Cloning and sequencing of a cyclodextrin glycosyltransferase gene from *Brevibacillus brevis* CD162 and its expression in *Escherichia coli*

Myung Hee Kim a,b, Cheon Bae Sohn a,* , Tae Kwang Oh b

a Department of Food and Nutrition, Chungnam National University, Yusung, Taejon 301-764, South Korea
b Microbial Enzyme Research Unit, Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Yusung, Taejon 305-600, South Korea

Received 15 April 1998; revised 20 May 1998; accepted 31 May 1998

Abstract

A cyclodextrin glycosyltransferase (CGTase) gene of *Brevibacillus brevis* CD162 was cloned into *Escherichia coli* using pUC19 as a vector. Determination of the nucleotide sequence showed the presence of an open reading frame of 2079 bp encoding a polypeptide of 693 amino acid residues, composed of a 20-amino acid signal sequence and a 673-amino acid mature enzyme. Neither a TATA- nor a TTGA-like sequence was observed within the cloned DNA fragment. However, the fragment was expressed in *Escherichia coli* by the lac promoter of pUC19 and 74% of the total activity was secreted into the fermentation medium. The amino acid sequence of the mature CGTase showed the highest homology of 86% to that of *Bacillus* sp. KC201. The CGTase purified to homogeneity from the recombinant *E. coli* exhibited the same properties as those of native CGTase from *Brevibacillus brevis* CD162 in terms of molecular mass, reaction conditions, stability and the production of cyclodextrins. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Brevibacillus brevis*; Cyclodextrin glycosyltransferase; Cyclodextrin glycosyltransferase gene; Cyclodextrin

1. Introduction

Cyclodextrin glycosyltransferase (CGTase, 1,4-α-D-glucan; 1,4-α-D-glucopyranosyltransferase, EC 2.4.1.19) produces cyclodextrins (CDs) from starch and related α-1,4-glucans through an intramolecular transglycosylation reaction [1]. Its products, α-, β-, and γ-CD, have an apolar cavity with a hydrophilic exterior and easily form inclusion complexes with hydrophobic substances. The formation of these inclusion complexes can impart new properties to the guest molecules, such as prolonged stability and increased water solubility. Therefore, these CDs have been used in foods, cosmetics, pharmaceuticals, etc. [2-4]. Most of the CGTases reported till now produce α-, and β-CD efficiently, but only γ-CD in a trace amount. Although the *B. subtilis* No. 313 CGTase produced γ-CD exclusively among CDs,
the yield of γ-CD from starch was only 2–3% [5]. A CGTase generating a large amount of γ-CD is of great interest in industrial applications because of its greater cavity size. Therefore, finding of a bacterium producing γ-CD in large quantities and overproduction of the CGTase has important industrial applications.

We have isolated a soil bacterium, Brevibacillus brevis CD162, producing γ-CD efficiently and characterized some of its enzymatic properties [6]. In this paper, we describe the cloning and sequencing of the gene (cgt) coding for CGTase and its expression in transformed Escherichia coli. The result of characterization of the enzyme expressed in the E. coli transformant and its comparison with the original enzyme are also presented.

2. Materials and methods

2.1. Bacterial strains, plasmid, enzymes, and media

B. brevis CD162 was routinely cultured in a medium containing 2.0% soluble starch, 0.75% yeast extract, 0.5% bacto peptone, 0.2% K2HPO4, 0.05% MgSO4·7H2O and 1.5% Na2CO3 (pH 10.2) at 30°C for 96 h. E. coli JM83 (F− ara Δ(lac-proAB) rspLΔ80d lacZΔM15), used as a cloning host, was cultured in Luria Bertani (LB) broth at 30 or 37°C. For solid media, 1.5% agar was added to LB broth. Plasmid pUC19 was used as a cloning and subcloning vector. Ampicillin (100 μg ml−1) was added to the medium to allow the growth of the plasmid-carrying strain. The enzymes used for DNA manipulation were obtained from the commercial sources and used under conditions recommended by the supplier. All chemicals were of the highest purity available.

