ELSEVIER

Carbohydrate Research 310 (1998) 211–219

CARBOHYDRATE RESEARCH

Purification and characterisation of cyclodextrin glycosyltransferase from *Paenibacillus* sp. F8

Kim L. Larsen, Lene Duedahl-Olesen, Hans Jørgen S. Christensen, Flemming Mathiesen, Lars H. Pedersen, Wolfgang Zimmermann *

Biotechnology Laboratory, Department of Civil Engineering, Aalborg University, Sohngaardsholmsvej 57, DK-9000 Aalborg, Denmark

Received 27 February 1998; accepted 30 June 1998

Abstract

Cyclodextrin glycosyltransferases (E.C. 2.4.1.19) (CGTases) are industrially important enzymes for the production of cyclodextrins (CD) from starch. While cyclomaltohexaose (α-CD), cyclomaltoheptaose (β-CD), and cyclomaltooctaose (γ-CD) are the most commonly reported products, the production of cyclomaltononaose (δ-CD) by CGTases has not been studied previously. A CGTase from *Paenibacillus* sp. F8 was purified and characterised. The molecular weight was estimated to be 72 kDa by SDS-PAGE. The pH optima of the enzyme were 7.5 for the cyclisation activity and 8.0 for the hydrolysis activity. The temperature optima for the cyclisation and hydrolysis activities were 50 and 60 °C, respectively. Ca^{++} had a stabilising effect on the enzyme activity. The initial production ratio of α-CD, β-CD, γ-CD, and δ-CD from soluble starch was 0.09:1:0.25:0.14. Prolonged incubation times resulted in a decreased ratio of δ-CD and, to a lesser extent, of γ-CD and a increased ratio of α- and β-CD compared to the other CD. Coupling experiments showed that δ-CD was more easily degraded by *Paenibacillus* sp. F8 CGTase compared to α-, β-, and γ-CD.

Keywords: Cyclodextrin glycosyltransferase; Purification; Characterisation; Production; Cyclomaltononaose

1. Introduction

Cyclodextrins (CD) have the ability to affect the stability, solubility, reactivity and bioavailability of a wide range of molecules and have found numerous applications in the agricultural, food, chemical, and pharmaceutical industries [1–5]. Furthermore, CD have shown to be valuable as selectivity reagents for the resolution of structural, positional, and stereo isomers in analytical chemistry [6,7].

CD are made from starch by cyclodextrin glycosyltransferases (E.C. 2.4.1.19) (CGTases) produced by various bacteria. CD have a minimum number of six α-(1→4)-linked D-Glc units and CD with up to 17 Glc units have been isolated and characterised [8–11]. Recent studies have shown that CGTases may be capable of producing cyclic amyloses with up to several hundred α-(1→4)-linked D-Glc units [12], similar to the α-(1→4)-glycosyltransferase from potato (E.C. 2.4.1.25) [13]. The large CD with more than 17 Glc units were

* Corresponding author. Fax: +45-9814-2555; e-mail: i5wz@civil.auc.dk

0008-6215/98/$—see front matter
PII S0008-6215(98)00178-5

produced by the CGTases initially and converted into smaller CD with 6 to 8 Glc units, classified as α-, β-, and γ-CD, respectively, after prolonged incubation times [12]. These CD constitute the majority of the cyclic products formed by CGTases after prolonged incubation with starch [1,2,14–16]. Most studies on larger CD have been centered on the purification and characterisation of these molecules. These studies date back to the pioneering work of French et al. [17,18], who have isolated for the first time large CD with 9 to 12 α-(1→4)-linked D-Glc units.

However, until recently, the existence of larger CD has not been proven unequivocally [8–11]. The large CD studied previously have been isolated from commercial CD preparations by multiple purification steps, including liquid chromatography [8–11,19,20]. Presently, only very limited information is available on the formation of large CD by CGTases.

Employing a new analysis method [21] we have shown that a range of crude CGTases from several bacterial strains produced δ-CD, which consists of 9 α-(1→4)-linked D-Glc units [22]. In this paper, we report the purification and characterisation of a CGTase from a newly isolated bacterial strain, *Paenibacillus* sp. F8 and present results on the production and degradation of δ-CD by this enzyme.

