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ORIGINAL PAPER

Cloning, extracellular expression and characterization of a predominant β-CGTase from *Bacillus* sp. G1 in *E. coli*

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Abstract The cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) gene from *Bacillus* sp. G1 was successfully isolated and cloned into *Escherichia coli*. Analysis of the nucleotide sequence revealed the presence of an open reading frame of 2,109 bp and encoded a 674 amino acid protein. Purified CGTase exhibited a molecular weight of 75 kDa and had optimum activity at pH 6 and 60°C. Heterologous recombinant protein expression in *E. coli* is commonly problematic causing intracellular localization and formation of inactive inclusion bodies. This paper shows that the majority of CGTase was secreted into the medium due to the signal peptide of *Bacillus* sp. G1 that also works well in *E. coli*, leading to easier purification steps. When reacted with starch, CGTase G1 produced 90% β-cyclodextrin (CD) and 10% γ-CD. This enzyme also preferred the economical tapioca starch as a substrate, based on kinetics studies. Therefore, CGTase G1 could potentially serve as an industrial enzyme for the production of β-CD.

Keywords *Bacillus* sp. G1 · Cyclodextrin · Cyclodextrin glucanotransferase · Extracellular expression · Predominant β-CGTase · Signal peptide

Abbreviations

CGTase	Cyclodextrin glucanotransferase
CD	Cyclodextrin

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Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19) is an industrially important enzyme that produces α-, β- and γ-CDs from starch. The α-, β- and γ-CD have closed ring structures with six, seven and eight glucose units, respectively, joined by α-1,4-glucosidic bonds. The doughnut-shaped CDs have an interior portion that is relatively apolar compared to water, and thereby can easily form inclusion complexes with many organic substances. CDs can therefore change the physicochemical properties of the guest molecule, such as its solubility and stability. As a result, CDs are extensively used in agriculture, chemicals, cosmetics, food and pharmaceuticals.

Cyclodextrin glucanotransferase has been discovered in a great number of *Bacillus* [2, 3], *Thermoanaerobacter* sp. [40], *Brevibacterium* [23] and *Thermoanaerobacterium* [11]. Most of the CGTase producing bacteria are naturally from the *Bacillus* genus and most of these *Bacillus* species produce the CGTase enzyme extracellularly [41], due to the functioning of signal peptide.

In the cyclization reaction, starch is cleaved and the intramolecular ends are joined to form closed circular structures. In addition to catalyzing this intramolecular reaction, CGTase is also involved in intermolecular transglycosylation (coupling and disproportionation) reactions. In general, CGTase also has weak hydrolytic action on starch.

Cyclodextrin glucanotransferases usually produce a mixture of α-, β-, and γ-CD, and the product ratio can vary depending on the source of bacteria, reaction time and conditions [39]. The type of CGTase producer can be classified as α-CGTase, β-CGTase or γ-CGTase, depending on the major type of CD produced by the CGTase reaction. All wild type CGTases are able to produce β-CD, however typically it is produced as part of a mixture of CDs. Qingsheng and Zimmermann [30] listed 31 different CGTases, only two of which served as strictly predominant β-CD producers. The results of this paper show that CGTase from *Bacillus* sp. G1 produced β-cyclodextrin predominantly. Additionally, under experimental conditions, no α-CD was formed. The yield could be further increased to 100% β-CD with the addition of 4% (v/v) Triton X-100 [33]. This interesting property makes CGTase from *Bacillus* sp. G1 industrially favorable for β-CD manufacturing. Expensive separation techniques are usually applied to isolate individual cyclodextrins from a mixture of α-, β-, and γ-CD. Since the proportion of β-CD produced is high, one attractive feature of CGTase from *Bacillus* sp. G1 would be to ease β-CD purification. Therefore it was necessary to clone and sequence the gene of this unique CGTase, and express it extracellularly using *E. coli* as host with the original *Bacillus* sp. G1 signal peptide, and to characterize the enzyme. The CGTase gene was expressed in *E. coli* at 37°C, and the majority of CGTase that was secreted into the medium was functionally active. This interesting excretory ability was accomplished by the *Bacillus* sp. G1 signal peptide which works well in *E. coli*, leading to easier purification steps. Heterologous recombinant protein expression in *E. coli* is commonly problematic causing intracellular localization and the formation of inactive inclusion bodies. This paper shows that this problem can be overcome by the *Bacillus* signal peptide.

Materials and methods

Bacterial strains and plasmids

The alkalophilic bacteria identified as *Bacillus* sp. G1 was isolated from local soil [7]. *Escherichia coli* JM109 [*end*A1, *rec*A1, *gyr*A96, *thi, hsd* R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ (*lac-pro*AB), (F′, *tra*D36, *pro*AB, *lac*I^{q}ZΔ M15)] was used as the host strain for expression purposes. The transformed cells were grown overnight in Luria-Bertani (LB) medium containing 100 μg/mL ampicillin at 37°C. Plasmid pTZ57R/T and pUC19 (Fermentas, Glen Burnie, MD, and Promega, Madison, WI) were used as cloning vectors.

