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Chemical modification of carboxylic residues in a cyclodextrin glucanotransferase and its implication in the hydrolysis/transglycosylation ratio of the α-amylase family

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Abstract

The product selectivity varies notably in the enzymes of family 13 of the glycosyl hydrolases, α-amylase family, despite their similar catalytic site (three COOH groups involved) and overall architecture. For example, α-amylases are strongly hydrolytic enzymes, whereas cyclodextrin glycosyltransferases (CGTases) are essentially transglycosylases. Chemical modification of the carboxylic groups (using a water soluble carbodiimide and three different amino nucleophiles) of CGTase from *Thermoanaerobacter* in absence or presence of a reversible inhibitor has been carried out. In contrast with most hydrolytic enzymes of the α-amylase family, in which carbodiimide modification produces an inactivation effect, the resulting CGTases kept residual activities in the range 22–50% for cyclization and coupling, and 39–80% for disproportionation and hydrolysis. In addition, the selectivity to cyclodextrins and the production of oligosaccharides were not significantly altered when tested under industrial conditions. By amino acid analysis and MALDI-TOF mass spectrometry, it was determined that 4–5 COOH residues were modified. The three carboxylic residues implicated in the active-site (Asp230, Glu258 and Asp329) have a very low water accessibility (<7 Å^2). This may help to explain the high transglycosylation/hydrolysis ratio of CGTases in comparison with other α-amylase enzymes.

Keywords: Cyclodextrin glycosyltransferase; α-amylases; Glucosyltransferase; Glycosyl hydrolase; Carbodiimide; Acarbose

1. Introduction

Cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19) belong to family 13 of the glycosyl hydrolases, called α-amylase family. It is formed by a large group of homologous $(\beta/\alpha)_8$-barrel proteins that degrade starch, such as α-amylases, α-glucosidases, pullulanases and isoamylases. Although the sequence similarity within this family is relatively low (<30%),

Abbreviations: CDs, cyclodextrins; CGTase, cyclodextrin glucanotransferase; *Tabium*, *Thermoanaerobacterium thermosulfurigenes EM1*; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; GEE, glycine ethyl ester; NME, norleucine methyl ester

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the active-site catalytic residues and the α-retaining bond cleavage mechanism are well conserved. However, their product selectivity varies notably in the α-amylase family. In fact, α-amylase is a strongly hydrolytic enzyme, whereas CGTase is basically a transglycosylase. It has been postulated that the transferase activity of CGTase evolved from an ancestral hydrolase [1,2]. CGTases display a very weak hydrolyzing activity (e.g. 3.0 U/mg for *Bacillus circulans* and 1.9 U/mg for *B. stearothermophilus* CGTases) compared with the α-amylases (typical values of 500–2000 U/mg).

Two highly thermostable CGTases have been isolated from thermophilic anaerobic microorganisms, belonging to *Thermoanaerobacterium thermosulfurigenes EM1* (*Tabium* CGTase) and to the genus *Thermoanaerobacter*, showing a great similarity (91%) in their sequences [3]. Both *Thermoanaerobacter* CGTase and *Tabium* CGTase exhibit a significant hydrolytic activity (82 and 30 U/mg, respectively) [3–5], which initially resulted in their misidentification as α-amylases.

CGTases produce cyclodextrins from starch through an intramolecular transglycosylation called cyclization. Apart from catalyzing this reaction, CGTase presents a coupling activity (opening of the rings of CDs and transfer to acceptors) and a disproportionation activity (transfer of linear maltooligosaccharides to acceptors). CGTases are capable—by disproportionation or coupling reactions—to use acceptors molecules of different nature, yielding gluco-conjugates of potential interest [6,7]. The hydrolytic activity of CGTases is in general much lower than the transglycosylation activities, even for the *Thermoanaerobacter* and *Tabium* CGTases.

The active-site of α-amylases and CGTases, located in the A domain, has three carboxylic residues involved in catalysis: Asp230, Glu258 and Asp329 (*Tabium* CGTase numbering). However, two main structural features differentiate CGTases from α-amylases: first, in all the CGTases structurally characterized, an aromatic residue (Phe or Tyr) in position 196 seems to play a central role as a cyclization axis during the CD-formation [8]; second, CGTases are larger, containing two additional domains (D and E). In addition, other factors may contribute to the differences in the ratio between hydrolysis and transglycosylation activities within this family. One of them is the existence of an extra acceptor subsite (S + 2) in CGTases, not present in amylases. It has been postulated that an induced-fit mechanism may take place after binding of sugars (but not water) at the acceptor subsites, which activates CGTases for catalysis [9].