2.2. DNA manipulation and cloning procedure

Total genomic DNA of B. brevis CD162 was isolated according to Mamur et al. [7], and general recombinant DNA manipulation was carried out according to published protocol [8]. B. brevis CD162 genomic DNA was partially digested with HindIII. The cloning vector, pUC19 was cleaved with HindIII and dephosphorylated with calf intestinal phosphatase. Genomic DNA fragments were then ligated with plasmid pUC19. The ligation products were used to transform E. coli JM83. The E. coli transformants were tested by the agar plate assay method for the expression of CGTase containing amyloytic activity. Agar plates contained 1% corn starch and ampicillin (100 μg ml−1) in LB medium. Recombinant transformants were examined for their ability to form clear zones, an indication of possible CGTase expression.

2.3. Assay of enzyme activity

CGTase activity was assayed as described by Kato and Horikoshi [5]. One unit of enzyme activity was defined as the amount of enzyme that forms 1 μmol of γ-CD from soluble starch in 1 min. Alkaline phosphatase was assayed by the method of Yamane et al. [9]. One unit of alkaline phosphatase activity is defined as that amount of enzyme preparation that gave 1 μmol of p-nitrophenol liberated per min. β-Galactosidase activity was determined according to a published protocol [10].

2.4. One-step purification of the CGTase from B. brevis CD162 and an E. coli transformant

CGTase from B. brevis CD162 was purified by a simpler method than the procedure described previously [6]. B. brevis CD162 was grown aerobically at 30°C for 96 h in the above described medium. Cells were removed by centrifugation and, ammonium sulfate was added to the supernatant to give 70% saturation. The precipitate was dissolved in 10 mM phosphate buffer (pH 7.0) and dialyzed against the same buffer. The resulting supernatant was adjusted with 1.5 M (NH4)2SO4, applied onto a fast protein liquid chromatography system equipped with a Phenyl Superose HR 5/5 column (Pharmacia), and then eluted with a linear gradient of 1.5 to 0 M (NH4)2SO4.

As for the E. coli transformant, the purification procedure of the extracellular CGTase was the same as that described for B. brevis CD162. CGTases of the intracellular and periplasmic fraction were obtained after centrifugation (10,000×g, 20 min) of the sonicated cells. The supernatant was stirred with 1% streptomycin sulfate (Sigma) for 1 h on ice, and then centrifuged at 10,000 rpm for
30 min. The resulting supernatant was adjusted to 1.5 M (NH₄)₂SO₄, and directly applied onto FPLC under the same conditions described above.

2.5. Nucleotide sequencing and computer analysis

The DNA sequence was determined using the di-deoxy-chain termination method of Sanger et al. [11]. The DNA and amino acid sequences were analyzed with the MACMOLLY program. The search for existing sequences similar to this cgt was done through GenBank using the BEAUTY program.

2.6. Electrophoresis and zymography

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [12]. After electrophoresis, the gel was kept overnight at 4°C in 50 mM phosphate buffer (pH 6.0) containing 2.5% Triton X-100. The gel was then washed twice in the same buffer without Triton X-100. The renatured gel was incubated in a reaction solution containing 1% soluble starch, 20 mM sodium acetate (pH 5.5) and 1 mM CaCl₂. The gel was rinsed with distilled water and then stained for starch-degrading activity with iodine solution (1% I₂, 10% KI and 50% ethanol).

2.7. Analysis of cyclodextrins

Analysis of cyclodextrins was performed with a minor modification by the method of Kitahata et al. [13] using High Pressure Liquid Chromatography (HPLC, Waters, USA). α-, β-, and γ-CD were separated under the following conditions: column, carbohydrate analysis part No. 83083; solvent system, water/acetonitrile 30:70; flow rate, 1 ml min⁻¹.