DNA manipulation and cloning procedures

The genomic manipulation of *Bacillus* sp. G1 was done according to the method of Ish-Horowitz and Burke [8]. DNA manipulations were performed according to the standard methods as described by Sambrook and Russell [32]. Degenerate oligonucleotides were designed based on conserved sequences among CGTases. The forward primer C1 was 5′-GGN GGN GAY TGG CAR GGN-3′ corresponded to the region ^{76}GGDWQG while the reverse primer C2 was 5′-CAT RTC RTG RTT RTC DAT RAA-3′ corresponded to ^{346}IDNHDMS (Fig. 1). The reaction contained 1 μg of *Bacillus* sp. G1 DNA, 200 pmol of each forward and reverse primer, 0.01 U/μL *Taq* Polymerase in 1× reaction buffer, 1.5 mM $MgCl_2$ and 0.2 mM of each dNTPs.

The PCR product was cloned into pTZ57R/T and was used as a probe to detect the CGTase gene by Southern hybridization. An approximately 6 kb *Xba*I fragment of the *Bacillus* sp. G1 genomic DNA was detected (data not shown). Both the genomic DNA from *Bacillus* sp. G1 and the cloning vector pUC19 were digested with *Xba*I and then ligated. The ligation products were used to transform *E. coli* JM109 cells. All the white colonies were tested by the agar plate assay method for their ability to form clear zones, indicating expression of the CGTase starch degrading activity. The agar plates contained 1% soluble starch and 100 μg/mL ampicillin in LB medium.

Nucleotide and protein sequence analysis

The search for existing sequences similar to the sequence obtained was done through the blastn and blastx programs provided by National Center for Biotechnology Information (NCBI) [1]. Nucleotide and amino acid sequences were compared with other sequences in GenBank and other databases using Clustal X [37].

Assay of enzyme activity

The cyclization activity of CGTase was measured according to the method established by Kaneko et al. [14] with slight modification. The reaction mixture containing 40 mg of soluble starch in 1.0 mL of 0.1 M phosphate buffer (pH 6.0) and 0.1 mL of enzyme solution was incubated at 60°C for 10 min. The reaction was stopped by the addition of 3.5 mL of 30 mM NaOH. Then, 0.5 mL of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 was added to the mixture. After 15 min, the reduction in colour intensity was