In this work, and continuing our contribution to the study of the molecular features determining CGTase specificity [3,7,10,11], chemical modification of carboxylic groups of CGTase from *Thermoanaerobacter* was carried out using 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide (EDC) and different amino nucleophiles. Experiments were performed with and without active center protection with acarbose, a reversible inhibitor. The number of modified COOH groups was evaluated both by MALDI-TOF mass spectrometry and amino acid analysis. The hydrolytic and transglycosylation activity of native and modified CGTases were analyzed. Results were correlated with water accessibility of the carboxylic side chains.

2. Materials and methods

2.1. Materials

CGTase from *Thermoanaerobacter* sp. 501 was kindly provided by Novozymes A/S. Taurine, glycine ethyl ester (GEE), norleucine methyl ester (NME), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, phenolphthalein, bromocresol green, malto-oligosaccharides (G1–G7), α-, β- and γ-cyclodextrin, methyl α-D-glucopyranoside, amyloglucosidase (EC 3.2.1.3) from *Aspergillus niger*, sinapinic acid and hydroxylamine were purchased from Sigma. Glucose GOD-Perid reagent, *p*-nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene (EPS) and α-glucosidase (EC 3.2.1.20) from *S. cerevisiae* were from Boehringer Mannheim. 3,5-Dinitrosalicylic acid (DNS) was from Fluka. Methyl orange was from Aldrich. Acarbose was kindly provided by Bayer. Partially hydrolyzed potato starch with an average degree of polymeration of 50 (Paselli SA2) was donated by Avebe B.A. (The Netherlands).

2.2. Enzyme purification

CGTase was purified by affinity chromatography using a column (10 × 120 mm) packed with

Sepharose-6FF (Pharmacia) covalently coupled to α-cyclodextrin. The gel was activated with α-cyclodextrin as described previously [12]. The column was washed with 200 ml of 10 mM sodium acetate buffer (pH 5.5) at 2.5 ml/min, and the bound CGTase was further eluted with 50 ml of the same buffer containing α-cyclodextrin (10 mg/ml). Fractions containing the CGTase were pooled and stored at −20 °C (in presence of α-CD). The concentration of protein was determined by Bradford Bio-Rad micro-assay with immunoglobulin G (IgG) as standard.

2.3. CGTase activity assays

The CGTase activities were measured at 85 °C by incubating appropriately diluted enzyme with substrate solutions in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. Reactions were followed during 5–10 min; at time intervals of 1 min aliquots were removed and assayed using the corresponding reagent.

2.3.1. Cyclization activity

The production of cyclodextrins was detected spectrophotometrically via the formation of inclusion complexes with several organic compounds. Paselli SA2 was used as substrate at final concentrations of 5% (w/v) for β- and γ-CD, and 2% (w/v) for α-CD. The α-CD was determined at 490 nm on the basis of its ability to form a stable, colorless inclusion complex with methyl orange [13]. The β-CD was determined at 552 nm on the basis of its ability to form a stable, colorless inclusion complex with phenolpthalein [8]. The γ-CD was determined measuring the color increase at 630 nm due to the formation of an inclusion complex with bromocresol green [14]. One unit of activity was defined as that catalyzing the production of 1 μmol of α-CD/β-CD/γ-CD per minute under the corresponding conditions.

2.3.2. Coupling

The reaction was based on the method described by Nakamura et al. [15]. The α- or β-cyclodextrins (2.5 mM) are used as donors and methyl-α-D-glucopyranoside (10 mM) as acceptor. The linear oligosaccharide formed in the reaction was converted into single glucose units by the action of amyloglucosidase. The amount of glucose was accurately detected with the glucose/GOD-Perid reagent. One unit of activity was defined as that catalyzing the conversion of 1 μmol of cyclodextrin per minute under the corresponding conditions.

2.3.3. Disproportionation

The reaction was based on the method described by Nakamura et al. [16]. *p*-Nitrophenyl-α-D-maltoheptaoside-4,6-*O*-ethylidene (3 mM) was used as a donor and maltose (10 mM) as acceptor. In this reaction, EPS is cleaved and the maltose is coupled to the free reducing end. The *p*-nitrophenol may be cleaved from the reaction product by the action of α-glucosidase. One unit of activity was defined as that catalyzing the release of 1 μmol of *p*-nitrophenol per minute under the corresponding conditions.

2.3.4. Saccharifying

Hydrolytic activity was assayed using 2% soluble starch as substrate, measuring the increase in reducing ends. The reducing power was accurately measured with dinitrosalicylic acid [17]. One unit of activity was defined as that catalyzing the release of 1 μmol of reducing end per minute under the corresponding conditions.

