Construction of a Chimeric Series of Bacillus Cyclomaltodextrin Glucanotransferases and Analysis of the Thermal Stabilities and pH Optima of the Enzymes

By TAKAHIRO KANEKO,* KI-BANG SONG,** TETSUO HAMAMOTO, TOSHIAKI KUDO and KOKI HORIKOSHI

Laboratory of Applied Bacteriology, The Riken Institute, Wako-shi, Saitama-ken 351-01, Japan

(Received 21 February 1989; revised 23 August 1989; accepted 4 September 1989)

The cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) gene from the alkalophilic Bacillus sp. strain no. 17-1 was cloned in Escherichia coli. The cloned CGTase gene consisted of a single open reading frame which would encode a polypeptide of 713 amino acids, and the first 27 amino acid residues comprised a signal peptide. The nucleotide sequence and the amino acid sequence of this CGTase (CGTase 17-1) gene had strong homology with those of the CGTase (CGTase 38-2) gene previously cloned in our laboratory from the alkalophilic Bacillus sp. strain no. 38-2, although the enzymic properties of the CGTase 17-1 were distinct from those of the CGTase 38-2. To analyse those enzymic properties further, we constructed 12 chimeric CGTases using three restriction nuclease sites and compared the enzymic properties of the chimeric CGTases. The N-terminal part of the enzyme was important for heat stability, and the pH-activity profile was influenced by both the N- and the C-terminal parts. A third segment was less important for enzymic properties.

INTRODUCTION

Cyclomaltodextrin glucanotransferase (EC 2.4.1.19, CGTase) produces a mixture of alpha-, beta- and gamma-cyclodextrins from starch or related carbohydrates. We have reported the enzymic properties of a CGTase produced by the alkalophilic Bacillus sp. strain no. 38-2 (Nakamura & Horikoshi, 1976), and analysed the nucleotide sequence of the CGTase (CGTase 38-2) gene from this strain (Kaneko et al., 1988). We have also isolated a CGTase (CGTase 17-1) from alkalophilic Bacillus sp. strain no. 17-1 (Yamamoto et al., 1972). Here we report the cloning of the CGTase gene from strain 17-1. We compare the gene and amino acid sequences of CGTase 38-2 and CGTase 17-1, and their properties, and describe the construction of chimeric enzymes using the CGTase genes from the two strains. Through analysis of the properties of the chimeric enzymes, the role of various enzyme segments in the structure and function of CGTase 38-2 and CGTase 17-1 proteins were examined.

METHODS

Bacterial strains and media. Alkalophilic Bacillus sp. strain no. 17-1 (ATCC 31007) was isolated in our laboratory from soil (Yamamoto et al., 1972). Escherichia coli K12 strain HB101 (F- hsd-20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44), was used as a host strain (Messing et al., 1981). The alkaline medium (pH 10) contained 1% (w/v) soluble starch, 0-5% yeast extract, 0-5% polypeptone, 0-1% K2HPO4, 0-02% MgSO4, 0-1M, and (sterilized separately) 1% Na2CO3. LB-starch medium (Maniatis et al., 1982) containing 0-2% soluble starch was used for growth of E. coli.

DNA isolation and cloning. Bacillus sp. 17-1 was grown aerobically at 37 °C in the alkaline medium described above. Chromosomal DNA was isolated by the method of Saito & Miura (1963). Plasmid DNA was purified by the method of Bolivar et al. (1977). A boiling method was used for rapid isolation of plasmids (Davis et al., 1980).

ABBREVIATION: CGTase, cyclomaltodextrin glucanotransferase.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M28053.
The cloning strategy was as follows. After complete digestion with PstI, plasmid pUC19 DNA (1 µg) and chromosomal DNA (3 µg) were mixed and ligated with T4 DNA ligase. The ligated DNA was used to transform E. coli HB101 by the method of Lederberg & Cohen (1974). The transformants were plated on the LB-starch plates, containing 50 µg ampicillin ml⁻¹. CGTase activity was detected as starch hydrolytic activity on LB-starch agar plates using I₂/KI indicator solution.