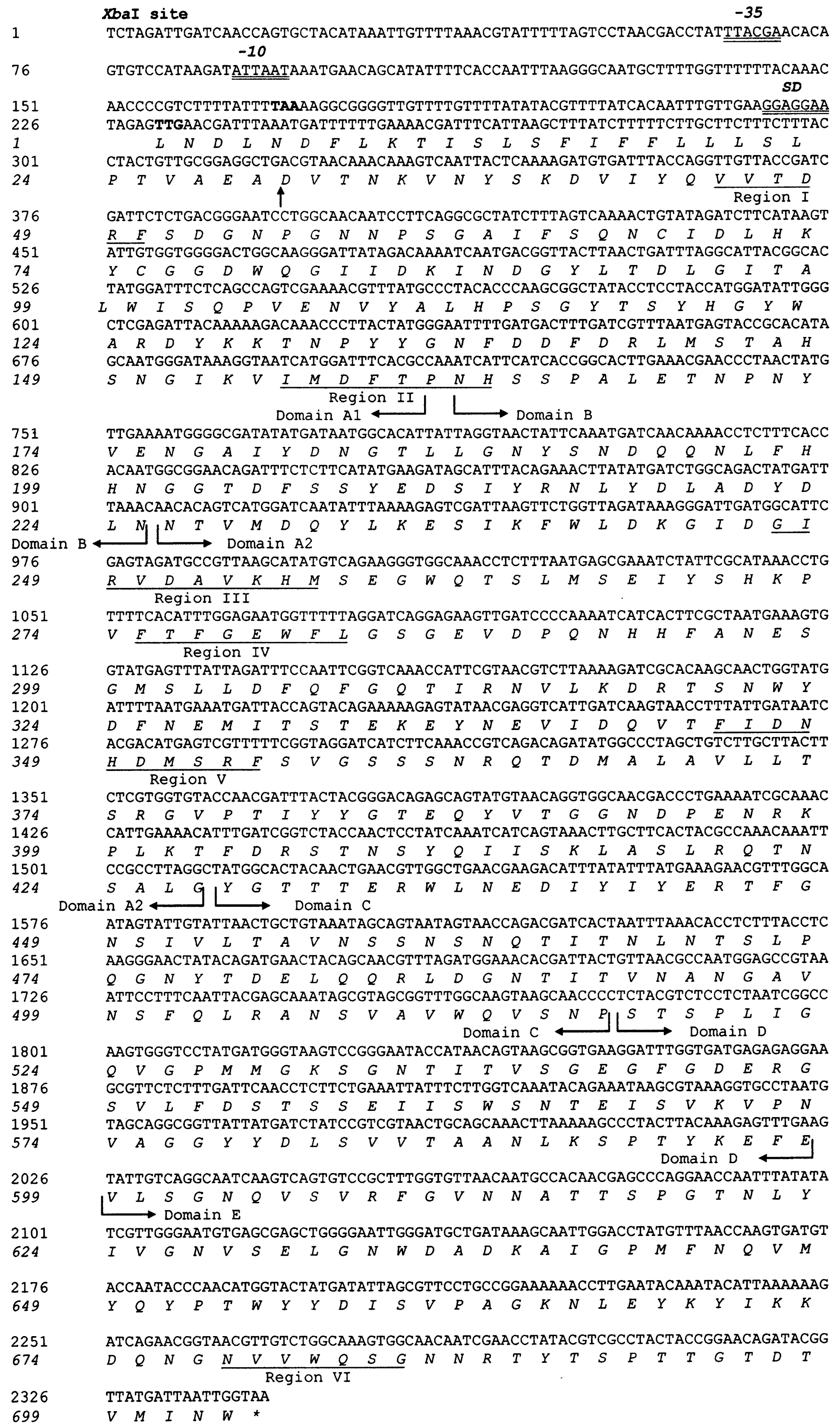

Fig. 1 Nucleotide sequence and deduced amino acid sequence of CGTase of *Bacillus* sp. G1. The flanking regions of the nucleotide sequence of the CGTase gene are shown. The possible -35 and -10 sequences in the promoter region and the possible ribosome-binding site, SD, are *double underlined*. The possible signal peptide cleavage site is indicated by a . The six highly conserved regions in different CGTases are *overlined*. The accession number in Genbank is AY770576

measured at 550 nm. One unit of enzyme activity was defined as the amount of enzyme which formed 1 μmol of β-CD per minute under the conditions mentioned above.

BCG assay [15] with modification was used to determine γ-CD cyclization activity. The assay was conducted by reacting 200 μL CGTase sample (or γ-CD standard) with 1 mL of 4% soluble starch in phosphate buffer (pH 6). After 20 min of incubation in 60°C water bath, 500 μL of 0.2 M HCl was added to stop enzymatic reaction. Subsequently, 200 μL of 0.05% (w/v) bromocresol green in 20% ethanol was added and left at room temperature for 20 min. After pipetting 2 mL of 1 M acetate buffer containing 30 mM citric acid (pH 4.2), samples were read at A_{630}. One unit of γ-CD forming activity was defined as the amount of enzyme that produced 1 μmol of γ-CD per min under these conditions.

Protein determination

Protein concentration was quantified by the method of Bicinchoninic Acid Assay using Pierce BCA Protein Assay^{TM} (Rockford, IL) with bovine serum albumin as the standard.

Cellular localization

The determination of CGTase localization was carried out according to Lee and Tao [20]. Culture was grown for 24 h in 200 mL LB/amp at 37°C. Cells were pelleted down by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was assayed for extracellulary enzyme. Cells were washed twice with half the volume of the broth culture using 0.9% (w/v) NaCl and then with the same volume of 25% (w/v) sucrose with 1 mM EDTA, and left for 10 min at room temperature. Cells were centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatant was assayed for periplasmic fraction enzyme activity. The cells were again suspended using 0.1 M phosphate buffer (pH 6.0) in the presence of 1 mM PMSF and EDTA to inhibit possible protease degradation. The intracellular fraction was the supernatant obtained from the sonication step after centrifugation.

Purification of recombinant CGTase

The method used to purify the recombinant G1 CGTase was similar to the procedure used to purify the wild type CGTase [33]. Crude enzyme was concentrated by ammonium sulfate precipitation at 70% saturation. The recombinant CGTase was subsequently purified using a α-CD-Sepharose 6B column. Bound CGTase enzyme was eluted with 1% α-CD in loading buffer. The eluted enzyme was then extensively dialyzed in 10,000 MWCO snake skin dialysis tubing (Pierce) against 0.1 M phosphate buffer (pH 6). The molecular weight of the purified enzyme was determined by SDS-PAGE.