2.4. Cyclodextrins production assay

The production of CDs and oligosaccharides was assayed under similar conditions to those used in industry. The CGTase (7 μg) was incubated at 85 °C with 25% (w/v) soluble starch in 10 ml of 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. At different times, aliquots of 300 μl were taken and mixed with 300 μl of 0.4 N NaOH in order to quench the reaction. Samples were centrifuged during 15 min at 10,000 × *g* and further analyzed by high-performance liquid chromatography (HPLC) using two Aminex HPX-42A columns (7.8 × 300 mm, Bio-Rad) put in series. Water was used as mobile phase (0.7 ml/min). The column temperature was kept constant at 85 °C. Detection was performed using a refraction-index detector.

2.5. Chemical modification of carboxylic groups

Purified CGTase (2.5 mg, 6.6 μM) was incubated in 5 ml of 10 mM MES buffer (pH 4.75)

containing 10 mM KCl at 25 °C with magnetic stirring. Solid amino nucleophile (taurine, GEE or NME) was added (198 mM final concentration). In those cases where protection of active-site was required, acarbose (pseudo-maltotetraose) was introduced (1.32 mM final concentration). The pH of reaction mixture was adjusted to 4.75 with 0.2 N HCl. Afterwards, solid EDC was added (19.8 mM final concentration) starting the reaction, and keeping pH at 4.75 with 0.2 N NaOH using a pH-stat (Radiometer). The final molar ratios were as follows: EDC/CGTase 3000:1; EDC/COOH groups 50:1; nucleophile/EDC 10:1. A total of 61 COOH groups in CGTase (43 Asp, 17 Glu and the C-terminal COOH) were considered for these calculations. Once the reaction was finished (approximately 3 h, based on NaOH consumption), the sample was treated with 0.5 M hydroxylamine (pH 7.0) for 5 h at 25 °C. The preparation was dialyzed extensively against 10 mM phosphate buffer (pH 7.0) at 4 °C to remove salt and excess of reagents. A blank was performed in the absence of carbodiimide. For acarbose experiments, a blank in presence of acarbose and amino nucleophile but in absence of EDC was prepared.

2.6. Circular dichroism spectra

Circular dichroism spectra were obtained using a Jasco J-720 Spectropolarimeter (Tokyo, Japan) in the range of 190–260 nm. A total of 150 μl of enzyme solution was deposited into the cell (0.5 mm). The protein concentration was ranging from 0.15 to 0.25 mg/ml.

2.7. Amino acid analysis

The amino acid composition of native and modified CGTases (1–5 nmol) was analyzed after acid hydrolysis (60 min at 155 °C in presence of 6 N HCl) using a Beckman 6300 aminoacid analyzer. Samples were evaporated and dissolved in 300 μl of 10 mM sodium citrate buffer (pH 2.0). The analyzer was calibrated with a commercial standard (Beckman) containing each amino acid (5 nmol/50 μl). The nucleophiles employed in the chemical modification were also tested under the same conditions of acid hydrolysis.

2.8. MALDI-TOF mass spectrometry

A volume of 5 μl of native (0.4 mg/ml) or modified (0.2 mg/ml) CGTases was mixed in a eppendorf tube with 5 μl of a matrix solution formed by saturated sinapinic acid in 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA). One microliter of this mixture was then deposited on a stainless steel probe tip and allowed to dry at room temperature for 5 min. Samples were measured on a Bruker (Bremen, Germany) Reflex II MALDI-TOF mass spectrometer equipped with an ion source with visualization optics and a N_2 laser (337 nm). Mass spectra were recorded in linear positive mode at 25 kV acceleration voltage with a delayed extraction time of 750 ns. A total of 200 spectra of single laser shots under threshold irradiance were accumulated. Only highly intense, well-resolved mass signals arising from 5 to 7 selected target spots were considered. The equipment was externally calibrated employing single, double and triple charged signals from a mixture of bovine albumin (66,430 Da) and cytochrome C (12,360 Da).

2.9. Calculations of the atomic accessible area

The accessibility for chemical modification of CGTase carboxylic residues was calculated using NACCESS software [18]. The calculations were made under the assumption that the three dimensional structure of *Thermoanaerobacter* CGTase (not solved) is similar to that of *Tabium* CGTase (91% identity in sequence). Fifty-six carboxylic groups (out of 61) occupy the same position in the amino acid sequences of both enzymes. For the water accessibility calculations, a *Thermoanaerobacter* sp. CGTase model was constructed based on the sequence of *Thermoanaerobacter* CGTase [19] and the PDB file 1A47.pdb from *T. thermosulfurigenes* CGTase structure. The model was constructed using DeepView and the Swiss-Model server.

