ethanol/H_2O/acetic acid (5:5:2:1, v/v/v/v). The TLC plate was dried at 120°C after visualized by dipping it into the solution composed of diphenylamine/aniline/acetone (1:1:50, w/v/v) and 85% (v/v) phosphoric acid in methanol.

Results

Cloning and Expression of Amy1680 in *E. coli*

The open reading frame of Amy1680 is 1,680 bp based on the results of 1% agarose gel electrophoresis (Fig. 1) and sequence. It means that Amy1680 would encode a single polypeptide of 560 amino acids. It indicated that an estimated molecular mass of Amy1680 was about 62 kDa based on the open reading frame of Amy1680. Consequences of restructuring plasmid PCR and double enzyme cut verification showed that I and Nhe I cut pET-Amy1680 into two fragments. One is the same size with PCR amplified fragments, and the other is the same size as pET-28a (+). The plasmid pET-28a (+) based on alkali ring structure of 5369 bp after double digestion, will form the base of a long chain 5369 bp, so there was a cleaning strip in the vicinity of agarose gel electrophoresis of 5369 bp. The bacterial fluid PCR and double enzyme digestion of the target gene in the vicinity of the 1680 bp appeared to clean the band, so that was the cloning of the successful connection.

The Optimal Temperature and pH for the Enzyme

Effect of temperature on the enzyme activity was shown in Fig. 2. Within 75°C, the enzyme activity increased with temperature increasing as Fig. 2a shown. However, when the temperature increased over 75°C, enzyme activity decreased rapidly. The recombinant enzyme assayed maximum activity was at 75°C, so the optimum temperature is absolutely 75°C.

The optimum pH for enzyme activity is 5.5 (Fig. 2b). There is a phenomenon worth mentioning that Amy1680 had a wide range of pH 4.5-6 and 8.5-9.5. It was high activity at pH 5.5 and 9.0. After repeated verification test, the result was still the same. The recombinant enzyme assayed maximum activity at 75°C, pH 5.5 and the results

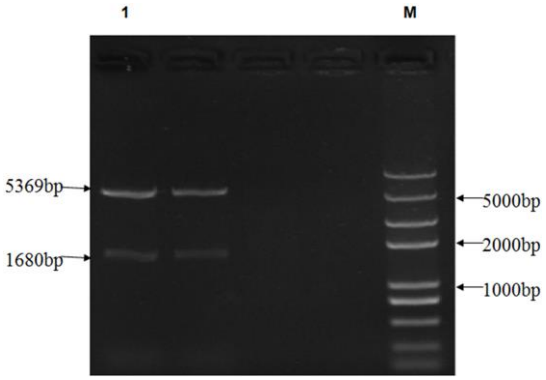

Fig. 1: Restructuring plasmid PCR and double enzyme cut verification. Marker 8000 (M) and pET-Amy1680 was digested with *BamH* I and *Nhe* I (1). The size of a band is consistent with the pET-28a (+), and the size of the other is consistent with the PCR product. The results showed that the expression vector was successfully linked

were 27.84 U/mL.

Thermal stability of the Amy1680 was estimated as shown in Fig. 2c. The initial activity remained was 85.3%, 76.4%, and 54.9%, respectively after pre-incubation of the enzyme for 1 h at temperatures of 75°C, 85°C and 95°C. The Amy1680 showed optimal activity at pH 5.5 and 75°C, remained 85.3% of its initial activity after 1 h pre-incubation. The enzyme activity decreased slightly which does not affect the industrial applications, due to its well thermal stability.

Effects of Metal Ions on the Amy1680 Activity

The influence of metal ions on the activity of Amy1680 was evaluated by measuring the remaining activity upon 1 and 5 m*M* concentrations of metal ions in the presence of pre-incubation (Fig. 3). When the Amy1680 was incubated with 5 m*M* K^+, Co^+, Ca^{2+}, Na^+, Ni^+, Zn^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Al^{3+}, the activity was retained at 136, 76, 105, 119, 0, 24, 2.5, 12, 27, 3.3 and 0% of the initial activity. Among metal ions tested: K^+, Na^+ and Co^{2+} enhanced the amylase activity, while Ca^{2+} did not have a significant effect on enzyme activity. However, metal ions including Ni^+, Cu^{2+}, Mn^{2+} and Al^{3+} at 5 m*M* completely inhibited the enzyme activity. In industrial production, Ca^{2+}-independency is considered as an advantageous feature of amylases applied in starch processing, because the addition and removal of calcium ion in subsequent stages would be omitted (Asoodeh *et al.*, 2014).

