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Cyclodextrin Formation by the Thermostable α-Amylase of *Thermoanaerobacterium thermosulfurigenes* EM1 and Reclassification of the Enzyme as a Cyclodextrin Glycosyltransferase

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Extensive characterization of the thermostable α-amylase of *Clostridium thermosulfurogenes* EM1, recently reclassified as *Thermoanaerobacterium thermosulfurigenes*, clearly demonstrated that the enzyme is a cyclodextrin glycosyltransferase (CGTase). Product analysis after incubation of the enzyme with starch revealed formation of α-, β-, and γ-cyclodextrins, as well as linear sugars. The specific activity for cyclization of this CGTase was similar to those of other CGTases, whereas the specific activity for hydrolysis was relatively high in comparison with other CGTases. Alignment of the amino acid sequence of the *T. thermosulfurigenes* enzyme with sequences from known bacterial CGTases showed high homology. The four consensus regions of carbohydrate-converting enzymes, as well as a C-terminal raw-starch binding motif, could be identified in the sequence.

Cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19) are able to convert starch into cyclodextrins (CDs), cyclic compounds which have the ability to form inclusion complexes with a wide range of small hydrophobic molecules used in the food, cosmetic, and pharmaceutical industries (35). Depending on the type of CD (with six, seven, or eight glucopyranose residues: α-, β-, or γ-CD, respectively) initially formed, the CD-forming enzymes are classified as α-, β-, or γ-CGTases (28). CGTases are known to catalyze four different transferase reactions: cyclization, coupling, disproportionation, and hydrolysis (5).

CGTases are functionally related to α-amylases (13, 17, 24), which hydrolyze starch into linear products. Although CGTases and α-amylases show a low overall degree of similarity in amino acid sequence, the predicted secondary-structure similarity is very high. Three-dimensional structures of CGTases closely resemble the $(\beta/\alpha)_8$-chain fold of α-amylases. The active sites of CGTases and α-amylases, located in the A domain, are rather similar (33). CGTases, however, possess two additional domains (D and E) absent from α-amylases. In agreement with this, CGTases generally have a molecular mass of 70 to 75 kDa, whereas α-amylases generally have a molecular mass of approximately 45 to 55 kDa (28). Domain E contains a raw-starch binding motif, but the exact functions of the D and E domains remain unknown (17, 34). At present, it remains unclear what determines the difference in product specificity between CGTases and α-amylases.

Highly thermostable CGTases may be applied for starch solubilization in the CD industry, eliminating the need for α-amylase pretreatment (32, 37). CGTases are produced by a variety of aerobic bacteria, including *Bacillus macerans*, *B. megaterium*, *B. circulans*, *B. stearothermophilus*, *B. ohbensis*, *B. alkalophilus*, *Klebsiella pneumoniae*, *K. oxytoca*, and *Micrococcus* sp. (28). None of these CGTases, however, are sufficiently thermostable for use in industrial starch liquefaction. In a group of thermophilic anaerobic microorganisms belonging to the genus *Thermoanaerobacter*, new CGTases which are characterized by a high thermostability have been found (25, 31, 32).

The thermophilic anaerobic microorganism *Clostridium thermosulfurogenes* EM1, recently reclassified as *Thermoanaerobacterium thermosulfurigenes* EM1 (19), forms at least two sets of thermostable starch-degrading enzymes: α-amylase and pullulanase (21, 30). In this paper, we describe the purification and characterization of a novel CGTase from *T. thermosulfurigenes* EM1, the first example of such an enzyme in this genus. We will show that this CGTase is highly heat stable in the presence of starch and is active over a wide pH range, making possible the use of this enzyme for starch liquefaction in the CD industry.

The same enzyme has been characterized previously as an α-amylase (30). The gene encoding this "α-amylase" has been cloned and expressed in *Escherichia coli*, and its primary structure has been determined (1, 8). We will show that the amino acid sequence of this "α-amylase" in fact is highly homologous to known bacterial CGTase sequences. Characterization of the purified enzyme clearly demonstrates that the enzyme is a CGTase, since product analysis after incubation of

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TABLE 1. Purification scheme of the CGTase from *T. thermosulfurigenes* EM1[a]

Fraction	Total activity (U)[b]	Total protein (mg)	Sp act (U/mg)[b]	Purification factor (fold)	Yield (%)
Cell-free culture broth	324	130	2.5	1	100
Affinity chromatography	225	2.9	88	35	79
Cell extract	516	3,300	0.16	1	100
Affinity chromatography	396	7.6	52	326	77
Heat treatment (15 min at 90°C)	258	3.1	83	519	50

[a] *E. coli* PC1990(pCT2) was used for production of the enzyme.
[b] Cyclization activity.

the enzyme with starch revealed formation of α-, β-, and γ-CDs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* JM109, carrying plasmid pCT2, a derivative of pUC18 containing the *amyA* gene of *T. thermosulfurigenes* EM1 (8), and *E. coli* PC1990, known to leak periplasmic proteins into the medium because of a mutation in the *tolB* locus (18), served as host strains. Plasmid isolation (alkaline lysis method), DNA handling, and calcium chloride transformation of *E. coli* were essentially carried out as described by Sambrook et al. (27). Plasmid DNA was isolated from *E. coli* JM109(pCT2) and transformed to *E. coli* PC1990. Transformants of *E. coli* PC1990 containing pCT2 and clearly expressing the *amyA* gene were selected on Luria broth agar (27) plates containing ampicillin (100 μg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside; 0.1 mM), and 10 g of potato starch per liter.

Enzyme purification. The CGTase from *T. thermosulfurigenes* EM1 was purified from cells of *E. coli* PC1990(pCT2). Cells were grown on Luria broth, enriched with 10 g of Casamino Acids, 10 g of tryptone, and 100 μg of ampicillin per liter and 0.1 mM IPTG, in a 3-liter fermentor at 30°C. Growth was monitored by measuring the optical density at 450 nm. When the optical density at 450 nm reached 2 to 3, an extra amount (30 g) of tryptone was added to the fermentor. Cells were harvested after 14 to 20 h of growth, by centrifugation (6,000 × *g*, 15 min), at an optical density at 450 nm of 11 to 13.

Extracellular CGTase was purified by applying the supernatant directly to an α-CD–Sepharose 6FF affinity column (16). After being washed with 10 mM sodium acetate buffer (pH 5.5), the bound CGTase was eluted with the same buffer supplemented with 10 mg of α-CD per ml.