Optimum pH and temperature

The optimum pH of the purified recombinant enzyme was determined by replacing 0.1 M phosphate buffer (pH 6.0) in the CGTase assay with the following buffers: 0.1 M sodium acetate buffer (pH 4–5), 0.1 M sodium phosphate buffer (pH 6–8) and 0.1 M glycine-NaOH buffer (pH 9–10). The reactions were carried out using the CGTase assay procedure mentioned above. The optimum temperature of the pure enzyme was determined by incubating the CGTase assay at different temperatures, ranging from 30–90°C for 10 min. Then the subsequent steps were done according to the CGTase assay described above.

pH and thermal stability

The pH stability of the recombinant enzyme was measured by incubating 0.1 mL purified enzyme with 0.2 mL of 0.1 M sodium acetate buffer (pH 4–5), 0.1 M phosphate buffer (pH 6–8) 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 an equal volume of 0.1 M sodium phosphate buffer, (pH 6.0), at different temperatures (40-90°C) for 30 min. Residual activities were measured with the standard CGTase assay as mentioned above.

Kinetic parameters

The K_m and V_{max} values for the pure enzyme were determined by incubating 0.1 mL purified CGTase with starches (soluble, potato, corn and tapioca) in 1 mL of 0.1 M phosphate buffer (pH 6.0). The assay was performed according to the standard enzyme assay method. K_m and V_{max} values were then determined from Hanes–Woof plot.

Analysis of cyclodextrins by high performance liquid chromatography (HPLC)

Approximately 8 U of purified CGTase and 1 mL 1% tapioca starch were incubated at the optimum temperature (60°C) and pH (phosphate buffer pH 6.0) for different durations, and then boiled for 10 min to stop the enzymatic reaction. The ratio of different cyclodextrins produced was analyzed using a Waters HPLC (Milford, MA) system with separation carried out using the Asahipak NH2P-504E column. Isocratic flow was set at 1 mL/min with 70:30 acetonitrile-water as mobile phase.

Results and discussion

Cloning of the CGTase gene from alkalophilic *Bacillus* sp. G1

PCR products of approximately 800 bp were successfully amplified from the genomic DNA of *Bacillus* sp. G1. Sequence analysis of the 831 bp nucleotide sequence, designated PP2, demonstrated that this product corresponded to 277 deduced amino acids. The sequence was subjected to a search against all known sequences in databases using blastn and blastx searches (data not shown). The amino acid sequence of PP2 exhibited high similarity with other CGTases, showing the highest similarity of 99% with *Bacillus* sp. KC201 CGTase [18].

Southern blotting was carried out with the genomic DNA of *Bacillus* sp. G1 digested with several restriction enzymes (result not shown). When probed with the 831 bp fragment (PP2), the results indicated that *Xba*I was the most suitable restriction enzyme for the construction of a genomic library for further full length CGTase gene screening. The *Xba*I digested fragments of genomic DNA from alkalophilic *Bacillus* sp. G1 were inserted into the *Xba*I site of pUC19 and then transformed into *E. coli* JM109 competent cells. One thousand white colonies were screened, and the positive colony was subjected to CGTase assay, which showed that the transformant possessed CGTase activity. The plasmid isolated from the transformant contained an approximately 5 kb fragment in the vector plasmid pUC19.

Nucleotide and amino acid sequences of the CGTase

Analysis of the nucleotide sequence of the cloned fragment revealed a large, uninterrupted, single open reading frame (ORF) encoding 2,109 bp, starting from nucleotide 231 to nucleotide 2,339. Interestingly, TTG, rather than ATG, appeared to be the start codon for this ORF. ATG at nucleotide 129 was initially thought to be the start codon, however, a stop codon (TAA) was identified at nucleotide 168 (Fig. 1) in a frame with the gene. This revealed that the aforementioned ATG could not possibly function as the starting codon. Often ATG is regarded as the "normal" start codon, whilst many organisms sometimes use other start codons as well, such as GTG, CTG or TTG. In some cases, especially in bacteria, these and other start codons can be utilized quite frequently [10]. Initiation codon ATG, GTG or CTG were not found downstream of the stop codon described above. However, three TTG codons could be identified (nucleotide 180, 231 and 252). The identification of the deduced start codon was aided by a Shine-Dalgarno (SD) sequence. GGAGGAA is believed to be the SD sequence as it is totally complementary to the 3′ end of 16S rRNA of *Bacillus* sp. G1. In bacteria, translation starts when ribosomes bind to the ribosome-binding site (also known as the SD sequence) which is normally adjacent to the start codon. In this case, the SD sequence was located 6 bp upstream from the second TTG. Due to this, the second TTG at nucleotide 231, which was adjacent to the SD, was hypothetically identified as the initiation codon.