Properties of the Purified Amy1680

The recombinant BL21(DE3) carried pET-Amy1680 was grown in LB liquid medium containing kanamycin. The

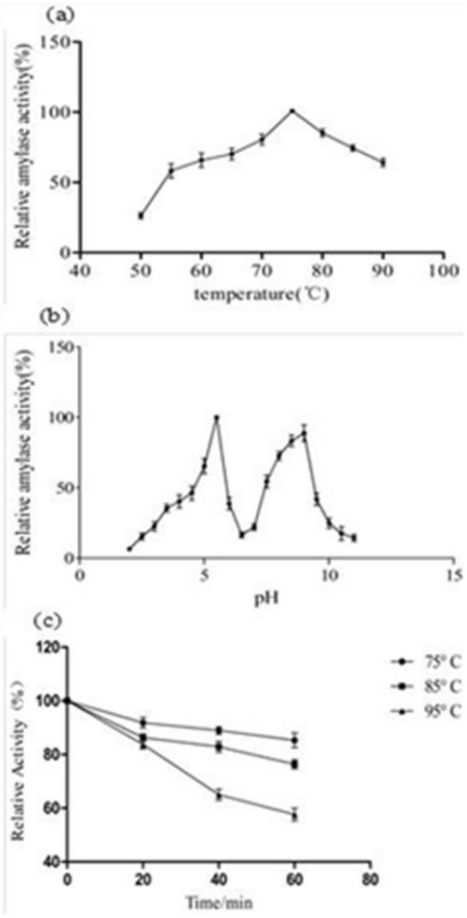

Fig. 2: (a) Effect of temperature on recombinant enzyme activity. The optimum temperature for enzyme reaction was 75°C. (b) Effect of pH on the activity of the recombinant enzyme. The optimum pH for enzyme reaction was 5.5. In addition, the enzyme activity was also high in enzyme reaction pH was 5.5. (c) Heat stability of the recombinant enzyme. Influence of 75°C (●), 85°C (■) and 95°C (▲) on recombinant enzyme stability

culture of 7 h was induced by adding IPTG at 30°C. The results were analyzed with SDS-PAGE shown in Fig. 4. The Amy1680 protein was written in BL21(DE3) harboring pET-Amy1680. The heat-labile protein contained in the crude enzyme was removed by heat treatment at 75°C for 30 min. Post heat treatment of the enzyme protein was further purified with HiTrap SP HP column chromatography. Moreover, the purified enzyme proteins compared with the marker showed that a clear band around 62 kDa was consistent with the size of the target protein. There was no doubt that the target gene was successfully expressed in the expression strain *E. coli.*

Hydrolysis Products of Amylase

Amy1680 was incubated in the starch solution at a

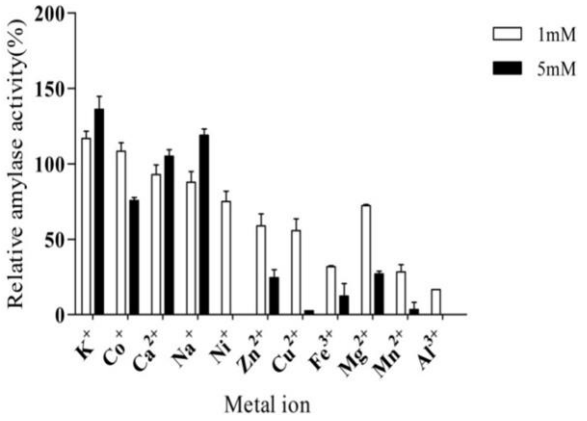

Fig. 3: Effect of metal ions on the activity of the recombinant enzyme. The influence of metal ions on the optimum conditions (75°C, pH 9.0) was determined. K^+ has a positive effect on enzyme activity