Cell-associated CGTase was purified from washed cells, resuspended in 10 mM sodium acetate buffer (pH 5.5), and disrupted by three passages through a French press (107 MPa; Aminco, Silver Spring, Md.). The crude extract obtained was applied to the affinity column, and fractions with CGTase activity were collected, incubated for 15 min at 90°C, and centrifuged (15,000 × *g*, 3 min) to remove all heat-denatured protein.

Gel electrophoresis and isoelectric focusing (IEF). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (15), using the Mini-protean II system (Bio-Rad, Veenendaal, The Netherlands). Molecular weights were estimated by using prestained SDS-PAGE standards of 18,500 to 106,000 molecular weight (Bio-Rad). Proteins were stained with Coomassie blue. Proteins displaying amylolytic activity were detected according to Lacks and Springhorn (14), with a few modifications. After SDS-PAGE was run, the gel was washed in 0.1 M sodium acetate solution (pH 6.5) supplemented with 5 mM calcium chloride for 2 h at room temperature and incubated in the same solution supplemented with 0.5% (wt/vol) soluble starch for 30 min at 60°C. After the gel was washed with deionized water, it was stained with a solution of iodine (0.05 M I_2; Merck, Amsterdam, The Netherlands) for 5 min.

IEF was performed with the Pharmacia-LKB Phastsystem (Uppsala, Sweden) according to the procedures recommended in the Phastsystem manual. For IEF, precast IEF 3-9 gels were used. The pI was estimated with the Pharmacia broad-range calibration kit (pI 3.50 to 8.65). Proteins were stained with Coomassie blue in the Phastsystem development unit.

Enzyme activities. Specific assays were used to determine the activities of the four different reactions catalyzed by CGTases (26).

The cyclization activity was determined by incubating diluted enzyme with 1% (wt/vol) Paselli SA2 (partially hydrolyzed potato starch with an average degree of polymerization of 50 [AVEBE, Foxhol, The Netherlands]) in a 10 mM sodium citrate (pH 5.9) solution (buffer A) for 10 min at 60°C. β-CDs formed were assayed on the basis of their ability to form a stable colorless inclusion complex with phenolphthalein, which usually is purple (36). One unit of CGTase activity is defined as the amount of enzyme able to produce 1 μmol of β-CD per min under standard conditions (pH 5.9 and 60°C).

The hydrolysis activity was determined by measuring the increase in reducing power during incubation with starch. None of the other reactions results in an increase in reducing sugars. Reducing sugar concentrations were determined with the dinitrosalicylic acid method according to Bernfield (3), with a few modifications. Diluted enzyme was incubated for an appropriate period of time with 1% (wt/vol) soluble starch (Merck) in buffer A at 60°C. One unit of hydrolysis activity is defined as the amount of enzyme producing 1 μmol of reducing sugars (as maltose) per min under standard conditions.

The coupling activity was determined by measuring the disappearance of β-CD in the presence of maltotriose (G3) or maltotetraose (G4). Diluted enzyme was incubated with 1% (wt/vol) β-CD and 1% (wt/vol) substrate in buffer A for 10 min at 60°C. The β-CD concentration was determined as described above. One unit of coupling activity is defined as the amount of enzyme able to convert 1 μmol of β-CD per min under standard conditions.

The disproportionation activity was determined by measuring the conversion of maltohexaose (G6) into other oligosaccharides. Diluted enzyme was incubated with 2% (wt/vol) G6 in buffer A at 60°C. The conversion of G6 was analyzed by high-performance liquid chromatography (HPLC) (see below). One unit of disproportionation activity is defined as the amount of enzyme able to convert 1 μmol of G6 per min under standard conditions.

Protein concentrations were determined by the method of Bradford, using the Pierce Coomassie protein assay reagent (Pierce Europe bv, Oud-Beijerland, The Netherlands).

Product analysis. Reaction mixtures containing 2% (wt/vol) soluble starch (from potato; Sigma Chemie GmbH, Deisenhofen, Federal Republic of Germany) or 2% (wt/vol) Paselli SA2 in buffer A were incubated at 60°C with 0.1 U of CGTase per ml. Samples were taken at convenient time intervals, and the reaction was stopped by cooling the mixtures on ice and adding 3 volumes of methanol. Precipitated material was spun down (3 min, 15,000 × *g*), and samples of the supernatant were analyzed by HPLC. For analysis of total reducing sugars, reactions were stopped with 1 volume of 1 M NaOH. CD formation was also measured under industrial production conditions (2) by incubation of 0.1 U of enzyme per ml with 10% (wt/vol) Paselli SA2 in buffer A at 60°C for 45 h.

CDs (α, β, and γ) and linear sugars were analyzed by HPLC, using a 25-cm Econosil-NH_2 10-μm column (Alltech Nederland bv, Breda, The Netherlands) eluted with acetonitrile-water (60:40, vol/vol) at 1 ml/min. The flow cell was set at 50°C, and products were detected by a refractive index detector (Waters 410; Waters Chromatography Division, Milford, Mass.).

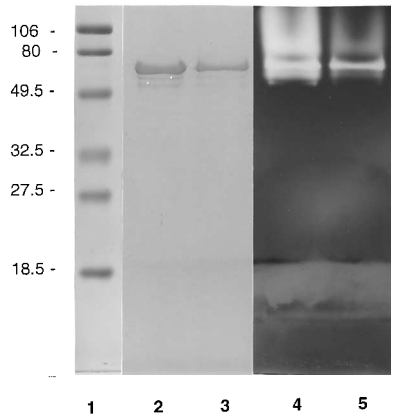

FIG. 1. SDS-PAGE of purified CGTase from *T. thermosulfurigenes* EM1. Lane 1, low-molecular-weight standards (10^3); lanes 2 and 3, proteins stained with Coomassie blue; lanes 4 and 5, detection of proteins displaying amylolytic activity. Lanes 2 and 4, purified extracellular CGTase; lanes 3 and 5, purified intracellular CGTase.