TTG is known to function as an initiation codon in *E. coli*, *Bacillus subtilis* and other prokaryotes [24, 31]. The distribution of start codons in *Bacillus subtilis* is 79.9% for ATG, 11.2% for TTG and lastly, 8.9% for GTG [6]. Interestingly, TTG functions as an initiator codon for CGTase genes also reported from *Bacillus* sp. A2-5a, *Bacillus ohbensis,* and *Bacillus* sp. E1 [27, 34, 41].

A putative promoter (TTACGA for the -35 region and ATTAAT for the -10 region) was observed. A palindromic sequence that could form a stable stem-and-loop structure (nucleotides 2,357 through 2,389) was 14 bp downstream of the stop codon of the ORF. The predicted signal peptide of the CGTase gene from *Bacillus* sp. G1 is from nucleotide 231 to 317. This region preceding the N-terminus of the mature enzyme showed the characteristics of a typical signal peptide. For a probable cleavage site to be acceptable, the residue in position -1 must be small (either Ala, Ser, Gly, Cys, Thr or Gln) and the residue in position-3 must not be aromatic (Phe, His, Tyr, Trp), charged (Asp, Glu, Lys, Arg) or large and polar (Asn, Gln). The amino acid sequence of this signal peptide consists of several positively charged amino acids followed by a run of core hydrophobic amino acids and a COOH-terminal alanine residue, and is consistent with the signal peptides from other Gram-positive bacteria [13, 17, 35]. The nucleotide sequence data of the isolated CGTase gene was submitted to the NCBI databases with accession number AY770576.

Comparison of amino acid sequence of *Bacillus* sp. G1 CGTase with other CGTases

The amino acid sequences derived from the cloned CGTase gene of *Bacillus* sp. G1 showed a significantly high similarity of 99.1% and identity of 99% with amino acid of CGTase from alkalophilic *Bacillus* sp. KC201. CGTase from *Klebsiella pneumoniae* Ma51 showed only 33.2% similarity and 28% identity with the sequence from *Bacillus* sp. G1. This was expected as the amino acid sequence of *Klebsiella pneumoniae* Ma51 CGTase showed only approximately 30% similarity with enzymes from *Bacillus*. Five domains and six highly conserved regions (labelled I–VI) could be identified in the amino acid sequence of *Bacillus* sp. G1. Three carboxylic acid groups, one glutamic acid and two aspartic acid residues are essential catalytic residues for CGTases. These residues in CGTase from *Bacillus* sp. G1 are Glu250, Asp222, and Asp321, which

are equivalent to Glu257, Asp229, and Asp328 in CGTase from *Bacillus circulans* strain 251 [19, 25].

Expression and localization of CGTase in *E. coli*

It was reported that most CGTases from *Bacillus* sp. expressed in *E. coli* end up in the periplasm rather than being secreted out to the culture medium due to a spatial barrier, i.e. an outer membrane [18, 20, 26, 34]. The cellular localization of the recombinant *Bacillus* sp. G1 CGTase expressed in *E. coli* was examined according to Lee and Tao [20]. About 62.3% of the expressed recombinant CGTase G1 was secreted out to the extracellular medium (Table 1). This is possibly due to the presence of a signal peptide preceding the CGTase gene encoding the mature CGTase protein. The signal peptide seems to work quite well in the *E. coli* host used to express the CGTase gene. Some of the enzyme activity was also observed in the periplasmic and the intracellular fractions. When the mature CGTase G1 gene (without signal peptide) was cloned into pQE and pWH expression vectors and transformed into *E. coli*, almost all of the CGTase activity was detected intracellularly (data not shown). It is interesting to note that even though CGTase from *Bacillus* sp. KC201 CGTase showed the closest similarity to *Bacillus* sp. G1 CGTase, over 60% of the enzyme activity of the recombinant CGTase from *Bacillus* sp. KC201 expressed in *E. coli* was found in the periplasmic fraction, in contrast to the CGTase from *Bacillus* sp. G1 expressed in *E. coli*. The authors eliminated the difference in extracellular expression levels due to protein folding, the number of disulfide bonds, and rare codon issues as possible causes for this discrepancy, since both CGTases are very similar in primary structure. Alignment of the signal peptide of both CGTases revealed that an identical sequence was present in the hydrophobic and carboxyl-terminal, but great variation was seen in amino region (Fig. 2).

Generally, growing *E. coli* at 37°C can cause proteins to accumulate as inclusion bodies. A common practice for expressing soluble protein using *E. coli* as a host is to lower the cultivation temperature to 15–20°C. Nevertheless, temperature shifting might not give an optimal yield of soluble protein and valuable time is lost due to prolonged incubation. A related work was published by Jeang et al. [9] wherein mature CGTase from *Bacillus macerans* was expressed in the *E. coli* system. Cells were grown at 20°C and no CGTase activity was found in culture medium, and inclusion bodies were claimed to form at higher cultivation temperatures. In this paper, all expression was carried out at 37°C and most of the enzyme activity was found in culture medium because of export assisted by the signal peptide. No obvious improvement in the expression level was seen at 35 or 30°C compared to 37°C. Growing the cells at temperatures lower than 30°C requires a prolonged incubation time to reach the same expression level as growing the cells at 37°C. The signal peptide from *Bacillus* sp. G1 seemed to work very well in *E. coli*.