3. Results and discussion

3.1. Chemical modification of CGTase COOH groups

Amidation of protein carboxylic groups proceeds in two steps [20]. First, the protonated COOH group

is activated to the *O*-acylisourea labile intermediate by reaction with EDC. Then, it reacts with an amine nucleophile yielding the amidated protein. Carbodiimide reacts not only with carboxylic acids but also with phenolic groups of tyrosine residues yielding *O*-arylisoureas [21]. To overcome this shortcoming, these adducts were decomposed by treatment with 0.5 M hydroxylamine, which regenerates the Tyr residues and inactivates the remaining EDC (under our conditions, the half-life for EDC in presence of hydroxylamine is 0.1–0.4 h [22]). Cysteine residues may also undergo irreversible carbodiimide modification [23]. However, we observed that the reaction of the sole SH group in *Thermoanaerobacter* CGTase (Cys585) with the specific reagents 2,2′-dipyridil disulphide and 5,5′-dithiobis (2-nitrobenzoic acid) did not take place (data not shown). This indicated that Cys585 was buried in the protein core, which was confirmed by further solvent accessibility analysis (relative and absolute accessibility values of 0).

CGTase from *Thermoanaerobacter* sp. 501 contains 61 carboxylic groups (43 Asp, 17 Glu and the C-terminal α-carboxylic group –Pro683–). We first studied the chemical modification of CGTase by EDC/nucleophile modification in the absence of an active-site protector. Asp230, Glu258 and Asp329 constitute the catalytic residues of CGTase [24]. Mutation of any of these residues leads to severely reduced catalytic activities [25]. We expected a complete elimination of the four CGTase activities if any of these residues was amidated. In fact, inactivation by EDC modification is very fast in glycosydases [26–28], including members of the α-amylase family [29,30].

A large molar excess of EDC with respect to enzyme (3000:1) was employed to favor chemical modification. Reactions were kept until completion as indicated by the NaOH consumption in the pH-stat. A blank was also performed in the absence of carbodiimide to assure that the experimental conditions of the process did not affect the enzyme activity.

The extent of chemical modification was studied both by amino acid analysis and MALDI-TOF mass spectrometry. By amino acid analysis, we calculated the number of modified residues based on the appearance of new peaks of taurine or norleucine, or the increase in the glycine peak (Fig. 1). By MALDI-TOF spectrometry, a mass of 75,621 Da was obtained experimentally for native CGTase. This value was slightly lower than the value of 75,626 Da calculated from the protein sequence (using the Expasy Molecular Biology Server). The degree of modification was calculated in view of the differences of molecular weights between native and modified species (Fig. 2). For each COOH residue amidated, a mass increase of 108.1 Da for taurine, 121.6 Da for GEE, and 163.7 Da for NME was expected. For example, in taurine-modified CGTase, the mass increase of 447 Da corresponds to incorporation of 4.1 taurine molecules through an amide bond in the CGTase (degree of substitution around 6.5%). With both techniques (amino acid analysis and MALDI-TOF), the estimated degree of substitution was comparable (Table 1). With the three nucleophiles used, the modification degree was in the range 6–8%, which implies that 4–5 carboxylic groups were modified.

Table 1
Modification degree of amidated *Thermoanaerobacter* CGTases using different amino nucleophiles

Nucleophile	Number of modified residues[a]	
	Amino acid analysis	MALDI-TOF
Taurine	4.4	4.1
NME	4.3	4.7
GEE	4.1	4.2

The number of modified residues was calculated by amino acid analysis and MALDI-TOF.

[a] Similar modification degrees were obtained for modified CGTases prepared from three independent experiments.

The low modification degree might be related to the reaction mechanism. EDC/COOH modification is a two step process limited by the nature of carbodiimide. EDC is an unstable compound with a short half-life and a high molecular size, which may result in steric hindrance. Besides, another side reactions (isomerization, hydrolysis) might take place reducing the effectiveness of the modification procedure.

To evaluate whether the secondary structure of the protein had been altered by the chemical modification process, circular dichroism analysis of native and modified CGTases was carried out. The native structure appeared to be maintained throughout the EDC/nucleophile reaction, since no significant changes were recognized in CD spectra after the modification (data not shown). Thus, the change in the transglycosylation to hydrolysis ratio might be