TABLE 2. Specific enzyme activities of CGTases from *T. thermosulfurigenes* EM1 and *B. circulans* 251

Strain	Sp act (U/mg)			
	Cyclization	Coupling	Disproportionation (± SD)	Hydrolysis
T. thermosulfurigenes EM1	86	74[a]	760 ± 50	23
B. circulans 251[b]	170[c]	73[d]	620 ± 50	3.5

[a] Activity was calculated as an average of G3 and G4 as second substrate.
[b] CGTase from *B. circulans* 251 (26).
[c] Activity was determined by incubating enzyme with 1% Paselli in 10 mM sodium citrate buffer (pH 6.0) at 50°C.
[d] Activity was determined by incubating enzyme with G4 as second substrate.

The total amount of reducing sugars was determined by the DNS method as described earlier. Maltose was used as a standard.

Thin-layer chromatography of saccharides was done with an ascending technique on 0.2-mm silica gel-coated aluminum sheets (type 60; Merck) as described previously (20). Reaction mixtures contained 2% (wt/vol) soluble starch, 20 mM sodium acetate buffer (pH 5.5), and 0.1 U of CGTase per ml. Mixtures were incubated at 60°C. Samples were drawn after various periods of time, mixed with 2 volumes of methanol, and centrifuged, and 5-μl portions of the supernatants were applied to a thin-layer chromatography sheet.

Ultrafiltration. Ultrafiltration experiments were performed with the Microcon system of Amicon (Amicon Division, Capelle a/d IJssel, The Netherlands).

Amino acid sequences. For determination of the N-terminal amino acid sequence, about 100 pmol of purified CGTase was dialyzed against 1% (vol/vol) acetic acid to remove all possible low-molecular-weight impurities and freeze-dried. The N-terminal amino acid sequence was determined at the Gas Phase Sequenator Facility, University of Leiden, Leiden, The Netherlands. The instrument used was an Applied Biosystems (Foster City, Calif.) model 470A protein sequencer equipped on-line with a model 120A phenylthiohydantoin analyzer.

Alignment of sequences was performed with a Macintosh Quadra 950 computer (program CLUSTAL V). Sequences were obtained from the EMBL database (accession numbers for CGTases: *T. thermosulfurigenes* EM1, M57580; *B. circulans* 251, A51661; *B. licheniformis*, X15752; *Bacillus* sp. strain 1011, A26678; *Bacillus* sp. strain 38-2, M19880; *B. ohbensis*, D90243).

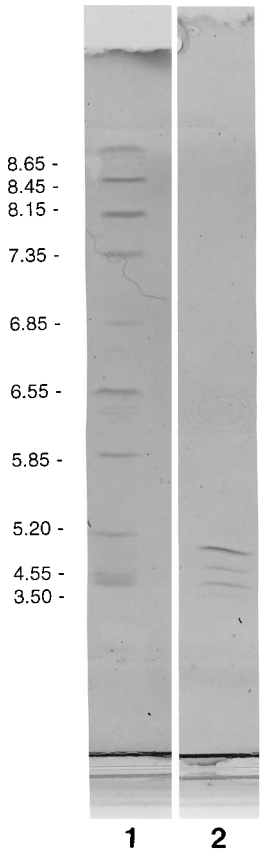

FIG. 2. IEF. Lane 1, broad-range pI calibration kit; lane 2, purified extracellular CGTase. pH standards are given on the left.

RESULTS

Purification of the CGTase. *E. coli* PC1990(pCT2), containing the α-amylase (CGTase)-encoding *amyA* gene of *T. thermosulfurigenes* EM1, was used for enzyme production. During growth on enriched Luria broth, CGTase was partially secreted into the culture fluid (61%) and partially remained cell associated (39%).

The cell-associated enzyme was purified 519-fold with a 50% yield by an affinity chromatography step (α-CD–Sepharose 6FF) and an additional heating step (15 min at 90°C) to remove all heat-labile proteins (Table 1). The extracellular CGTase was purified 35-fold in a single step, with a yield of 79%. The specific cyclization activity of the purified extracellular CGTase after affinity chromatography was 88 U/mg, which is slightly higher than for the purified cell-associated CGTase (83 U/mg).

The CGTase was purified to apparent homogeneity from both fractions (Fig. 1). Two additional minor bands were observed in both fractions on SDS-PAGE stained with Coomassie blue. These minor bands were observed again on SDS-PAGE stained for amylolytic activity and are most probably CGTase degradation products. *E. coli* PC1990 (without plasmid) did not display any amylolytic activity in the supernatant or in the cells (data not shown).

CGTases are known to catalyze four different reactions with starch. The purified enzyme indeed possessed cyclization, coupling, and disproportionation as well as hydrolyzing activity. In Table 2, the data for the enzyme from *T. thermosulfurigenes* are shown and compared with the activities of a well-characterized CGTase (26).

Size, subunit structure, and isoelectric point. The CGTase displayed a molecular weight of 68,000 on SDS-PAGE (Fig. 1), which is in agreement with the value of 75,100 predicted for the mature protein on the basis of the gene sequence (8). No difference in molecular weights could be detected between the excreted CGTase and the intracellular CGTase. The calculated mass of the signal sequence of the enzyme was 3.5 kDa (8), a difference which should be visible on SDS-PAGE. Apparently in both fractions the signal sequence is cleaved off, indicating that the cell-associated CGTase is present in the periplasmic space.

IEF of the enzyme revealed one major band with an isoelectric point of 5.0 and three minor bands with isoelectric points of 4.3, 4.4, and 4.6 (Fig. 2).

The CGTase passed through an ultrafiltration membrane with a molecular weight cutoff of 100,000. This indicated that the active enzyme consists of a single unit of 68 kDa.

Effect of temperature and pH on activity. The pH optimum of the CGTase for cyclization activity was broad, in the range of pH 4.5 to 7.0 (Fig. 3A). A lower and sharper pH optimum was observed for the hydrolyzing activity, in the range of pH 4.0 to 4.5. Maximum CGTase cyclization activity was observed at 80 to 85°C, and maximum hydrolyzing activity was noted at 90 to 95°C (Fig. 3B).