**Amino acid sequence and amino acid composition.** The CGTase enzyme was purified as described by Nakamura & Horikoshi (1976). The N-terminal amino acid sequence was determined by the method of Edman & Henschen (1975) using a Protein/Peptide Sequencer model 477A, and the PTH analyser model 120A system (Applied Biosystems). Amino acid composition was determined as described by Spackman et al. (1958) with a Hitachi amino acid analyser, model 835, after hydrolysis with 6 M-HCl (Moore & Stein, 1963).

**DNA sequencing.** Sequencing was done using the diideoxy chain-termination reaction (Sanger et al., 1977) by the exonuclease deletion method (Henikoff, 1984) with pUC18 and pUC19.

**Construction of the chimeric enzymes.** Plasmid pUCP1, containing the CGTase gene from alkalophilic Bacillus sp. 38-2, was obtained in the present study (see Results and Fig. 1). The 3.6 kb HindIII fragment containing the CGTase gene from alkalophilic Bacillus sp. 38-2 (Kaneko et al., 1988) was inserted into pUC19, the orientation of the CGTase gene being the same as that of pUCP1. The resulting plasmid (CGTase⁺, Ap+', 0.6 kb) was designated pCS100.

Using three aligned restriction nuclease sites, the NdeI, Eco47III and BalI sites in both the CGTase 17-1 gene (pUCP1) and the CGTase 38-2 gene (pCS100), 12 chimeric CGTase genes were constructed (see Figs 5 and 6). Plasmid pCGT1 contained a chimeric gene (SUUU). 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively. For example, 'SUUU' indicates the chimeric enzyme in which the N-terminal segment was derived from CGTase 38-2 gene and the second, third, and fourth segments were derived from CGTase 17-1. Plasmid pCGT1 was constructed by ligation of a 3.6 kb NdeI(1)–NdeI(II) fragment [N-terminal segment (IS) of the CGTase 38-2 gene and most of plasmid pUC19] of pCS100 and a 2.3 kb NdeI(2)–NdeI(3) fragment [the second, third and fourth segments (2U, 3U, 4U) of the CGTase 17-1 gene] of pUCP1.

Plasmid pCGT2 (SSSU) was constructed by ligation of a 4.7 kb Eco47III(II)–Eco47III(I) fragment (1S, 2S) of pCS100 and a 3.0 kb Eco47III(I)–SmaI fragment (3U, 4U) of pUCP1. Plasmid pCGT3 (SSSU) was constructed by ligation of a 4.6 kb SmaI–BalI fragment (1S, 2S, 3S) of pCS100 and a 2.0 kb BalI(2)–BalI(3) fragment (4U) of pUCP1. Plasmid pCGT4 (SSUS) was constructed by ligation of a 5.7 kb BalI(3)–BalI(2) fragment (1S, 2S, 3U) of pCGT2 and a 1.5 kb BalI–SmaI fragment (4S) of pCS100. Plasmid pCGT5 (USSS) was constructed by ligation of a 4.5 kb NdeI(1)–NdeI(2) fragment (1U) of pUCP1 and 2.5 kb NdeI(II)–NdeI(I) fragment (2S, 3S, 4S) of pCS100. Plasmid pCGT6 (USSU) was constructed by ligation of a 5.5 kb SmaI–BalI fragment (1U, 2S, 3S) of pCGT5 and a 2.0 kb BalI(2)–BalI(3) fragment (4U) of pUCP1. Plasmid pCGT7 (USUU) was constructed by ligation of a 4.5 kb NdeI(1)–NdeI(2) fragment (1U) of pUCP1 and a 2.3 kb NdeI(II)–NdeI(3) fragment (2S, 3U, 4U) of pUCP1. Plasmid pCGT8 (SSSU) was constructed by ligation of a 5.5 kb SmaI–BalI fragment (1U, 2S, 3U, partially digested with BalI) of pUCP1 and a 1.5 kb BalI–SmaI fragment (4S) of pCS100. Plasmid pCGT9 (USSU) was constructed by ligation of a 5.2 kb SmaI–Eco47III fragment (1U, 2S, partially digested with Eco47III) of pUCP1 and a 1.4 kb Eco47III(II)–Eco47III(I) fragment (3S, 4S) of pCS100. Plasmid pCGT10 (USUS) was constructed by ligation of a 6.8 kb BalI(3)–BalI(2) fragment (1U, 2S, 3U) of pCGT7 and a 1.5 kb BalI–SmaI fragment (4S) of pCS100. Plasmid pCGT11 (UUUS) was constructed by ligation of a 3.6 kb NdeI(II)–NdeI(I) fragment (1S) of pCS100 and a 2.5 kb NdeI(2)–NdeI(I) fragment (2S, 3U, 4S) of pCGT8. Plasmid pCGT12 (SSSU) was constructed by ligation of a 3.6 kb NdeI(II)–NdeI(I) fragment (1S) of pCS100 and a 2.1 kb NdeI(2)–NdeI(I) fragment (2S, 3S, 4S) of pCGT9.