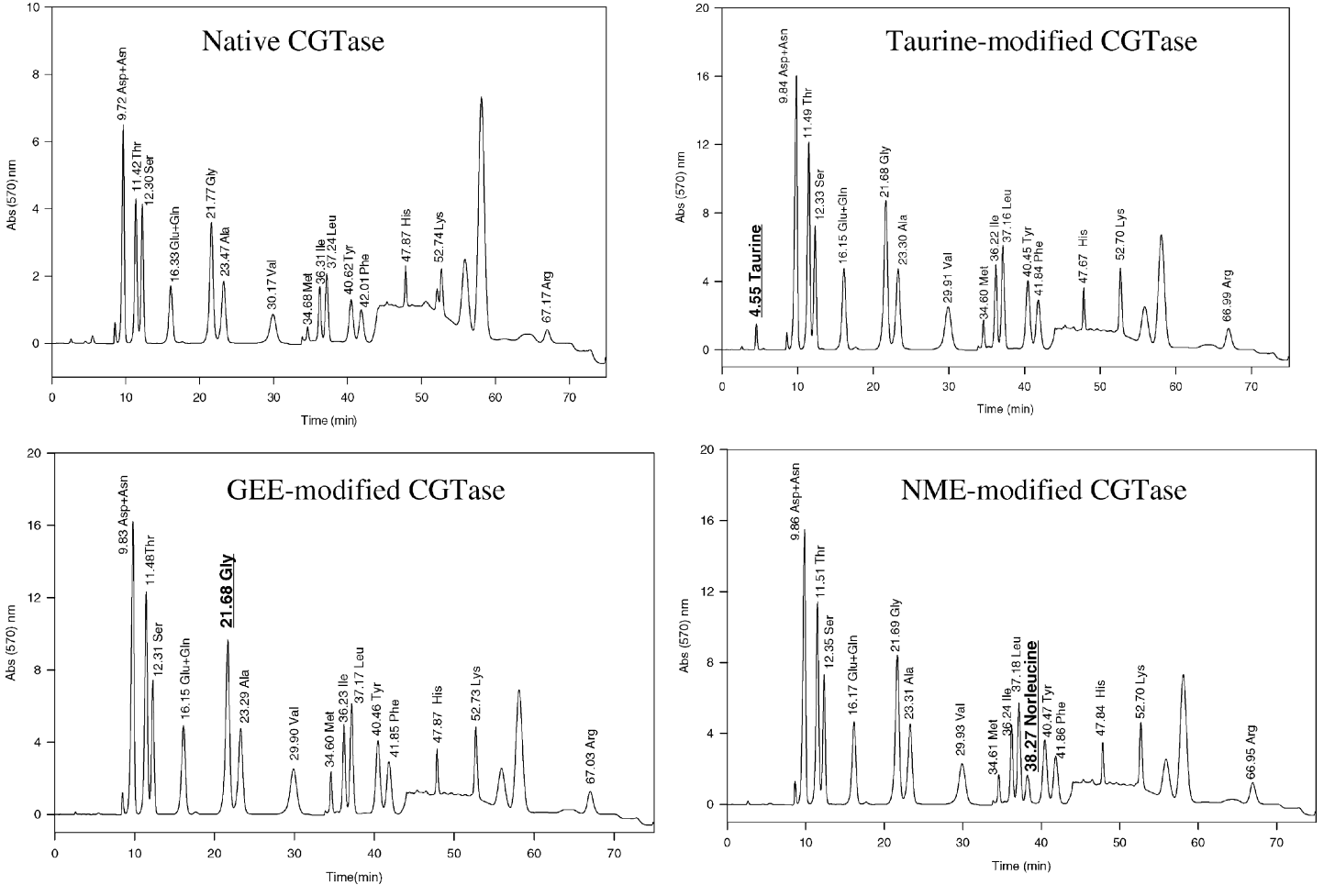

Fig. 1. Amino acid analysis of native and chemically-modified CGTases.