Thermal stability of the CGTase. Calcium (10 mM) and starch (1% [wt/vol] Paselli) were both found to enhance the thermal stability of the enzyme at 90°C (Fig. 4A). The stability of the CGTase at various temperatures was determined in the presence of starch (Fig. 4B). At 70°C almost no activity was lost

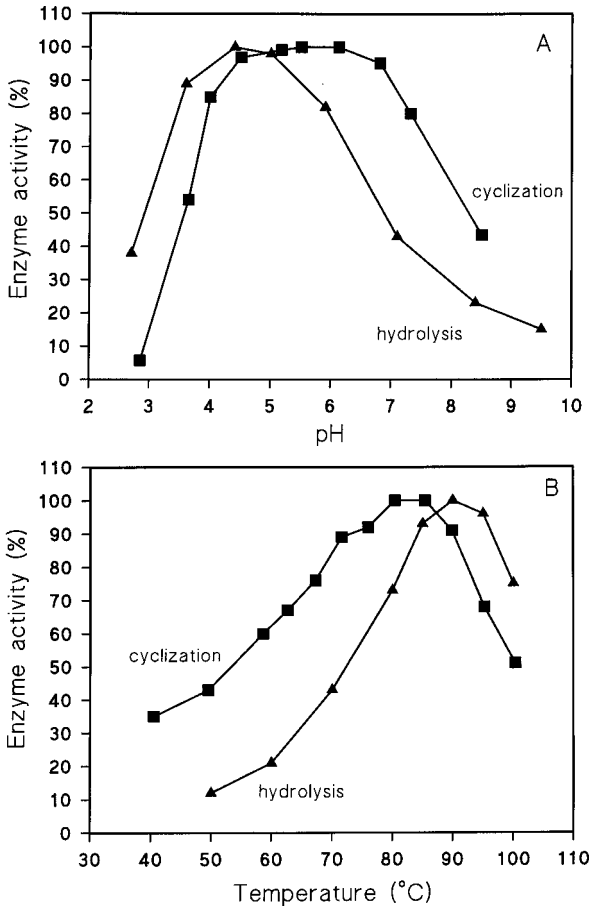

FIG. 3. Effect of pH (A) and temperature (B) on activity of CGTase.

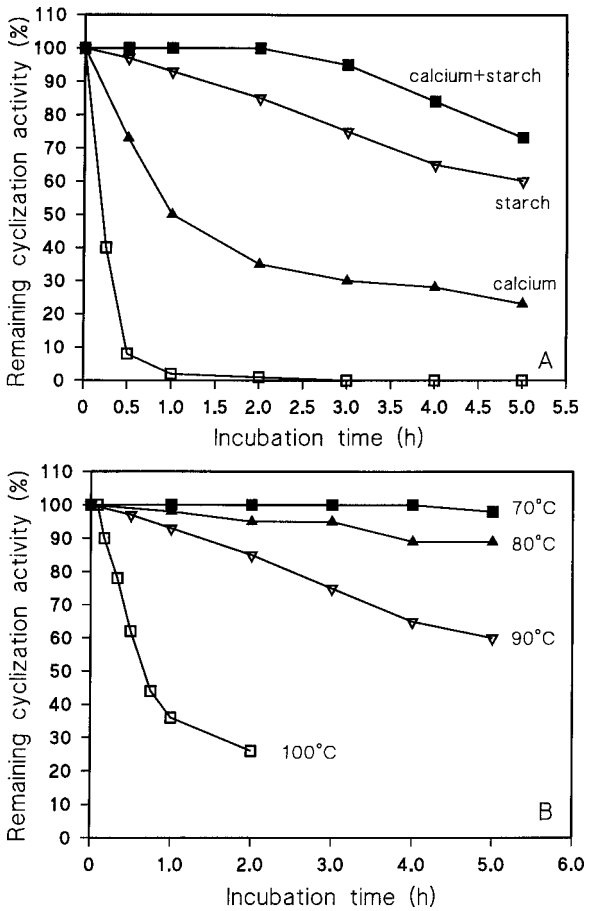

FIG. 4. Thermal stability of CGTase. (A) Effect of starch and calcium on the thermal stability of the CGTase at 90°C. □, 0.1 M sodium citrate (pH 5.9; buffer A), supplemented with 10 mM calcium chloride (▲), 1% (wt/vol) Paselli (▽), or 10 mM calcium chloride and 1% (wt/vol) Paselli (■). (B) Effect of temperature on stability of the CGTase in the presence of 1% (wt/vol) Paselli.

after 5 h of incubation; at 80°C only 10% of the activity was lost, and at 90°C only 35% was lost. At 100°C the enzyme displayed a half-time of inactivation of 30 min.

CD production. Production of CDs was monitored over time by incubation of CGTase with 2% (wt/vol) Paselli SA2 (Fig. 5A). α-CD was produced as the major compound in the first 100 min of the reaction; thereafter, β-CD was produced as the major compound. A maximum yield of 60% conversion of starch into CDs could be obtained after 2 h; the product ratios at that point were 43% α-CD, 46% β-CD, and 11% γ-CD (Fig. 5A).

Under industrial CD production conditions, CGTase is incubated for prolonged periods of time, with high concentrations of starch (2). Thus, incubations were done with 10% (wt/vol) Paselli SA2 for 48 h (Fig. 5B). A maximum yield of 38% conversion of starch into CDs could be obtained after 8 h. At that point CD ratios were 33% α-CD, 54% β-CD, and 13% γ-CD. Formation of linear sugars from 10% Paselli was also monitored over time. After 8 h, 2% of the starch had been converted into linear sugars, reaching a maximum of 5% after 30 h of incubation.

Saccharides formed from 2% (wt/vol) soluble starch were also analyzed by thin-layer chromatography (Fig. 6). Formation of α-, β- and γ-CDs over time was clearly visible. Initially, α-CD was the predominant product formed. Formation of some of the linear sugars over time was also clearly visible.

Comparison of amino acid sequences. As a control, the N-terminal amino acid sequence of the purified extracellular CGTase from *T. thermosulfurigenes* EM1 was determined. The first five amino acids could not be analyzed because of contaminations in the sample. The next 15 amino acids could be identified and were exactly the same as for the α-amylase (CGTase)-encoding *amyA* gene of *T. thermosulfurigenes* EM1 (1) (data not shown).

Alignment of the amino acid sequence of the *amyA* gene product from *T. thermosulfurigenes* with known sequences of thermostable α-amylases (7) resulted in identity scores of around 30% with *B. stearothermophilus* and *B. licheniformis* α-amylases (data not shown). The amino acid sequence of the CGTase of *T. thermosulfurigenes* EM1 was found to be highly homologous to bacterial CGTase amino acid sequences (Fig. 7). Similarity/identity scores ranged from 71%/57% to 78%/69%. In contrast to α-amylases, CGTases are known to possess five domains, and all five domains could be identified in the sequence of the enzyme (Fig. 7). The COOH-terminal region of the enzyme was found to be highly homologous with the raw-starch binding domain of glucoamylase (34).