Most of the cloned CGTases from other strains were found to be secreted into the periplasm of the host, with the exception of *Bacillus* sp. E1, *Brevibacillus brevis* CD162 and *Bacillus firmus* var. *alkalophilus* CGTases. Approximately 68.5% of the recombinant CGTase from *Bacillus* sp. E1 expressed in *E. coli* was secreted into the culture medium [41], while recombinant CGTase from *Brevibacillus brevis* CD162 [16] and *Bacillus firmus* var. *alkalophilus* CGTase [28] expressed in *E. coli* secreted 74.3 and 67.0% of the recombinant protein, respectively, into the culture medium. Figure 2 shows the different regions of signal peptides from selected CGTases.

Table 1 Distribution of CGTase activity in different fractions of *E. coli* harboring the CGTase gene from *Bacillus* sp. G1

	Cell fraction		
	Extracellular	Periplasm	Intracellular
CGTase activity (unit/ml)	35.71	14.00	7.63
Fractions of total activity (%)	62.28	24.42	13.30

CGTase activity on the basis of culture broth

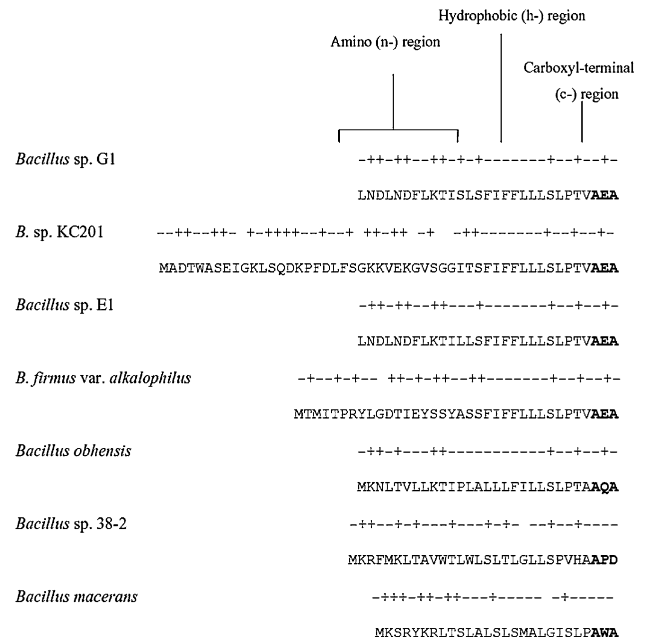

Fig. 2 Identifiable domains of the signal peptide of secretory proteins—the amino (n-) region, the hydrophobic (h-) region and the carboxyl-terminal (c-) region in selected CGTases. The hydrophilic amino acids are marked (*plus*) and the hydrophobic amino acids are marked (*minus*). The amino acids indicated in bold are the typical Ala-X-Ala cleavage site

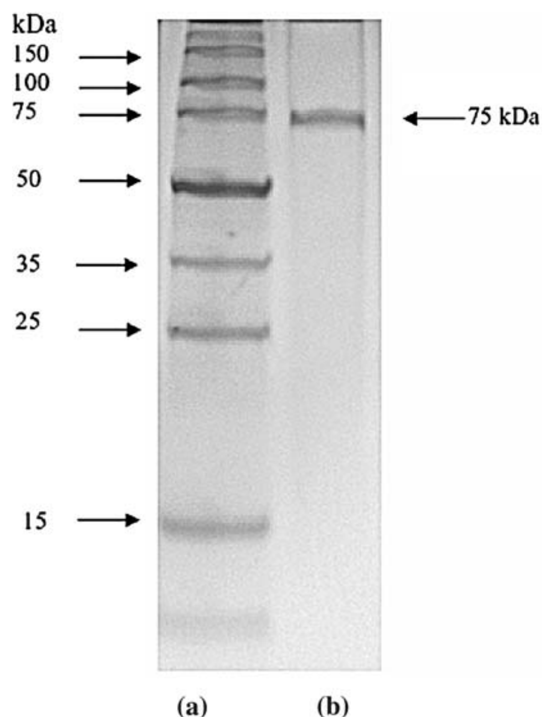

Fig. 3 SDS PAGE. **a** Promega Broad Range MW Markers. **b** Recombinant CGTase

Purification and characterization of recombinant G1 CGTase

Recombinant CGTase G1 was purified using a α-CD Sepharose column. A single protein band was observed by SDS-PAGE with an estimated size of 75 kDa (Fig. 3), which was in good agreement with wild type *Bacillus* sp. G1 CGTase [33]. The theoretical molecular weight calculated from amino acid sequence using DNASIS MAX 1.0 was 75,389 Da. Globular size of the recombinant enzyme was found to be 79.2 kDa using a Superdex 75 gel filtration chromatography column. A similar size was determined for the wild type enzyme.

