

Purification and characterization of recombinant enzyme CGTase G1

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The cyclodextrin glucanotransferase (CGTase) gene from *Bacillus* sp. G1 was successfully isolated and cloned into *Escherichia coli*. The CGTase gene was expressed in *E. coli* and approximately 62% of CGTase enzyme was secreted into the medium. The recombinant CGTase was purified to homogeneity using ammonium sulfate precipitation followed by α -cyclodextrin-bound-epoxy-activated Sepharose 6B affinity chromatography. The purified recombinant CGTase exhibited a single band of 75kDa on SDS-PAGE and globular size was determined to be 79kDa by gel filtration chromatography. The enzyme has an optimum temperature of 60°C and optimum pH of 6.0. Phosphate buffer pH 6.0 was found to be more preferred compared to citrate or MES buffer at same pH value. Stability of the recombinant CGTase G1 covered over a wide pH range, from pH 6 to pH 10 where as it has a half-life of 30 minutes at 60°C. Stability and half life was able to be increased by additional of CaCl₂. K_m and V_{max} value for recombinant CGTase from *Bacillus* sp. G1 was calculated to be 0.468 mgml⁻¹ and 64.1mg β -CD respectively. After 16 hours incubation at 60°C, the yield for cyclodextrin production from tapioca starch as the substrate were 90% for β -cyclodextrin and 10% for α -cyclodextrin without adding any selective agents.

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Introduction

Cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19) is an industrial applicable enzyme that produces α -, β - and γ -cyclodextrins (CDs) from starch through an intramolecular transglycosylation reaction. The α -, β - and γ -CD have closed circular structures consist of six, seven and eight glucose units respectively. The applications of CDs are very broad. CDs are able to form inclusion complexes with a large variety of compounds which may improve chemical and physical properties of the guest molecule. CDs have wide application in today's industries such as food, cosmetics, chemicals, environmental, agricultural, cosmetics, pharmaceuticals and toiletries.

The market for cyclodextrin is big, in 1998 alone, global cyclodextrin consumption was around 6,000 metric tons, with an annual growth rate of 15-20% (McCoy M., 1999). There are three major types of cyclodextrin, namely α -CD, β -CD and γ -CD. The cheapest cyclodextrin is β -CD. It costs around US\$ 3-4/kg. For α -CD it is about US\$ 20-25/kg while γ -CD is roughly US\$ 80-100/kg.

CGTase G1 was originally produced by *bacillus* sp. G1, isolated locally from soil. Purification and characterization of the wild type protein was published (Ho *et al.*, 2005). Gene for CGTase G1 was successfully cloned into *Escherichia coli*. In this paper, purification and characterization of the recombinant CGTase is presented.

Materials and Methods

Purification of recombinant CGTase

Method used to purify recombinant G1 CGTase is similar to the wild type purification (Ho *et al.*, 2005). Purification of recombinant CGTase was carried out at 4°C. Crude enzyme was concentrated by ammonium sulfate precipitation at 70% saturation prior loaded into α -CD-Sepharose 6B column. After washing the column with more than 7 column volumes of 0.01 M acetate buffer pH 5.5, bound CGTase enzyme was eluted with 1% α -CD in loading buffer. The eluted enzyme was extensively dialyzed in 10,000 MWCO snake skin dialysis tubing (Pierce) against 0.1 M Potassium Phosphate buffer pH 6.

Optimum pH and temperature

The optimum pH of the pure recombinant enzyme was determined by replacing 0.1 M phosphate buffer pH 6.0 in the CGTase assay with different buffers from pH 4-10. The reactions were carried out using the CGTase assay (Kaneko *et al.*, 1987). The optimum temperature of the pure enzyme was determined by incubating the reaction mixture of CGTase assay in different temperatures, ranging from 40°C - 90°C for 10 min. Then the reaction was done according to the CGTase assay.