**CGTase assay.** CGTase activity was measured as dextrinizing power using amylose as a substrate, according to the method of Fuwa (1954), with a slight modification. In the standard assay, the reaction mixture, containing 50 µl enzyme solution (suitably diluted with distilled water) and 0.3 ml 0.2% amylose in 10 mM-phosphate buffer (pH 6.0), was incubated at 60 °C for 10 min. The reaction was stopped with 0.5 ml of 1 M-acetic acid and 0.5 ml 0.02% 1,0,2-2% KI solution. Distilled water was added to make up a final volume of 10 ml, and absorbance at 700 nm was measured. One unit of enzyme was defined as the amount of enzyme which produced a 10% reduction in the intensity of blue colour of the amylose–iodine complex per minute under the conditions described.

**Enzymes and chemicals.** RNAase, lysozyme, and ampicillin (Ap) were purchased from Sigma. Restriction endonucleases, DNA polymerase I and T4 ligase were obtained from Toyobo, Osaka, Japan. The M13 sequence kit and exonuclease deletion kit were from Takara Syuzo, Kyoto, Japan. All other chemicals used were of reagent grade.

**RESULTS AND DISCUSSION**

**Cloning and sequencing of the CGTase gene from alkalophilic Bacillus sp. strain no. 17-1**

Cloning in E. coli. About 4 × 10⁴ colonies of the transformants were screened; one transformant showed amylolytic activity on LB-starch plates using the I₂/KI indicator, and
Comparison of chimeric CGTase

I

Fig. 1. Restriction map of plasmid pUCP1. Restriction sites are indicated, and their coordinates given in kb. The medium and thick lines indicate the cloned fragment from alkalophilic Bacillus sp. 17-1. The thick line indicates the fragment containing the CGTase gene.

![Restriction map](image)

Fig. 2. Strategy for sequencing the CGTase gene of alkalophilic Bacillus sp. 17-1. A detailed restriction map of the EcoRI-MboI region of pUCP1 encoding the CGTase gene and the sequencing strategy are shown. The arrows below the line represent the direction and extent of sequence determinations done by the dideoxy chain-termination method of Sanger et al. (1977). Extra MboI sites were located at positions 249, 306, 667, 676, 739, 1036, 1192, 1338, 1505, 1725, 1834, 2022 and 2203 in Fig. 3. The MboI site at position 2422 (Fig. 3) is shown here.

showed CGTase activity using a paper chromatography method (Kobayashi et al., 1984). The plasmid thus obtained was designated as pUCP1; it contained a 5.5 kb insert of Bacillus sp. 17-1 DNA. A restriction map of the 5.5 kb insert of pUCP1 is shown in Fig. 1.