The purified enzyme had the highest β-CD cyclization activity at 60°C in kalium phosphate buffer (pH 6.0), using soluble starch as a substrate (Fig. 4). Further studies were conducted using buffers which have good buffering capacity at (pH 6.0) based on their pK_a value for reactions. Approximately 50% of the CGTase activity decreased when citric buffer was used compared to phosphate buffer. However, sodium citrate buffer pH 6.0 was extensively used in other CGTase assays, including CGTase from *Bacillus circulans* 251 and *Thermoanaerobacterium thermosulfurigenes* EM1 [29, 40]. For reactions carried out using MES buffer, less than a 10% decrease in CGTase activity was observed. In another case, it was also reported that the activity and product specificity of commercial CGTase Toruzyme® could be altered easily using simple buffer changes [12].

The recombinant enzyme was found to be not stable in acidic conditions. At pH 4.0 the enzyme completely lost its ability to produce β-CD. Since the wild type protein was originally from alkalophilic bacterial, the recombinant enzyme retained activity at higher pH conditions. The recombinant CGTase G1 was stable over a wide pH range (pH 6.0–10.0) with a gradual loss of activity at acidic pH. It had a half-life of approximately 30 min at 60°C, and the cyclization activity was decreased by almost 80% at 70°C. CGTase from *Thermoanaerobacterium thermosulfurigenes* strain EM1 (*Tabium*) has the highest thermostability with a half-life of 15 min at 90°C [40]. Other CGTases from mesophilic bacterial such as BC 251 have a half life of 9.7 min at 60°C [21], and the half life for CGTase from *Brevibacillus brevis* CD162 and *Bacillus ohbensis* was 30 min at 55°C [16, 34]. It was found that the recombinant CGTase G1 is slightly more thermostable compared to most of the CGTases originally produced by mesophilic strains.

The kinetic parameter K_m for recombinant CGTase *Bacillus* sp. G1 was calculated as 0.47 mg/mL when soluble starch was used as the substrate. The higher K_m values indicated that the recombinant enzyme had a relatively low affinity to soluble starch, comparatively to potato, corn and tapioca starch (Table 2). Different K_m values have been reported for various CGTases, namely recombinant CGTase *Thermococcus* sp. B1001, 1.45 mg/mL [38]; *B. firmus*, 1.21 mg/mL [5]; *K. pneumoniae* AS-22, 1.35 mg/mL [4]; and *Bacillus agaradhaerens*, 21.2 mg/mL [22]. As shown in Table 2, it was estimated that when using different types of starches, the turnover number (k_{cat}) varies slightly except when tapioca starch was used. Turnover number of the CGTase enzyme represents the maximum number of β-CD cyclization reactions per active site per second. The data in Table 2 also shows that the efficiency (specific constant k_{cat}/K_m equal to 1,000) of CGTase G1 to convert starch to β-CD was best achieved when tapioca starch was utilized. When soluble starch and potato starch were used, the k_{cat}/K_m value were almost the same (36 and 39 respectively). However, the value doubled when corn starch was utilized as a substrate. The k_{cat}/K_m value indicates the energy level of the transition-state with respect to the free enzyme and free substrate. Lower transition-state energy results in a higher k_{cat}/K_m value. This simply means that CGTase G1 can utilize tapioca starch more efficiently compared to other starches. In other words, the conversion (percentage of CD produced per mg of substrate) from starch to cyclodextrin would be higher when tapioca is used. In most β-CD producing processes, potato and corn starch are generally used, however, the global price for these starches is rising. Since CGTase G1 reacted most

