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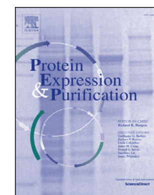

Cloning, heterologous expression, and enzymatic characterization of a novel glucoamylase GlucaM from *Corallococcus* sp. strain EGB

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The gene encoding a novel glucoamylase (GlucaM) from the *Corallococcus* sp. strain EGB was cloned and heterologous expressed in *Escherichia coli* BL21(DE3), and the enzymatic characterization of recombinant GlucaM (rGlucaM) was determined in the study. The *glucaM* had an open reading frame of 1938 bp encoding GlucaM of 645 amino acids with no signal peptide. GlucaM belongs to glycosyl hydrolase family 15 and shares the highest identity 96% with the GH15 glucoamylase of *Corallococcus coralloides* DSM 2259. The rGlucaM with His-tag was purified by the Ni^{2+}-NTA resin, with a specific activity from 3.4 U/mg up to 180 U/mg, and the molecular weight of rGlucaM was approximately 73 kDa on SDS-PAGE. The *K*m and *V*max of rGlucaM for soluble starch were 1.2 mg/mL and 46 U/mg, respectively. rGlucaM was optimally active at pH 7.0 and 50 °C and had highly tolerance to high concentrations of salts, detergents, and various organic solvents. rGlucaM hydrolyzed soluble starch to glucose, and hydrolytic activities were also detected with amylopectin, amylase, glycogen, starch (potato), α-cyclodextrin, starch (corn and potato). The analysis of hydrolysis products shown that rGlucaM with α-(1–4),(1–6)-D-glucan glucohydrolase toward substrates. These characteristics indicated that the GlucaM was a new member of glucoamylase family and a potential candidate for industrial application.

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1. Introduction

Glucoamylase (α-1,4-D-glucanglucohydrolase; EC 3.2.1.3), an industrial enzyme which hydrolyzes 1,4-linked α-D-glucosyl residues successively from the non-reducing ends of oligo-polysaccharide chains containing starch, amylose, amylopectin and glycogen with the release of D-glucose, have been isolated from a wide variety of microorganisms [1,2]. Glucoamylase is second in worldwide distribution and sales among industrial enzymes [2]. The primary commercial application of glucoamylase is the production of glucose syrups from starch [3]. These syrups can be used for fermentation, production of crystalline glucose, or as a starting material for fructose syrups [4].

Glucoamylases are present almost exclusively in filamentous fungi and then in bacteria and yeasts [5]. In order to meet the requirements of different industrial processes, the demand for novel glucoamylase with high activity and sufficient stability has increased. Diverse of glucoamylases with special characterization have been isolated, e.g., novel glucoamylases from *Aspergillus awamori* and *Aspergillus niger* [6,7]; a thermostable glucoamylase with specific activity 80 U/mg from *Thermoanaerobacter tengcongensis* MB4 [8]; glucoamylase GLL1 from *Saccharomycopsis fibuligera* [9]; glucoamylase TmGlu1with specific activity 8.0 U/mL from the *Tricholoma matsutake* [10] and glucoamylase with specific activity 9.0 U/mg from pathogen *Sclerotinia sclerotiorum* [11]. However, the specific activity of these glucoamylases is quite low. Besides, the limitations in cultivation of special organism and poor heterogeneous expression due to the genetic differences between the isolated strains and the existing expression systems remain obstacles for their widespread industrial application.

Very little research has carried out to characterize the enzymes produced by myxobacteria. Few of these enzymes have been characterized, including proteases from *Myxococcus virescens* [12], α-amylases from *Corallococcus coralloides* D [13]. Therefore bacterial glucoamylases with higher activity and novel characterization are still needed to fulfill the industrial requirement of starch

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processing. In this study, we first report the cloning and expression of the glucoamylase gene *glucaM* from *Corallococcus* sp. strain EGB. Upon purification and characterization, the recombinant glucoamylase showed special characteristics compared with others.

2. Materials and methods

2.1. Strains and plasmids

Corallococcus sp. strain EGB (CCTCC No. M2012528) [14] was used to clone glucoamylase gene; *Escherichia coli* DH5α and BL21(DE3) (Invitrogen Co. Ltd., Shanghai, China) were employed for recombinant plasmid construction of the glucoamylase gene. The pMD19^T vector (TaKaRa Biotechnology Co. Ltd., Dalian, China) was utilized for gene cloning and DNA sequencing, and the pET-29a(+) plasmid (Novagen Co. Ltd., Shanghai, China) was used as the expression vector for heterologous expression of glucoamylase in *E. coli* BL21 (DE3).

2.2. Gene cloning, sequencing, and construction of expression vector

Genomic DNA was extracted from *Corallococcus* sp. strain EGB cells using the method as described by Avery and Kaise [15]. Based on the DNA sequence of annotated hypothetical glucoamylase gene (Gene ID: CP003389) of *C. coralloides* DSM 2259. The gene encoding glucoamylase was cloned by PCR amplification with the a primers pair of GlucaM-F (5′-CATATGGACCTCTACAGCGCCGC-3′) and GlucaM-R (5′-CTCGAGAGCCGCCATCCATTGAGT-3′). The amplified PCR fragment was sequenced by Invitrogen Co. Ltd. (Shanghai, China). The PCR products were digested with *Nde* I and *Xho* I and inserted into pET29a (+) digested with the same enzymes to produce the pET29a-GlucaM recombinant plasmid. The pET29a-GlucaM was then transformed into *E. coli* BL21 (DE3) for gene expression.