To determine the origin of the DNA inserted in pUC19, the 5.5 kb insert DNA was biotinylated (Langer et al., 1981), and hybridized to PstI-digested chromosomal DNA of Bacillus sp. 17-1 and E. coli HB101, immobilized on a nitrocellulose sheet (Southern, 1975). The biotinylated segment hybridized to a 5.5 kb chromosomal DNA segment of Bacillus sp. 17-1 identical in length to that contained in pUCP1. No complementary sequences were detected in E. coli chromosomal or plasmid DNA fragments.

**DNA sequence.** We determined the DNA sequence of a 2.9 kb EcoRI-MboI region of pUCP1 (Fig. 2). The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. Analysis of the sequence showed that there was a single open reading frame of 2139 bp which could encode a polypeptide of 713 amino acids. The SD sequence AGGAGG, which is highly complementary to the 3' end of B. subtilis 16S rRNA (Murray & Rabinowitz, 1982), was observed 6 bp upstream of the initiation codon.

**Determination of amino acid composition and N-terminal amino acid sequence.** The N-terminal amino acid sequence of CGTase 17-1 was determined up to the 17th residue to be NH₂-Ala-Pro-Asp-Thr-Ser-Val-Ser-Asn-Lys-Gln-Asn-Phe-Ser-Thr-Asp-Val-Ile. This amino acid sequence was identical to that deduced from the DNA sequence starting at nucleotide position 82 in Fig. 3. The initial 27 amino acid residues (residues -27 to -1) seemed to represent a signal
Comparison of chimeric CGTase

Fig. 3. Nucleotide sequence of the CGTase gene from alkalophilic Bacillus sp. 17-1. The DNA sequence is given in the direction 5′ to 3′, numbered from nucleotide 1 at the putative initiation site. The proposed ribosome-binding site (Shine–Dalgarno, SD) is underlined with a dotted line. The predicted amino acid sequence is given below the DNA sequence. The deduced position of processing of the signal peptide is indicated by an arrow. The boxed amino acids have been determined by automated Edman sequencing of the purified CGTase.

Table 1. Amino acid composition predicted from the DNA nucleotide sequence of the CGTase from alkalophilic Bacillus sp. 17-1 compared with that determined from the purified enzyme

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>From DNA sequence</th>
<th>Amino acid analysis (mol%)</th>
<th>Amino acid</th>
<th>From DNA sequence</th>
<th>Amino acid analysis (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>31</td>
<td>4.5</td>
<td>5.2</td>
<td>Gly</td>
<td>65</td>
</tr>
<tr>
<td>His</td>
<td>13</td>
<td>1.9</td>
<td>2.1</td>
<td>Ala</td>
<td>62</td>
</tr>
<tr>
<td>Arg</td>
<td>19</td>
<td>2.7</td>
<td>3.1</td>
<td>Cys</td>
<td>2</td>
</tr>
<tr>
<td>Trp</td>
<td>13</td>
<td>1.9</td>
<td>0.2</td>
<td>Val</td>
<td>48</td>
</tr>
<tr>
<td>Asx*</td>
<td>101</td>
<td>14.7</td>
<td>14.7</td>
<td>Met</td>
<td>13</td>
</tr>
<tr>
<td>Thr</td>
<td>66</td>
<td>9.5</td>
<td>8.9</td>
<td>Ile</td>
<td>38</td>
</tr>
<tr>
<td>Ser</td>
<td>42</td>
<td>6.1</td>
<td>5.9</td>
<td>Leu</td>
<td>39</td>
</tr>
<tr>
<td>Glx*</td>
<td>43</td>
<td>6.3</td>
<td>6.0</td>
<td>Tyr</td>
<td>32</td>
</tr>
<tr>
<td>Pro</td>
<td>27</td>
<td>3.9</td>
<td>4.8</td>
<td>Phe</td>
<td>32</td>
</tr>
</tbody>
</table>

–, Not detected.
*Asx represents Asp and Asn; Glx represents Glu and Gln.

peptide which was removed during the secretion process. Thus, the mature CGTase comprised 686 amino acids and had an $M_r$ of 74410. The amino acid composition of CGTase 17-1 was calculated from the deduced amino acid sequence and compared with the composition of purified CGTase. Molar ratios obtained by these analyses were closely consistent (Table 1).