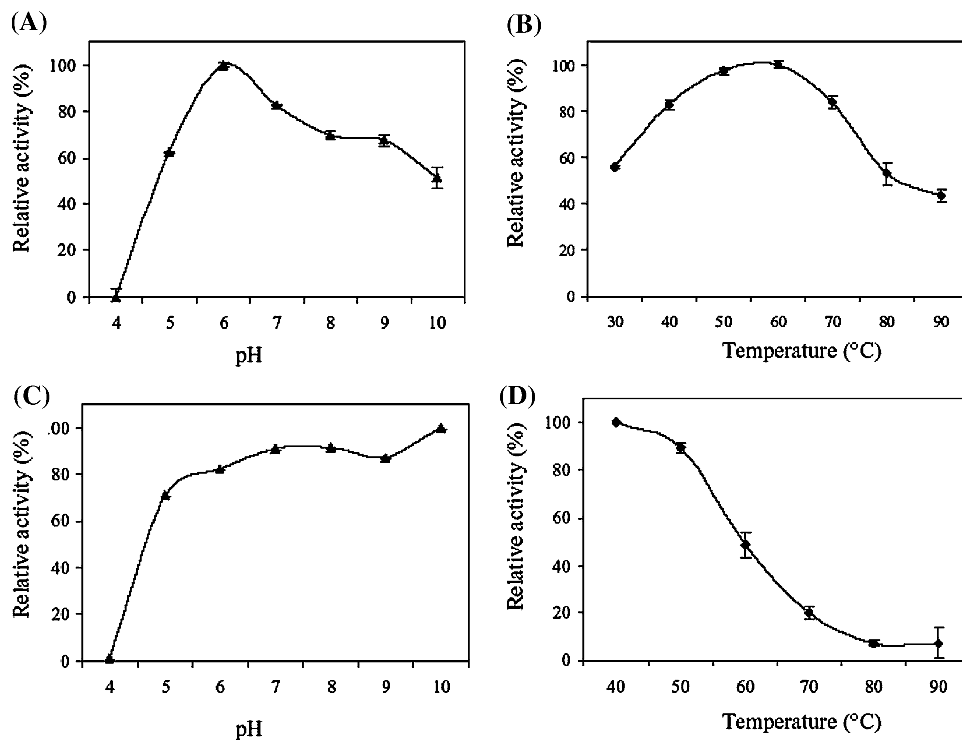

Fig. 4 Properties of recombinant CGTase produced by *E. coli*. **a** Optimum pH. **b** Optimum temperature. **c** pH stability. **d** Thermal stability

Table 2 Effects of different starches to the kinetic parameters of recombinant CGTase G1

Starch type	K_m (mg/ml)	V_{max} (mg β-CD/ml per min)	k_{cat} (s^{-1})	Efficiency k_{cat}/K_m
Soluble	0.47	64.1	17	36
Potato	0.38	56.5	15	39
Corn	0.17	57.5	15	87
Tapioca	0.02	53.3	20	1,000

efficiently with tapioca starch, a less expensive starch, this characteristic would probably make the enzyme attractive in a production scale.

Product specificity analysis with HPLC

Purified recombinant CGTase was reacted with 1% tapioca starch at optimum pH and temperature for various periods, and the reactions were stopped by boiling. Cyclodextrins that were produced were analyzed by HPLC. The results in Fig. 5 showed that β-CD was predominantly produced. HPLC results also revealed that the amount of γ-CD was higher at the beginning of the time course, and eventually decreased in prolonged incubations. Changes in the thermodynamic equilibrium of balancing hydrolysis activity and cyclization reactions for β-CD and γ-CD might be a possible explanation [36]. Recombinant G1 CGTase might be able to hydrolyze γ-CD in the reverse mode to cyclization, also known as a coupling reaction. After 16 h of reaction time, approximately 90% of the total cyclodextrins produced was β-CD, and 10% was γ- CD. We emphasize again that no α-CD was produced using CGTase G1. This interesting property makes CGTase from *Bacillus* sp. G1 industrially favorable for β-cyclodextrin manufacturing.

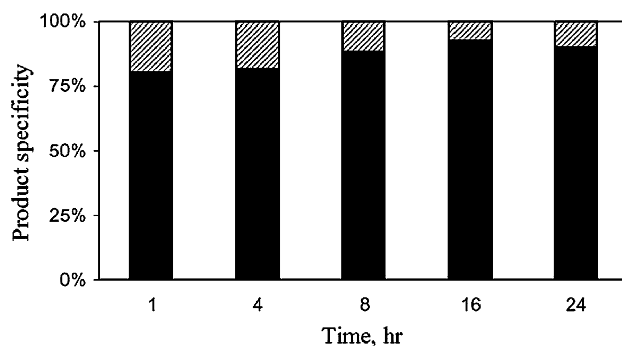

Fig. 5 CD production time course. The reaction was carried out at 60°C in 0.1 M phosphate buffer. Only β-CD and γ-CD were produced with no trace of α-CD detected (*filled square* β-CD, *square with diagonal* γ-CD)

Conclusion

The CGTase gene from alkalophilic *Bacillus* sp. G1 has been successfully cloned and expressed in pUC19. With the assistance of a signal peptide, the recombinant CGTase was expressed extracellularly into the culture medium, suggesting

that *Bacillus* sp. G1 is a good model for further studies on the mechanism of secretion of CGTase from *E. coli*. The elucidated characteristics of the enzyme indicated that this enzyme would be suitable to applications producing β-CD.

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