The four consensus regions of carbohydrate-converting enzymes (23, 33), containing active-site residues and residues involved in substrate binding, were found to be conserved in the amino acid sequence of the CGTase from *T. thermosulfurigenes* EM1 (Table 3). These regions were also found to be conserved in sequences from the other bacterial CGTases.

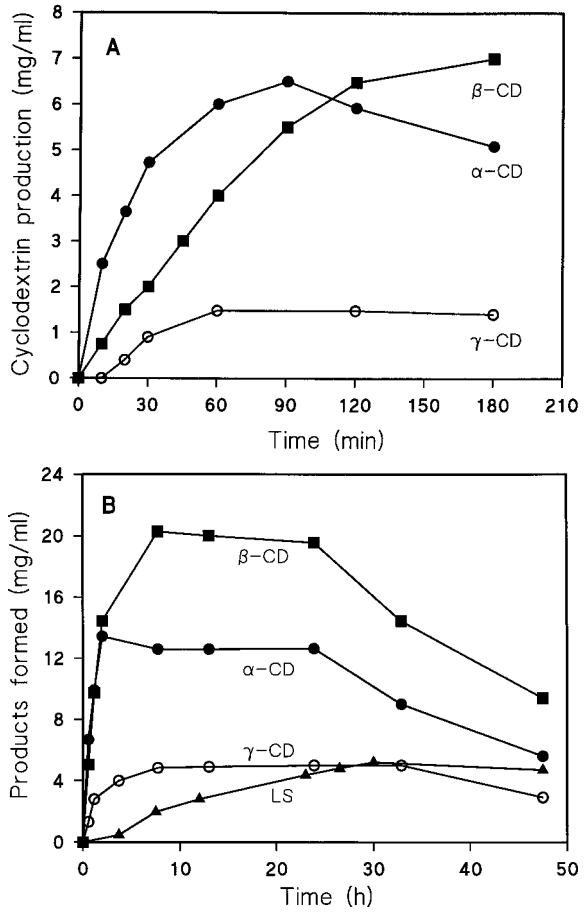

FIG. 5. Products formed during incubation of purified CGTase from *T. thermosulfurigenes* with 2% (wt/vol) (A) and 10% (wt/vol) (B) Paselli SA2. Concentrations of reducing sugars were determined by the DNS method. The total amount of linear sugars (LS) was calculated from the increase in reducing power.

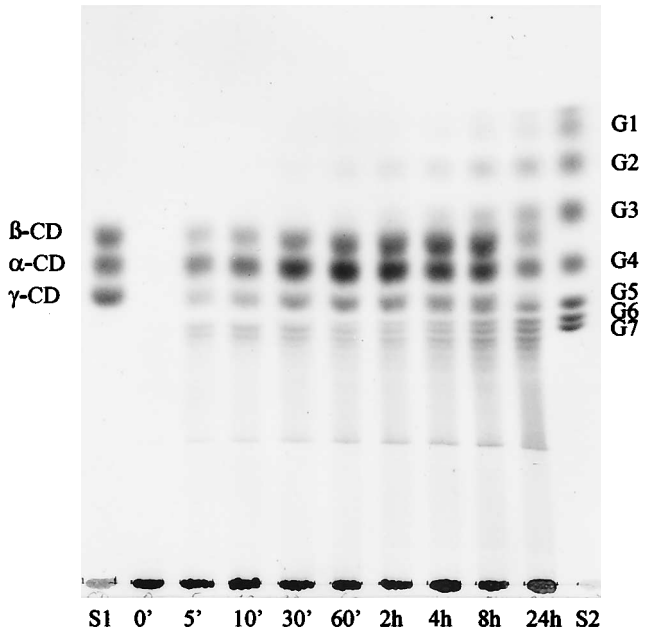

FIG. 6. Thin-layer chromatography of saccharides. Samples were taken at various times (lanes 2 to 10) during incubation of purified *T. thermosulfurigenes* CGTase with 2% (wt/vol) soluble starch. A mixture of α-, β-, and γ-CDs (0.1% [wt/vol] each) was applied to lane 1 (S1); standards of linear oligosaccharides (glucose to maltoheptaose, 0.1% [wt/vol] each) were applied to lane 11 (S2).

X-ray studies and site-directed mutagenesis experiments (13, 17, 24) have identified Asp-255, Glu-283, and Asp-354 (*Thermoanaerobacterium* numbering) as catalytic residues in CGTases. These residues were found to be conserved in the amino acid sequence of the *Thermoanaerobacterium* CGTase.

DISCUSSION

The high amino acid sequence homology of the α-amylase (CGTase) from *T. thermosulfurigenes* EM1 with known bacterial CGTases (Fig. 7) and its determined molecular weight of 68,000 (Fig. 1) both suggest that the enzyme is a CGTase. The raw-starch binding motif appears to be present in most CGTases but not in α-amylases (34) and was found also in the amino acid sequence of the *T. thermosulfurigenes* enzyme (Fig. 7).

Characterization of the purified enzyme clearly demonstrates that the enzyme is a true CGTase. Product analysis after incubation of the enzyme with starch showed the formation of α-, β- and γ-CDs (Fig. 5 and 6). In fact, the enzyme is an α-CGTase, since α-CD was initially and predominantly formed from starch. Despite this, the enzyme is an unusual type of CGTase, since besides CDs, linear sugars were also formed from starch (Fig. 5B and 6). Specific activities for cyclization, coupling, disproportionation, and hydrolysis were compared with those of the CGTase from *B. circulans* 251 (Table 2). Coupling and disproportionation activities were comparable. Cyclization activity was higher by a factor of 2 for the *B. circulans* CGTase. Hydrolyzing activity, however, was higher by a factor of 6 for the *Thermoanaerobacterium* CGTase. The wild-type CGTase of *B. circulans* 251 did not produce linear sugars from 10% Paselli (26), whereas the CGTase from *T. thermosulfurigenes* converted 5% of the starch into linear sugars (Fig. 5B).