3. Results and discussion

The HindIII fragments of chromosomal DNA from B. brevis CD162 DNA were inserted into the HindIII site of pUC19, and then transformed into competent cells of E. coli JM83. About 2×10⁴ colonies of the transformants were screened and one colony showed strong amylolytic activity on LB-corn starch plates. The product analysis of carbohydrates by the method of Kato and Horikoshi [5] showed that the transformant possessed the CGTase activity. The plasmid isolated from the transformant, designated as pCGMH162, contained the 3.7 kb DNA fragment on the vector plasmid pUC19 (Fig. 1). Southern blot analysis [14] of the B. brevis CD162 chromosomal DNA with the cloned DNA fragments as hybridization probes proved the existence of a single cgt in the genome (data not shown). The pCGMH162 subjected to restriction mapping was subcloned into the plasmid pUC19 for nucleotide sequence analysis. pCGMH163 containing a HindIII–PstI (2.7 kb) fragment and pCGMH164 containing a HindIII–SalI (2.3 kb) fragment encoded the cgt gene. The pCGMH164 was used for further nucleotide sequencing.

The nucleotide sequence of the 2.3 kb insert in pCGMH164 was determined (Fig. 1). Initially, neither a promoter sequence nor a translation initiation codon was observed within the cloned DNA fragment. However, by further sequence analysis, we confirmed that the cloned cgt was expressed in E. coli from the lac promoter of pUC19. Computer analysis revealed an open reading frame (ORF) encoding 693 amino acid residues with a calculated molecular mass of 77393 Da. The first 4 amino acids (−20 to −17) containing the translational initiation codon ATG, were derived from the vector pUC19. Since the amino acid sequence deduced from the DNA sequence matched the N-terminal amino acid sequence of the mature CGTase of B. brevis CD162 (amino acids +1 through +15; underlined in Fig. 1), it was concluded that the ORF encodes the structural gene for a CGTase. The amino acid sequence from −20 to −1 was deduced to be a signal peptide involved in secretion of the protein, and the mature CGTase contained 673 amino acids corresponding to a molecular mass of 75262 Da. In addition, a palindromic sequence that could form a stable stem-and-loop structure (+2138 to +2175) was followed by a T-rich sequence which is characteristic of the β-independent transcriptional termination signal [15]. The nucleotide sequence data have been submitted to the GeneBank Nucleotide Sequence Database and assigned the accession number AF011388.

The deduced amino acid sequence of the CGTase was compared with sequences in the GenBank using the BEAUTY program. The most similar enzyme
was the CGTase from *Bacillus* sp. KC201 [16], which had 86% identity. Also, it showed 84 and 73% identity to those of *Bacillus* sp. E1 [17] and *B. ohbensis* [18], respectively. However, the *B. brevis* CD162 CGTase showed a relatively low homology, less than 53%, with CGTases from other *Bacillus* sp.
The portion of the signal peptide derived from the original \( \text{cgt} \) (\(-1 \) to \(-16\)) revealed 69% identity with those of \textit{Bacillus} sp. KC201 [16] and \textit{Bacillus} sp. E1 [17], and 38% with \textit{B. ohbensis} [18], and showed the characteristics of a typical signal peptide [19].

CGTases contain at least six highly conserved regions (A, B, C, D, E, F). Regions A and F were believed to be involved in the cyclization of maltotetraose during the transglycosylation reaction of CGTases [20,21]. Regions B, C, D and E are conserved in amylases such as \( K\)-amylase, glucoamylase, maltotetraose-forming enzyme.
and raw-starch digesting amylase, which are known to be the active and/or substrate-binding sites of starch hydrolyzing enzymes [22] (Fig. 2). As shown in Fig. 2, the amino acid homology was very low in the A and F region of CGTases of other microorganisms including B. brevis CD162 and α-amylase of B. subtilis [23], whereas a relatively high homology was observed in the B, C, D and E regions. However B. circulans α-amylase [24] showed strong homology to the A, B, C, D, and E regions of CGTases. These observations add support to the suggestion of Sin et al. [18] and del-Rio et al. [25] that CGTase and α-amylase genes have evolved from a common ancestral gene.