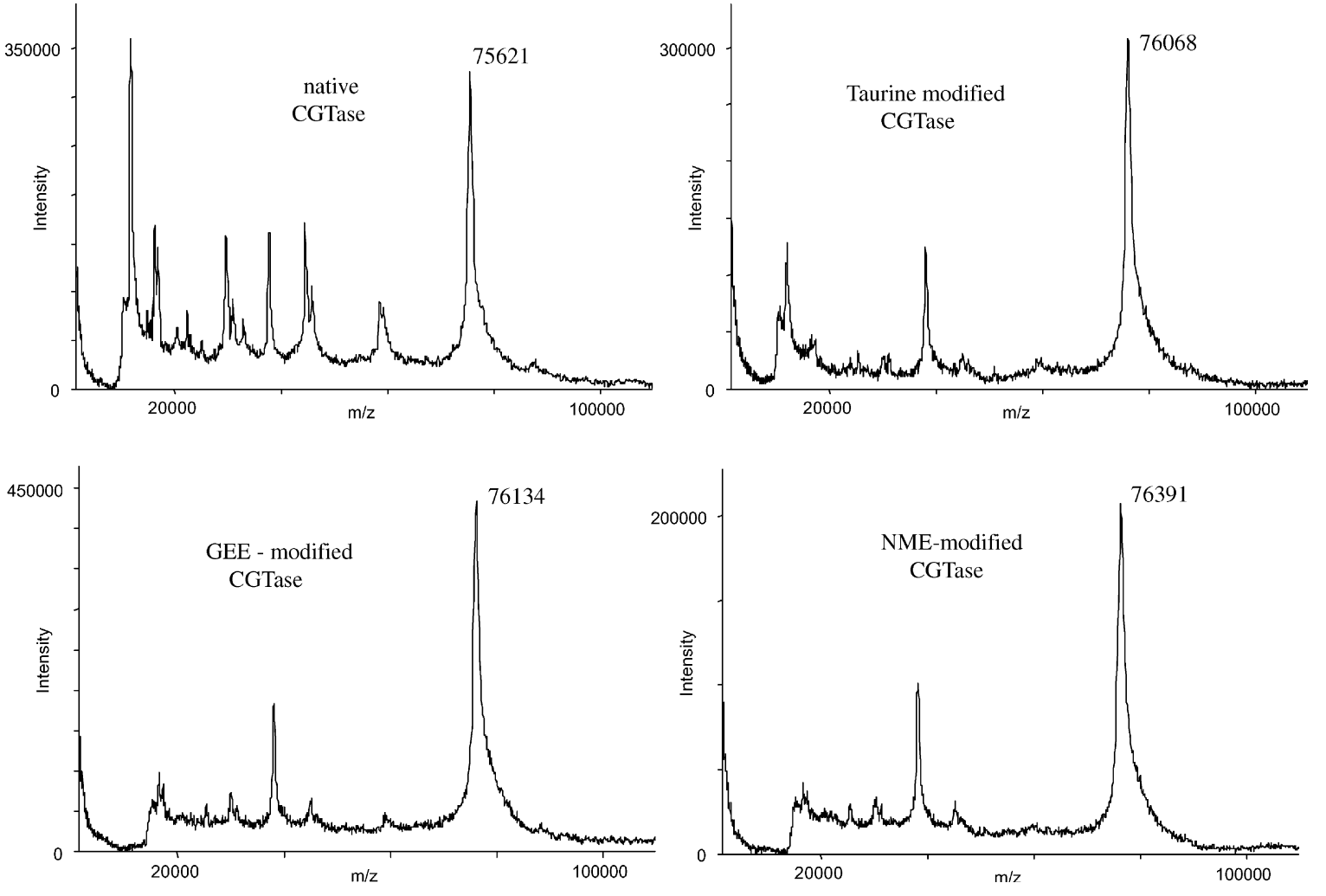

Fig. 2. MALDI-TOF mass spectra of native and chemically-modified CGTases.

Table 2
Initial activities of native and amidated *Thermoanaerobacter* CGTases[a]

CGTase	Activity (U/mg protein)						
	Cyclization			Coupling		Saccharifying	Disproportionation
	α	β	γ	α	β		
Native	300	223	131	332	68	85	1195
Taurine-modified	110	72	37	165	26	65	953
NME-modified	90	67	40	85	28	56	470
GEE-modified	77	60	33	74	24	56	501

[a] Conditions described in Section 2.

attributable only to chemical modification and not to an altered secondary structure of the protein.

3.2. Effect of chemical modification on CGTase activities

The different activities—cyclization, coupling, disproportionation and saccharifying—of native and modified CGTases were evaluated after modification (Table 2). All the assays refer to initial activities (5–10 min) to avoid the interferences between the different reactions. The γ-coupling assay could not be evaluated because we detected interaction between γ-CD and assay reagents (amyloglucosidase), which disturbed the measurements. Surprisingly, chemical modification did not inactivate the enzyme, and only a reduction of activity was observed. Regardless the nucleophile used, all the activities catalyzed by CGTase suffered a decrease. The rates of CDs formation were 25–37% with respect to activity of native glycosyltransferase, and the coupling activity values were 22–50% with respect to native enzyme. A lower decrease of activity was obtained for those reactions not involving cyclodextrins. Thus, disproportionation and hydrolysis activity values were in the range 39–80% and 65–76%, respectively, of those displayed by the native enzyme.

We also analyzed the behavior of native and modified CGTases at long reaction times, under conditions similar to those used in the industry. From the production assay, we observed that neither the CDs selectivity nor oligosaccharides production were significantly altered after modification (Fig. 3). For modified CGTases, the CDs maximal production was achieved at about 48 h, compared with the 24 h required for native CGTase, as a consequence of its higher initial activities. Nevertheless, the molar ratio α-CD/β-CD/γ-CD was not varied (for example, 2.6/5.1/1 for taurine-modified CGTase, and 2.8/5.4/1 for the native enzyme).

The nature of the nucleophile, taurine, GEE or NME, allowed us to evaluate two parameters: the charge of the modified residues (negative for taurine, and neutral for GEE and NME), and the side chain size. In general, taurine-modified CGTase, in which the negative charge of the original COOH groups is conserved, showed higher activities compared with those of their modified counterparts. As a consequence of the transformation of the negatively charged carboxylic groups into neutral moieties, a slight shift of the isoelectric point from neutral (p*I* 6.3) to moderately acid values (p*I* 5.9) was observed (data not shown). Taurine-modified CGTase kept the same isoelectric point as the native enzyme.