Modelling studies and alignments of amino acid sequences have suggested that residue 221 (*Thermoanaerobacterium* numbering) is present in CGTases at a dominant position in the center of the active-site cleft (6, 26). In most CGTases the residue is an aromatic amino acid (Tyr or Phe), whereas in α-amylases the residue is much smaller (Gly, Ser, or Val) (23). Penninga et al. (26) constructed mutants of the *B. circulans* 251 CGTase in which this aromatic residue had been replaced by a nonaromatic residue. This resulted in a switch-over to synthesis of linear maltooligosaccharides from starch, which indicated that the aromatic amino acid residue at this position is of crucial importance for an efficient cyclization reaction. In the case of the CGTase from *T. thermosulfurigenes*, the residue is a phenylalanine (Fig. 7), indicating that other factors are responsible for the relatively high hydrolyzing activity of the enzyme. At present, it remains unclear what determines the difference in product specificity between CGTases and α-amylases. It is expected that we will gain more information about this from determination of the three-dimensional structure of the *T. thermosulfurigenes* enzyme, allowing detailed comparisons of structures of both α-amylase and other CGTase proteins. Crystals of the CGTase from *T. thermosulfurigenes* suitable for X-ray diffraction have been obtained (5a).

There is one other example of an α-amylase-CGTase enzyme in the literature (10). The α-amylase-CGTase from *Bacillus* sp. strain B1018 was initially characterized as an α-amy-

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T. thermosulf.  M----KKTF-KLILVLMLSLTLVFGL--TAPIQAASDTAVSNVVNYSTDV  43
B. circ. 251    M-----KKFLKSTAALAVGLSLTFGLF--SPAQAAPDTSVSNKQNFSTDV  43
B. lichen.      MFQMAKRVLLSTTLTFSLLAGSALPFLPASAIYADADTAVTNKQNFSTDV  50
B. spec. 1011   M-----KRFMKLTAVWTLWLSLTLGLL--SPVHAAPDTSVSNKQNFSTDV  43
B. spec. 38-2   M-----KRFMKLTAVWTLWLSLTLGLL--SPVHAAPDTSVSNKQNFSTDV  43
B. ohben.       MKNLT--VLLKT-------IPLALLLFILLSLPTAAQADVTNKVNYTRDV  41
                *       .              . .    .  . ... *.*  *.. **

T. thermosulf.  IYQIVTDRFVDGNTSNNPTGDLYDPTHTSLKKYFGGDWQGIINKINDGYL  93
B. circ. 251    IYQIFTDRFSDGNPANNPTGAAFDGTCTNLRLYCGGDWQGIINKINDGYL  93
B. lichen.      IYQVFTDRFLDGNPSNNPTGAAFDGTCSNLKLYCGGDWQGLVNKINDNYF 100
B. spec. 1011   IYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGYL  93
B. spec. 38-2   IYQIFTDRFSDGNPANNPTGAAFDGSCTNLRLYCGGDWQGIINKINDGYL  93
B. ohben.       IYQIVTDRFSDGDPSNNPTGAIYSQDCSDLHKYCGGDWQGIIDKINDGYL  91
                ***. **** **. .*****  .    . *  * ******...**** *.

T. thermosulf.  TGMGVTAIWISQPVENIYAVLPDSTFGG--STSYHG-WARDLRE-QSYFG 139
B. circ. 251    TGMGVTAIWISQPVENIYSII---NYSGVNNTAYHGYWARDFKKTNPAYG 140
B. lichen.      SDLGVTALWISQPVENIFATI---NYSGVTNTAYHGYWARDFKKTNPYFG 147
B. spec. 1011   TGMGITAIWISQPVENIYSVI---NYSGVNNTAYHGYWARDFKKTNPAYG 140
B. spec. 38-2   TGMGITAIWISQPVENIYSVI---NYSGVHNTAYHGYWARDFKKTNPAYG 140
B. ohben.       TDLGITAIWISQPVENVYALHP-SGY-----TSYHGYWARDYKRTNPFYG 135
                ...*.**.********...      .     *.*** **** .  .. .*

                                  I
T. thermosulf.  SFTDFQNLINTAHAHNIKVIIDFAPNHTSPASETDPTYAENGRLYDNGTL 189
B. circ. 251    TIADFQNLIAAAHAKNIKVIIDFAPNHTSPASSDQPSFAENGRLYDNGTL 190
B. lichen.      TMTDFQNLVTTAHAKGIKIIIDFAPNHTSPAMETDTSFAENGKLYDNGNL 197
B. spec. 1011   TMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNL 190
B. spec. 38-2   TMQDFKNLIDTAHAHNIKVIIDFAPNHTSPASSDDPSFAENGRLYDNGNL 190
B. ohben.       DFSDFDRLMDTAHSNGIKVIMDFTPNHSSPALETDPSYAENGAVYNDGVL 185
                   **  *. .**.  **.*.**.***.***   . ..**** .*..* *
                        Domain A1 ◄┘ └► Domain B

T. thermosulf.  LGGYTNDTNGYFHHYGGTDFSSYEDGIYRNLFDLADLNQQNSTIDSYLKS 239
B. circ. 251    LGGYTNDTQNLFHHNGGTDFSTTENGIYKNLYDLADLNHNNSTVDVYLKD 240
B. lichen.      VGGYTNDTNGYFHHNGGSDFSTLENGIYKNLYDLADLNHNNSTIDTYFKD 247
B. spec. 1011   LGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVYLKD 240
B. spec. 38-2   LGGYTNDTQNLFHHYGGTDFSTIENGIYKNLYDLADLNHNNSSVDVYLKD 240
B. ohben.       IGNYSNDPNNLFHHNGGTDFSSYEDSIYRNLYDLADYDLNNTVMDQYLKE 235
                .* *.** .  *** **.***. *..**.**.**** .  .*.  .* *.*
                                  Domain B ◄┘ └► Domain A2
                      II                                  III
T. thermosulf.  AIKVWLDMGIDGIRLDAVKHMPFGWQKNFMDSILSYRPVFTFGEWFLGTN 289
B. circ. 251    AIKMWLDLGIDGIRMDAVKHMPFGWQKSFMAAVNNYKPVFTFGEWFLGVN 290
B. lichen.      AIKLWLDMGVDGIRVDAVKHMPQGWQKNWMSSIYAHKPVFTFGEWFLGSA 297
B. spec. 1011   AIKMWLDLGVDGIRVDAVKHMPFGWQKSFMATINNYKPVFTFGEWFLGVN 290
B. spec. 38-2   AIKMWLDLGVDGIRVDAVKHMPFGWQKSFMSTINNYKPVFNFGEWFLGVN 290
B. ohben.       SIKLWLDKGIDGIRVDAVKHMSEGWQTSLMSDIYAHEPVFTFGEWFLGSG 285
                .**.*** *.****.******. *** . *  .    *** *******