It is well known that CGTases produced from Bacillus sp. are usually secreted into the culture medium. However, most of the CGTases expressed in E. coli, except those of K. pneumoniae [26] and Bacillus sp. E1 [17] are present in the periplasm due to another spatial barrier, the outer membrane. Therefore, the cellular localization of the CGTase expressed in E. coli carrying pCGMH164 was examined by the method of Lee et al. [27]. E. coli was aerobically grown in LB broth for 16 h at 30°C and extracellular, periplasmic and cellular CGTase activities were assayed. As shown in Table 1, the CGTase activities in the extracellular, periplasmic and cellular fractions were 1.53, 0.42 and 0.11 unit ml⁻¹, respectively, showing that 74% of the total activity was found in the extracellular space. To test the nature of enzyme excretion, the distribution of a periplasmic protein, alkaline phosphatase, and a cytoplasmic protein, β-galactosidase, were also determined. These enzyme activities were not detected in the extracellular fraction while most of β-galactosidase and alkaline phosphatase were located in the cytoplasmic and periplasmic fractions, respectively. Hence, this showed that the CGTase did not seem to be derived from cell lysis of transformants harboring pCGMH164. These results are quite different from the other Bacillus CGTases expressed in E. coli. Binder et al. [26] and Yong et al. [17] earlier reported that CGTase excretion may result from an unknown structural feature. The mechanism of excretion of the cloned CGTase is not yet understood, but it can be presumed that an intrinsic structural property of recombinant CGTase is responsible for the extracellular excretion of CGTase from E. coli. The Bacillus sp. CGTase published by Yong et al. might possess structural properties similar to our CGTase because of high amino acid homology (84%). This unique feature of the E. coli CGTase might be a good model system to study excretion of the enzyme in gram negative E. coli.

Table 1
Localization of the CGTase, alkaline phosphatase, and β-galactosidase activities in E. coli JM83 (pCGMH164)

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Extracellular activity (U ml⁻¹)</th>
<th>Periplasmic activity (U ml⁻¹)</th>
<th>Cytoplasmic activity (U ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGTase</td>
<td>1.53</td>
<td>0.42</td>
<td>0.11</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>0</td>
<td>3.1</td>
<td>0.9</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0</td>
<td>0</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Enzyme activities were calculated on the basis of culture broth volume.
We compared enzymatic properties of native CGTase and *E. coli* CGTase obtained by one-step purification using a Phenyl Superose HR 5/5 column. The molecular mass (Fig. 3) and pI (data not shown) of both enzymes were estimated to be approximately 75 kDa, and 6.3, respectively. Both enzymes displayed optimum activity at 55°C and were stable up to 50°C, with 36% of the original activity retained after heat treatment at 60°C for 30 min. The effects of pH on both the CGTase activity and stability were also determined. They exhibited an optimum activity at pH 8.0 and were stable over a wide pH range (pH 5.5–9.0) with a gradual loss of activity at higher alkaline or acidic pH values (Fig. 4). Studies on CD production from starch under the usual reaction conditions [28] showed that native CGTase yielded 1.3% for α-CD, 33.9% for β-CD, and 9.9% for γ-CD, while the recombinant CGTase produced 1.1% for α-CD, 33.2% for β-CD, and 9.9% for γ-CD which was comparable to the parent CGTase. Therefore the results indicate that *E. coli* CGTase has the same enzymatic features to *B. brevis* CD162 in re-

---

**Fig. 4.** Properties of the CGTases produced by *B. brevis* CD162 and *E. coli* carrying pCGMH164. (A) Optimum pH. (B) Optimum temperature. (C) pH stability. (D) Thermal stability. In (A) and (C), 0.2 M succinate-0.2 M Na₂B₄O₇ buffer (pH 4.0–5.5), 0.1 M KH₂PO₄–0.2 M Na₂B₄O₇ buffer (pH 6.0–9.0), 0.2 M Na₂CO₃–0.2 M Na₂B₄O₇ buffer (pH 9.5–11.0), and 0.1 M Na₂HPO₄–0.1 M NaOH buffer (pH 11.5–12.0) were used. The residual activities were measured after the enzymes were kept at the pH indicated for 1 h at room temperature (C), or at various temperatures for 30 min at pH 8.0 (D).
spect to molecular mass, pH, optimum reaction conditions, stability and product analysis of the enzyme reaction.

In this study, CGTase from B. brevis CD162 has the merit of producing relatively large amounts of γ-CD compared to other Bacillus CGTases [5,16,18]. Also, the transformant harboring pCGMH164 extracellularly expressed CGTase as in its parental strain. For further industrial application, overexpression and protein engineering of our CGTase are being studied.

References