The above data seemed to address that the chemical modification was not taking place in any of the three catalytic COOH groups. Thus, the reduction in specific activity could be related to a decrease in the substrate binding affinity, derived from the change in size and/or charge of certain COOH residues. To confirm this, a similar chemical modification experiment was carried out protecting the catalytic triad in the active pocket (Asp230, Glu258, Asp329) with the pseudotetrasaccharide acarbose, a strong α-amylase and CGTase inhibitor [31]. The structure of acarbose is very similar to that of maltotetraose. A molar excess 200:1 of acarbose with respect to CGTase was employed to assure active-site protection [32]. A blank in presence of acarbose and amino nucleophile, but in absence of EDC, was prepared. In this control, no significant decrease of activity with respect to the native enzyme was observed, suggesting that the possible presence of

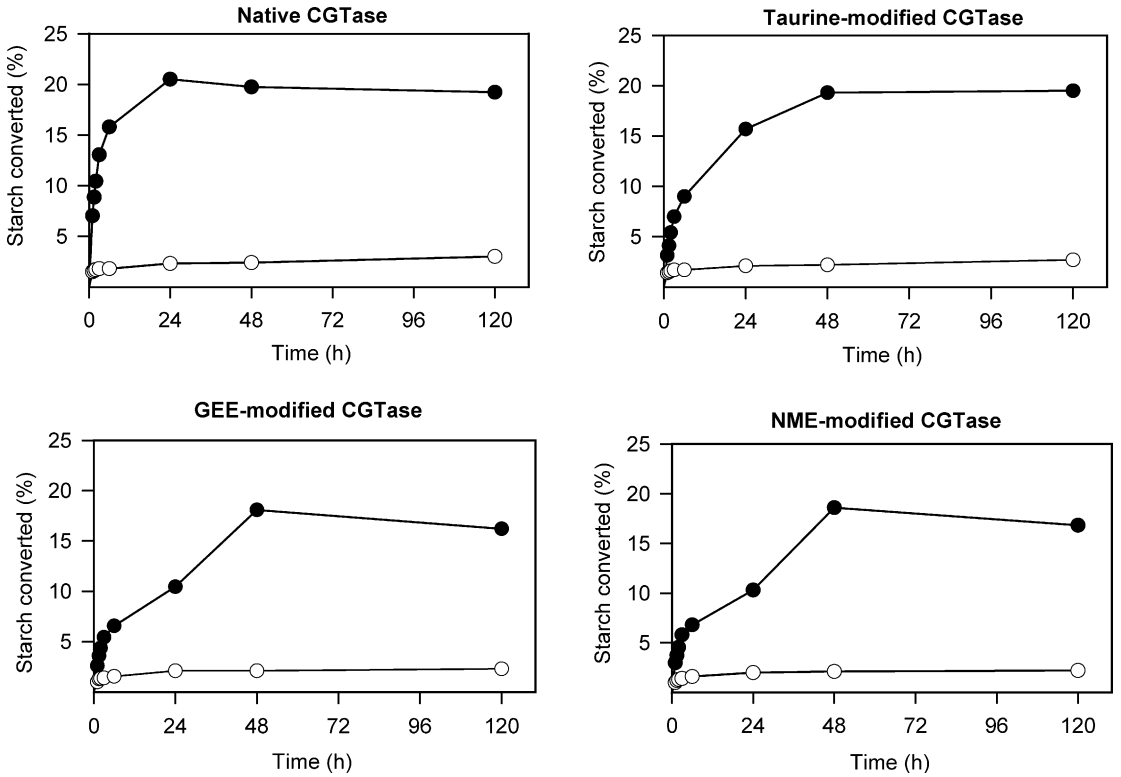

Fig. 3. Production assay of native and modified CGTases: (●) cyclodextrins; (○) oligosaccharides (G1–G7). Conditions described in Section 2.

some remaining acarbose (not removed by the extensive dialysis) was not affecting substantially our specific activity values.

The number of COOH groups amidated and the residual activities were closely similar to those experiments carried out without acarbose. This fact is indicating that chemical modification takes place preferentially in COOH groups not involving the catalytic site and the near subsites (protected by acarbose).

3.3. Solvent accessibility of COOH groups in CGTase

The accessibility of *Thermoanaerobacter* CGTase carboxylic residues was calculated using NACCESS software (Table 3). The program calculates the atomic accessible area when a probe (of the same radius as water, 1.4 Å) is rolled around the Van der Waal's surface of a macromolecule, and hence it is referred as solvent accessible surface. The relative accessibility of each residue is defined with respect to the accessibility of the same residue type in an extended ALA-x-ALA tripeptide [33]. Those residues more exposed to the media (therefore less buried) are more likely to undergo chemical modification because steric hindrances are reduced.