T. thermosulf.  EIDVNNTYFANESGMSLLDFRFSQKVRQVFRDNTDTMYGLDSMIQSTASD 339
B. circ. 251    EVSPENHKFANESGMSLLDFRFAQKVRQVFRDNTDNMYGLKAMLEGSAAD 340
B. lichen.      APDADNTDFANESGMSLLDFRFNSAVRNVFRDNTSNMYALDSMLTATAAD 347
B. spec. 1011   EISPEYHQFANESGMSLLDFRFAQKARQVFRDNTDNMYGLKAMLEGSEVD 340
B. spec. 38-2   EISPEYHQFANESGMSLLDFPFAQKARQVFRDNTDNMYGLKAMLEGSEVD 340
B. ohben.       EVDPQNHHFANESGMSLLDFQFGQTIRDVLMDGSSNWYDFNEMIASTEED 335
                    .   ************ *    *.*. * .   * .  *. ..  *

                      IV
T. thermosulf.  YNFINDMVTFIDNHDMDRFY-NGGSTRPVEQALAFTLTSRGVPAIYYGTV 388
B. circ. 251    YAQVDDQVTFIDNHDMERFHASNANRRKLEQALAFTLTSRGVPAIYYGTE 390
B. lichen.      YNQVNDQVTFIDNHDMDRFKTSAVNNRRLEQALAFTLTSRGVPAIYYGTE 397
B. spec. 1011   YAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSE 390
B. spec. 38-2   YAQVNDQVTFIDNHDMERFHTSNGDRRKLEQALAFTLTSRGVPAIYYGSE 390
B. ohben.       YDEVIDQVTFIDNHDMSRFSFEQSSNRHTDIALAVLLTSRGVPTIYYGTE 385
                *  . * ********* **       *  . ***  *******.****.
```

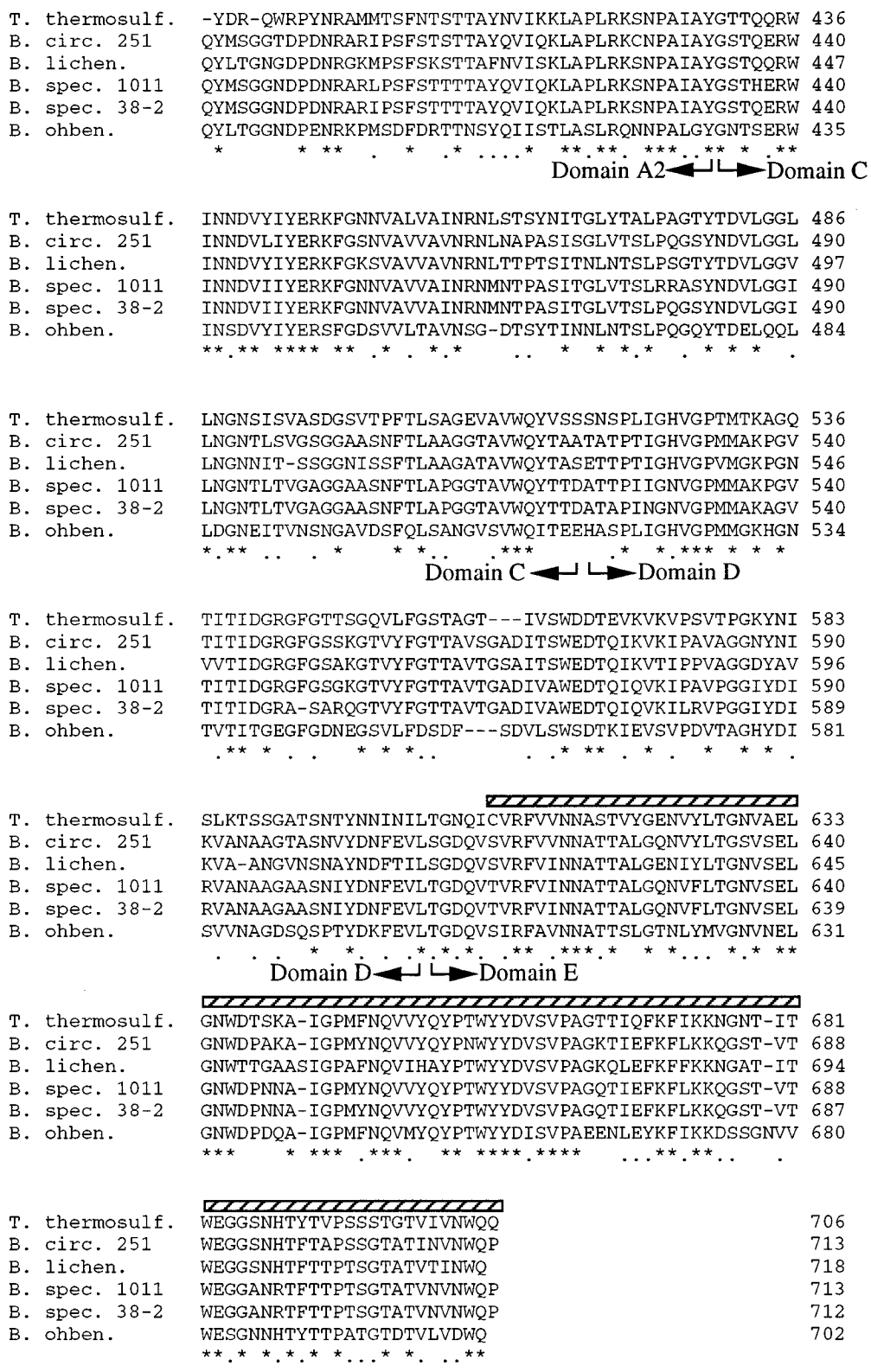

FIG. 7. Alignment of amino acid sequences of bacterial CGTases. Consensus regions I to IV of carbohydrate-converting enzymes (22, 23) are numbered and marked by a solid bar, and the region with similarity to the raw-starch binding domain (34) is marked by a hatched bar. An asterisk indicates amino acid identity, a point indicates a conservative replacement, and the hyphens indicate gaps introduced to optimize the alignment. The start and end of the A to E domains of CGTases are marked (9). Residues 221 (*Thermoanaerobacterium* numbering), assumed to be at a dominant position in the center of the active-site cleft (6, 26), are boxed. T. thermosulf., *T. thermosulfurigenes* EM1 (1); B. circ. 251, *B. circulans* 251 (17); B. lichen., *B. licheniformis* (9); B. sp. 1011, *Bacillus* sp. strain 1011 (12); B. sp. 38-2, *Bacillus* sp. strain 38-2 (11); B. ohben., *B. ohbensis* (29).

TABLE 3. Consensus regions I to IV of amylases[a] in CGTases[b]

CGTase from:	Consensus																											
	Region I							Region II									Region III					Region IV						
	Residue	D	A	V	I	N	H	Residue	G	F	R	L	D	A	A	K	H	Residue	E	V	I	D	Residue	F	V	D	N	H	D
T. thermosulfurigenes (1)[c]	161	D	F	A	P	N	H	251	G	I	R	L	D	A	V	K	H	283	E	W	F	L	349	F	I	D	N	H	D
B. circulans 251 (17)	162	D	F	A	P	N	H	252	G	I	R	M	D	A	V	K	H	284	E	W	F	L	350	F	I	D	N	H	D
B. licheniformis (9)	169	D	F	A	P	N	H	259	G	I	R	V	D	A	V	K	H	291	E	W	F	L	357	F	I	D	N	H	D
Bacillus sp. strain 1011 (12)	162	D	F	A	P	N	H	252	G	I	R	V	D	A	V	K	H	284	E	W	F	L	350	F	I	D	N	H	D
Bacillus sp. strain 38-2 (11)	162	D	F	A	P	N	H	252	G	I	R	V	D	A	V	K	H	284	E	W	F	L	350	F	I	D	N	H	D
B. ohbensis (29)	157	D	F	T	P	N	H	247	G	I	R	V	D	A	V	K	H	279	E	W	F	L	345	F	I	D	N	H	D
K. pneumoniae (4)	160	D	Y	A	P	N	H	249	A	I	R	I	D	A	I	K	H	287	E	W	F	G	358	F	M	D	N	H	D

[a] References 22 and 23.
[b] Numbering of the amino acid sequences of the enzymes starts at the amino-terminal amino acid of each protein, including the signal peptide.
[c] Reference is given in parentheses.

lase but has strong sequence homology with CGTases and a molecular weight of 78,000 and produces β-CD from starch. These data show that the enzyme is in fact a CGTase. It would not be surprising to find more examples in which reclassification of α-amylases is necessary.