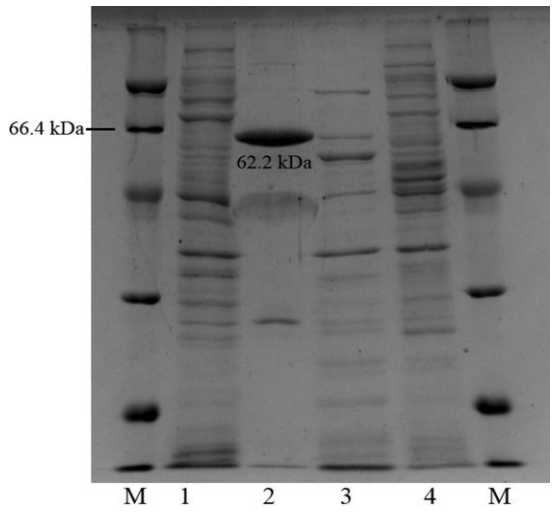

Fig. 4: SDS-PAGE analysis of Amy1680. Protein size standard (M), cellular proteins from *E.coli* before recombination (1), proteins after cation exchange chromatography (2), the Amy1680 protein of heat treatment for 30 min (3), Amy1680 protein before heat treatment (4)

concentration of 1% (w/v) at 75°C for 5 h. The results of enzymatic hydrolysis products by TCL analysis are presented in Fig. 5. The final product of the starch by the Amy1680 is glucose.

Discussion

Since it has been known that hyperthermophilic and thermophilic bacteria are good sources of heat-resistant α-amylase, research has been focused on heat-resistant α-amylases. In recent years, there have been thermophilic bacteria, acid-stable and heat-resistant α-

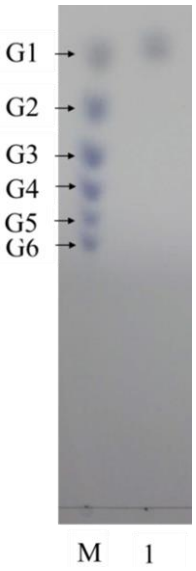

Fig. 5: Hydrolysis pattern of Amy1680 on soluble starch. The standards (M) was Maltooligosaccharide containing glucose (G1), maltose (G2), maltotriose (G3) maltotetraose (G4), maltopentaose (G5), maltohexaose (G6). The sample was soluble starch (1), and Amy1680 was incubated with the soluble starch at a concentration of 1% (w/v) at 75°C for 5 h. The final hydrolyzate is glucose

amylases reported (Cipolla *et al.*, 2012). *Thermotoga neapolitana* DSM 4359 is a novel source of acid-stable α-amylases, which has important significance in research. In earlier reports, Ca^{2+} is usually an enhancement to the power of amylases activity. Moreover, reports of Ca^{2+}-independent enzyme productions are gradually increased (Emampour *et al.*, 2015). It is realistic to focus on a broad temperature and pH range of activity, suitable thermostability, and make extra efforts on Ca^{2+}-independent α-amylase productions (Asoodeh *et al.*, 2014). Parts of thermophilic microorganisms respond to these technical requirements. Because of production capacity and harsh conditions of the wild bacterium, it is a wise movement to use genetic engineering methods to get the corresponding α-amylase. Not only Genencor but also Novozymes lead the global enzyme market in the enzyme preparation industry. However, the two companies' products are costly (Banerjee *et al.*, 2010). Consumers are in urgent need of some enzyme preparation both affordable and high quality.

Conclusion

In this study, recombinant α-amylase has high activity in acidic and alkaline conditions. Moreover, Amy1680 does not need Ca^{2+} to increase the stability. These properties make the enzyme appropriate to fermentation,

paper, detergents and other industries. So Amy1680 is worthy of further study and exploration in the future.

Acknowledgments

This work was partly supported by Science and Technology Department of Jilin Province, China, for providing financial support (20150203003NY; 20140519011JH).

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(Received 06 March 2018; Accepted 27 March 2018)