Comparison of the nucleotide sequences and deduced amino acid sequences of the CGTase genes of alkalophilic Bacillus sp. strain no. 17-1 and strain no. 38-2. We previously cloned and analysed the nucleotide sequence of the CGTase gene of Bacillus sp. 38-2 (Kaneko et al., 1988). Fig. 4 shows the nucleotide sequence and amino acid sequence alignment between the CGTase 17-1 and...
Comparison of chimeric CGTase

Table 2. Comparison of CGTase 17-1 and CGTase 38-2

<table>
<thead>
<tr>
<th></th>
<th>CGTase 17-1</th>
<th>CGTase 38-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid residues</td>
<td>713</td>
<td>712</td>
</tr>
<tr>
<td>(Signal peptide)</td>
<td>(27)</td>
<td>(27)</td>
</tr>
<tr>
<td>( M_s ) of mature enzyme</td>
<td>74140</td>
<td>75160</td>
</tr>
<tr>
<td>Homology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid</td>
<td>77%</td>
<td>83%</td>
</tr>
<tr>
<td>Heat stability*</td>
<td>53 °C</td>
<td>65 °C</td>
</tr>
<tr>
<td>pH-activity profile</td>
<td>narrow</td>
<td>broad</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6</td>
<td>6, 9</td>
</tr>
</tbody>
</table>

* Temperature at which 50% loss of activity occurred (see Fig. 7 and text).

CGTase 38-2 genes. In the amino acid sequences, 593 (83%) of the aligned amino acids were identical. The overall homology of the aligned nucleotide sequence was 77%. However, the enzymic features, especially the heat stability and optimum pH, of the two CGTases were distinct (Table 2). To analyse these differences further, we constructed chimeric CGTases using the two genes and compared their enzymic properties.

Construction and comparison of chimeric CGTases

Construction of chimeric CGTases. As described in Methods, 12 chimeric CGTase genes were constructed (Fig. 5). Schematic representations of the chimeric CGTases are shown in Fig. 6. E. coli HB101 cells harbouring the various chimeric genes were grown aerobically in 10 ml LB broth for 24 h at 37 °C, then harvested and sonicated. The lysates were dialysed against running tap water and these crude extracts were used as the enzyme preparations. Their activities were 270–440 units per ml broth; E. coli harbouring pUC19 had no dextrinizing activity. SDS-polyacrylamide gel electrophoresis and Western blot analysis indicated that the 14 enzyme proteins (CGTase 17-1, CGTase 38-2 and 12 chimeric CGTases) had almost the same \( M_s \), as expected.
Fig. 5. Construction of chimeric CGTases. Restriction endonuclease sites which were used for construction of chimeric CGTases are indicated. The thick lines indicate the fragments containing the CGTase genes from alkalophilic Bacillus, and thin lines indicate the vector plasmid pUC 19.

Fig. 6. Schematic representation of chimeric CGTase genes. The restriction endonuclease sites used were NdeI, Eco47III and BanI, and their amino acid residues were numbered taking initiation Met as 1. 'S' and open boxes represent regions from CGTase 38-2; 'U' and filled boxes represent regions from CGTase 17-1.

**Heat stability of chimeric CGTases.** With respect to the heat stability of the chimeric CGTases, their N-terminal segment was most important. As shown in Fig. 7, among the 14 CGTases, those enzymes (S---) which contained the N-terminal segment derived from CGTase 38-2 were more stable than those (U---) which contained the N-terminal segment derived from CGTase 17-1 ('S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively). The second segment also had some role in increasing heat stability. Among the enzymes (S---)
Comparison of chimeric CGTase

Fig. 7. Comparison of heat stability of chimeric CGTases. Each enzyme in 10 mM-phosphate buffer (pH 6.0) was treated at 40, 50, 55, 60, 65, and 70 ºC for 30 min. The residual activities were measured by the standard method (see Methods). The temperatures in each figure represent the point of 50% loss of activity. 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively (see Fig. 6).

whose first (N-terminal) segment was from CGTase 38-2, those chimeric CGTases (SS--) which contained a second segment from CGTase 38-2 were most stable. The third and the fourth segment apparently did not contribute to heat stability.