Table 3 shows that the accessibility of the three carboxylic residues in the active-site (Asp230, Glu258 and Asp329) is very low (<7 Å^2). This seems to imply that the carboxylic triad is not easy to be modified, as a consequence of the buried region where it is located.

For this reason, other COOH groups involved in different subsites, no too far from the active center and of higher accessibility, could be implicated in the chemical modification process. From Table 3, one can conclude that Asp37 (69 Å^2 solvent accessibility), Asp171 (75 Å^2), Asp331 (41 Å^2) and Glu646 (58 Å^2) are possible candidates for chemical modification. In particular, Asp171 is located in a loop close to the region where substrate binding at S-6 and S-7 takes place.

Table 3
Water accessibility of the polar head of carboxylic residues of CGTase from *Thermoanaerobacter* sp.

Residue	Accessibility		Residue	Accessibility		Residue	Accessibility		Residue	Accessibility	
	Å^2	%		Å^2	%		Å^2	%		Å^2	%
Asp3	1.3	2.4	Glu154	9.3	12	Asp267	12	23	Asp417	16	29
Asp15	0.8	1.5	Asp160	31	57	Glu276	32	42	Glu422	1.4	1.8
Asp23	0.9	1.8	Asp171	75	122	Asp283	0.1	0.2	Asp458	0	0
Asp27	12	21	Glu188	4.2	5.5	Asp296	26	47	Glu486	7.9	10
Asp37	69	126	Asp189	2.8	5.2	Asp299	23	43	Asp518	0.4	0.7
Asp40	10	19	Asp197	16	29	Asp305	19	35	Glu542	34	44
Asp53	0	0	Asp200	2.1	3.9	Asp314	37	68	Asp543	31	56
Asp63	17	32	Asp202	21	38	Asp320	5.4	10	Glu545	8.3	11
Glu81	16	21	Asp209	4.0	7.3	Asp326	2.5	4.6	Glu599	13	17
Asp89	13	24	Asp221	39	72	Asp329	6.6	12.1	Glu609	0	0
Asp105	0.3	0.6	Asp225	4.3	8	Asp331	41	76	Asp614	31	56
Asp118	34	62	Asp230	0	0	Glu343	5.2	6.9	Asp636	3.9	7.1
Asp136	3.2	5.8	Asp245	6.7	12	Glu363	0	0	Glu646	58	76
Glu147	38	50	Glu258	4.1	5.4	Asp371	18	34	Glu660	0	0
Asp149	27	49	Glu265	31	41	Asp382	30	55	Asp680	36	67

Absolute and relative accessibility in Å^2 and %, respectively.

However, further studies become necessary (by saturation mutagenesis and screening) to clarify if those residues could play an important role in the transglycosylation activity of *Thermoanaerobacter* CGTase.

Apart from the existence of a cyclization axis in the catalytic site, and the presence of two more domains in CGTases with respect to α-amylases, new significant features are arising to point out the differences in the hydrolysis/transglycosylation ratio within the α-amylase family. From our experiments and accessibility calculations, it seems to be clear that the active-site region of CGTase has a low water accessibility. The existence of this apolar environment may help to explain the high transferase activity of CGTase in comparison with α-amylases. In similar EDC modifications of glycosyl hydrolases, it is common that the activity is completely lost even at very low degrees of modification [26–30], which suggests that the COOH groups at the catalytic site are well exposed to the medium. In this context, Mattson et al. [34] indicated that an extremely high excess of EDC with respect to protein (6770:1) was necessary to achieve inactivation of a CGTase from *B. circulans* var. *alkalophilus*. However, no information about the number of COOH groups amidated was provided.

In the last few years, site-directed mutagenesis experiments have been performed to convert CGTase into a starch hydrolase [9]. These experiments are based on mutations of aromatic residues in subsites close to the catalytic site (e.g. S + 2), which are known to be involved in transglycosylation activity. Only an acetylated *Thermoanaerobacter* CGTase with 127 U/mg [3], and the recent mutant F260E from *Tabium* CGTase with 177 U/mg [9] have shown better hydrolytic activities than native *Thermoanaerobacter* CGTase. The inverse has also been attempted, i.e. the transformation of an α-amylase (Novamyl) into a CGTase [35]. In this case, the strategy was based on steric parameters, i.e. the introduction by site-directed mutagenesis of an aromatic residue to act as a cyclization axis, and the removal of a loop likely to be a steric hindrance for cyclization.

Further studies are required in order to accomplish the design of CGTases and other members of the α-amylase family towards one specific application. For that purpose, the combination of rational and irrational design (e.g. directed molecular evolution), along with the development of new computational tools, will be a very valuable approach.

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