Comparison of our data with data published about the characterization of the α-amylase from *T. thermosulfurigenes* EM1 is difficult since experimental setups were not the same. Haeckel and Bahl (8), as well as Madi et al. (21), characterized the enzyme by using crude extracts. Spreinat and Antranikian (30) used purified enzyme. They found a temperature optimum for hydrolysis activity of 80°C (or 90°C in the presence of calcium) and a pH optimum of 4.5 to 5.5. Data presented in this paper differ slightly. The specific hydrolysis activity of the enzyme reported by Spreinat and Antranikian was 9.5 U/mg, whereas we found 23 U/mg. In this case, the purity of the enzyme is important, as well as the assay procedure.

On SDS-PAGE we observed three bands displaying amylolytic activity, and with IEF we observed four bands, suggesting the presence of possible degradation products of the CGTase. Spreinat and Antranikian (30) observed three active amylase bands on PAGE (native) and four bands with IEF. The reasons for the appearance of these multiple forms of the enzyme remain unknown.

TABLE 4. Comparison of amino acid compositions of the CGTase from *T. thermosulfurigenes* EM1 (without signal peptide) and the CGTase from *Thermoanaerobacter* sp. (25)

Amino acid	No. of residues in sequence	
	T. thermosulfurigenes EM1	*Thermoanaerobacter* sp.
Ala	37	42
Arg	22	19
Asn	59	55
Asp	43	43
Cys	1	1
Gln	24	22
Glu	15	17
Gly	61	63
His	11	11
Ile	43	40
Leu	38	39
Lys	22	23
Met	13	16
Phe	32	32
Pro	24	32
Ser	57	43
Thr	71	76
Trp	14	14
Tyr	42	44
Val	50	49
Total	679	681

New thermostable CGTases have also been found in thermophilic anaerobic microorganisms belonging to the genus *Thermoanaerobacter* (25, 31, 32). According to Lee et al. (19), both genera can be initially distinguished by the reduction of thiosulfate to elemental sulfur in the case of *Thermoanaerobacterium* and to H_2S in the case of *Thermoanaerobacter*. The characteristics of the *Thermoanaerobacter* CGTases are very similar to those presented in this paper. The *Thermoanaerobacter* CGTase characterized by Norman and Jörgensen (25, 32) displayed a temperature optimum of 90 to 95°C and a pH optimum of 5.0 to 6.5 (starch hydrolyzing activities). With 10% (wt/vol) corn starch, 30% of the substrate was converted into CDs (36% α-CD, 44% β-CD, and 20% γ-CD). Unfortunately, the sequence of this CGTase has not been published. The overall amino acid compositions of the *Thermoanaerobacter* (25) and *Thermoanaerobacterium* CGTase enzymes show relatively minor differences (Table 4). Another CGTase from this genus was characterized by Starnes (31). This CGTase, produced by *Thermoanaerobacter thermohydrosulfuricus* (formerly *Clostridium thermohydrosulfuricum* [19]) displayed a temperature optimum of 80 to 90°C and a pH optimum of 4.0 to 6.5. CD formation from 25% (wt/vol) starch was studied, of which 8% was converted into CDs (35% α-CD, 44% β-CD, and 21% γ-CD).

The *T. thermosulfurigenes* CGTase is highly thermostable in the presence of starch (Fig. 4A and B). Because of this, the enzyme could provide advantages to the CD industry. In the industrial production of CDs, the first step is liquefaction of starch with a thermostable α-amylase (37). This step can be omitted when this CGTase is used, since it is sufficiently thermostable to solubilize starch at high temperatures.

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