2.3. Expression and purification of the recombinant enzyme

E. coli BL21(DE3) harboring the pET29a-GlucaM was screened in LB agar plate containing 50 μg/ml of kanamycin. The strain was cultured in LB media containing 50 μg/ml of kanamycin at 37 °C to OD_{600} of 0.5–0.6. Protein expression was induced at 18 °C with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM for 24 h. The cells were harvested by centrifugation at 12,000 rpm for 10 min. The cells was then re-suspended in an equilibration buffer (50 mM Tris-HCl, pH 7.0) at 4 °C and disrupted by ultrasonic disruption (Sonicator 201 M, Kubota, Japan) for 10 min. The obtained suspension was centrifuged at 15,000 rpm for 20 min. The recombinant GluaM with C-terminal His-tagged was purified from the soluble fraction with Ni^{2+}-NTA resin (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purified glucoamylase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [16].

2.4. Glucoamylase activity assay

Glucoamylase activity assay was carried out using the method of measuring the formation of reducing sugars released during starch hydrolysis [14]. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.0) containing 0.5% (*w*/*v*) soluble starch and 1 μL diluent enzyme solution at 50 °C for 10 min. The amount of released reducing sugar was determined by the dinitrosalicylic (DNS) acid method [17]. One unit of glucoamylase hydrolytic activity was defined as the amount of enzyme needed to release 1 μmol of reducing sugar per minute. Protein concentrations were estimated according to the method of Bradford [18].

All determinations were performed in three replicates, and the control experiment without glucoamylase was carried out under the same conditions. R version 3.1.1 (Vanderbilt University, USA) was used for the statistical analysis. The one-way ANOVA test was used, and a p value of 0.05 was deemed significant.

2.5. Kinetic parameters of the rGlucaM (K_m and V_{max})

The effect of concentration(0.1–10 mg/mL starch) on glucoamylase activity was evaluated in 50 mM of Tris-HCl buffer (pH 7.0) at 50 °C. The kinetic parameters (Michaelis-Menten constant, K_m, and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk [19].

2.6. Enzymatic characterization of rGlucaM

Studies on the influence of temperature and pH were conducted with the rGlucaM. The effect of pH on the activity of the rGlucaM was determined in various 50 mM buffers with starch as the enzyme substrate (citrate buffer, pH 3.0–6.0; sodium phosphate buffer, pH 6.0–7.0; Tris-HCl buffer, pH 7.0–9.0; and Glycine-NaOH buffer, pH 9.0–10.0) by maintaining a constant temperature of 50 °C. The enzyme activity was calculated at pH ranging from 3.0 to 10.0. pH stability assays were performed by incubating aliquots of the enzyme at a pH range of 3.0–10.0 for 24 h at 4 °C and the residual activity was then measured under standard assay condition. The effect of temperature on the enzymatic activity was determined by assaying with starch at a temperature range of 20–80 °C for 1 h. Assays were performed as described above.

To determine the effect of salt concentration on rGlucaM activity, an enzyme assay was performed using the standard assay described above with NaCl and KCl at final concentrations between 1 and 4 M.

To analyze the effects of various metal ions and chemicals on the activity of r GlucaM, the activity of rGlucaM was determined at 50 °C for 10 min with appropriate metal salts at final concentrations of 1 mM and 5 mM. To determine the effects of EDTA (10 mM and 20 mM), dimethyl sulfoxide (DMSO), methanol, ethanol, acetonitrile (10% and 20%), and various concentrations of surfactants (Triton X-100, SDS), the procedure used was the same as that for metal ions. Activity in the absence of any additives was taken as 100%.

Substrate specificity of rGlucaM was determined by measuring the enzyme activity towards various polysaccharides and maltooligosaccharides. All reactions were conducted in 50 mM Tris-HCl buffer (pH 7.0) at 50 °C for 10 min with 1% (*w*/*v*) substrate.

2.7. Catalytic properties of the glucoamylase

To detect the hydrolysis pattern of the purified glucoamylase, the hydrolysis products of soluble starch caused by the enzyme were detected by thin-layer chromatography (TLC) as described previously [20]. Appropriately diluted enzyme was incubated with 0.5 mL of 0.5% soluble starch, amylose, amylopectin and glycogen in 50 mM Tris-HCl buffer (pH 7.0). The reaction was carried out at 50 °C for 1 h. Thin-layer chromatography was performed in a solvent system of *N*-butyl alcohol/carbinol/water (4:2:1), and the spots were visualized by spraying the silica gel plate (10 × 20 cm, Merck, Germany) with H_2SO_4/methanol (1:1, *v*/*v*) followed by heating at 90 °C for 10 min [21].

The hydrolysis products of rGlucaM towards oligosaccharides were detected by thin-layer chromatography (TLC) as described elsewhere [20]. Oligosaccharides (G4, G3, G2), at a final

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