The pH and thermal stability

The pH stability of the recombinant enzyme was measured by incubating 0.1 ml pure enzyme with 0.2 ml of 0.1 M sodium acetate buffer (pH 4-5), 0.1 M sodium phosphate buffer (pH 6-7) and glycine-NaOH buffer (pH 9-10), respectively at 60°C, without substrate for 30 min. Then, the remaining activity of the enzyme was assayed by the standard assay method. The temperature stability of the enzyme was measured by incubating 0.1 ml pure enzyme with equal volume of buffer 0.1 M sodium phosphate buffer, pH 6.0 at different temperatures (40°C - 90°C) for 30 min. Standard CGTase assay was performed to determine their residual activity.

Kinetic parameters

The K_m and V_{max} values for the pure enzyme were determined by incubating 0.1 ml purified CGTase with soluble starch in 1 ml 0.1 M phosphate buffer pH 6.0. Assay were carried out according to standard enzyme assay method. K_m and V_{max} values were then determined from Hanes-Woof plot.

Analysis of Cyclodextrin by HPLC

Enzyme-substrate mixtures were incubated at optimum temperature for different duration and were subsequently boiled for 10 minutes to stop the enzymatic reaction. The ratio of different cyclodextrins produced was analyzed using Waters HPLC system with Asahipak NH2 as column. Isocratic flowrate was set at 1 ml.min⁻¹ with 70:30 acetonitrile-water as the mobile phase. The column temperature was maintained at 30°C.

Results and Discussion

The purified enzyme has the highest β -CD cyclization activity at pH 6.0 and 60°C using soluble starch as substrate. The recombinant enzyme was found not stable in acidic condition. Since the wild type protein was originally from alkalophilic bacterial, the recombinant enzyme was found able to retain activity at higher pH conditions. The recombinant G1 was stable over a wide pH range (pH 6.0-10.0) with gradual loss of activity at acidic pH. It has a half-life of approximate 30 minutes at 60 °C and cyclization activity lost of almost 80% at 70 °C. For CGTase with known 3D structures, CGTase from *Thermoanaerobacterium thermosulfurigenes* strain EM1 (*Tabium*) has the highest thermostability with a half-life of 15 minutes at 90°C (Wind *et al.*, 1995). Other CGTase from mesophilic bacterial such as BC 251 has a half life of 9.7 minutes at 60°C (Leemhuis *et al.*, 2004), $t_{1/2}$ CGTase from *Brevibacillus brevis* CD162 (Kim *et al.*, 1998) and *Bacillus ohbensis* (Sin *et al.*, 1991) was 30 minutes at 55°C.

The kinetic parameters K_m and V_{max} for recombinant CGTase *Bacillus* sp. G1 was calculated to be 0.468 mgml⁻¹ 64.1mg β -CD respectively. These values indicated that the recombinant enzyme has a relatively high affinity to soluble starch, comparatively more active than other reported CGTases since it needs much lower concentration of substrate to achieve half of V_{max} . Different K_m values have been reported for various CGTases, namely recombinant CGTase *Thermococcus* sp. B1001 (Tomoko, 2000), 1.45 mgml⁻¹; *B. firmus* (Gawande, 1999), 1.21 mgml⁻¹; *K. pneumoniae* AS-22 (Gawande, 2001), 1.35 mgml⁻¹; *Bacillus agaradhaerens* (Martins, 2002), 21.2mgml⁻¹.

Analysis of cyclodextrins using HPLC for purified recombinant enzyme showed that β -CD was predominantly produced. After 16 hours of reaction time, approximately 90% of total cyclodextrins produced was β -CD, and 10% γ -CD. No α -CD was detected in any part of the time course. The CGTase of *Bacillus* sp. G1 has the highest homology and identity to the CGTase of alkalophilic of *Bacillus* sp. KC201 and both enzymes are predominant β -CD producer, with no α -CD produced. However, the ratio of β -CD to γ -CD produced by CGTase *Bacillus* sp. KC201 was reported 6.2:1 (Kitamoto, 1992), relatively, G1 CGTase was found to produce higher percentage of β -CD.

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