The homologies of the first, second, third and fourth segments were 86.1%, 86.0%, 81.4% and 77.7%, respectively. It is interesting that the heat stability was changed distinctly by substituting the two most homologous segments, the first and second segments. Thus, heat stability may be influenced by only a few amino acid substitutions. Several properties are known to influence the heat stability of proteins. Insertions of helix-breaking amino acids (proline, threonine, etc.) into an alpha-helix decreases heat stability, and insertion of helix-forming amino acids (glutamic acid, alanine, etc.) into an alpha-helix increases heat stability. The secondary structures of the two CGTases were predicted by Chou-Fasman's method and the numbers of helix-forming and helix-breaking amino acid residues in the alpha-helix regions were compared. There were fewer helix-breaking and more helix-forming amino acid residues in the alpha-helix region of CGTase 17-1 than those of CGTase 38-2, suggesting that the number of helix-breaking and helix-forming amino acids may not be the primary influence on heat stability of the CGTases. Hydrophobic interaction inside the protein molecule is also important in stabilizing protein structure. Comparing the first and second segments of CGTase 38-2 and CGTase 17-1, amino acid substitution from CGTase 17-1 to CGTase 38-2 increased its hydrophobic properties a little. These substitutions might be important for heat stability.
Fig. 8. Comparison of pH-activity profiles of chimeric CGTases. The pH was adjusted with the following buffer systems: sodium acetate (pH 4.0, 5.0, and 6.0); potassium phosphate (pH 5.0, 6.0, 7.0, 8.0, and 9.0); glycine/NaOH (pH 8.0, 9.0, 10.0, and 11.0). 'S' and 'U' represent regions from CGTase 38-2 and CGTase 17-1, respectively (see Fig. 6).

pH-activity profiles of chimeric CGTases. The pH-activity profile depended on both the N-terminal and C-terminal segments. As shown in Fig. 8, the chimeric CGTases (S--S) which contained the N- and C-terminal segments derived from CGTase 38-2 showed two pH optima, at pH 6 and pH 9, and those enzymes (U--U) which contained the N- and C-terminal segments from CGTase 17-1 showed only one pH optimum, at pH 6. Those chimeric enzymes which contained the N-terminal segment from CGTase 38-2 and the C-terminal segment from CGTase 17-1 (S--U), or which contained the N-terminal segment from CGTase 17-1 and the C-terminal segment from CGTase 38-2 (U--S), showed intermediate pH profiles. There was no change of pH profile upon substitution of the third segment except with the chimeric enzymes USSS and USUS.

Yamane et al. (1984) reported that the α-amylase produced by B. natto (B. subtilis) IAM1212 had a deletion of about 100 amino acid residues near the C-terminal region, but this deletion did not affect enzyme activity. Fukumori et al. (1987) reported that the cellulase of alkalophilic Bacillus sp. no. 1139 had a size of 92 kDa and that the large C-terminal part (about 46 kDa) of the cellulase was not necessary for enzyme activity. In contrast, the CGTases studied here consisted of about 720 amino acid residues, and in our unpublished findings CGTases lacking 30 amino acid residues from the C-terminal end showed no enzyme activity. These results suggest that the C-terminal part of the enzyme is important for CGTase activity. The change of pH-activity profile on substitution of the C-terminal segment might be related to this phenomenon.

Our analysis of the chimeric CGTases suggests that the N- and/or C-terminal segment(s) might be important for the CGTase specificity. The third segment might not be essential for the enzyme specificity.
We would like to thank Mr Wayne Bellamy for his critical reading of this manuscript.
This research was partially supported by Grants from Life Science Promotion, The Riken Institute and from the Ministry of Education, Science, and Culture of Japan.

REFERENCES