2. Results and discussion

Identification of Paenibacillus *sp. F8*.—According to its cellular fatty acid composition, the strain showed similarity to the *Paenibacillus* group. A 98.7% similarity to *Paenibacillus pabuli* according to partial 16SrDNA sequence comparisons was found, however this result was not in correlation with the physiological characteristics of this isolate. It was therefore not possible to identify strain F8 as one of the described species within the *Paenibacillus* group.

Purification of the CGTase.—Preliminary results showed that *Paenibacillus* sp. F8 did not produce measurable amounts of CGTase when grown on Glc or soluble starch as carbon source. When grown on substrates such as whole rice, rolled oats and wheat bran, CGTase activities was produced. CGTase was purified by ammonium sulphate precipitation and affinity chromatography on β-CD Sepharose 6B (Table 1). The ammonium sulphate precipitation step was used to concentrate the enzyme preparation. Since it lead to a loss of 67% of the enzyme activity, this step was omitted in the final purification scheme. Eighty-eight per cent of the enzyme activity added to the β-CD Sepharose 6B gel could be recovered by elution with an α-CD solution. α-CD was chosen because, in contrast to β- and γ-CD, it did not interfere with the coomassie brilliant blue based protein determination method of Bradford (1976) [23]. A 52-fold purification was achieved in the two steps. The CGTase preparation was concentrated by use of a dialysis membrane resulting in a 2.2-fold purification (Table 1). The CGTase was further purified using gel filtration. The CGTase was eluted as a single symmetrical peak and contained the highest protein content of all fractions (Fig. 1). The large fraction of UV-absorbing material, which eluted between 15 to 22 mL did not contain proteins when analysed with the Bradford protein assay. The gel filtration step yielded an additional 1.36-fold purification resulting in a total 157-fold purification. The proteins in each purification step were analysed by sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE) under non-reducing conditions (Fig. 2). The β-CD affinity purification step removed most of the non-CGTase protein in the ammonium sulphate precipitated fraction. Gel filtration chromatography could be used as final polishing step. A coomassie brilliant blue dyed

Table 1
Purification of the CGTase from *Paenibacillus* sp. F8

Step	Volume (mL)	Protein (μg/mL)	Protein total (μg)	mUnits/mL	mUnits total	Specific activity (Units/mg protein)	Yield (%)	Purification factor
Culture supernatant	1875	145	272000	28.1	52600	0.19	100	1
Ammonium sulphate precipitation (70%)	37	3420	127000	473	17500	0.14	33	0.72
β-CD Sepharose 6B	60	26.1	1560	258	15500	9.93	29	52.3
Dialysis	3.2	215	687	4790	15300	22.3	29	115
Gel filtration	128	4.14	530	126	16100	30.3	30	157

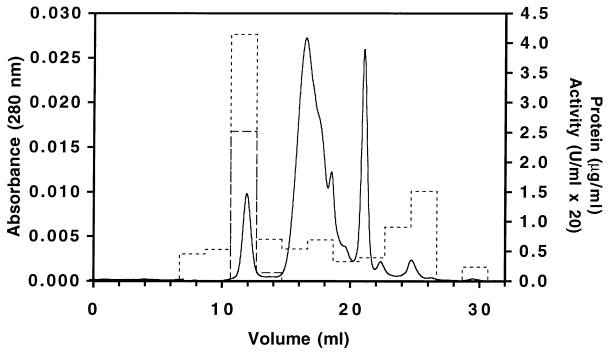

Fig. 1. Gel chromatographic separation of *Paenibacillus* sp. CGTase. 50 μl sample containing 240 mUnits of CGTase was applied. Solid line: absorbance at 280 nm, Dotted line: protein content, Dashed line: CGTase activity.

sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel of the purified CGTase fraction showed the presence of only one protein band with an apparent molecular weight of 72 kDa indicating that the additional peak at 4.6 min detected by SDS-CGE of the same fraction was due to an artefact.

Characterisation of the CGTase.—The effect of pH on the cyclisation activity and the hydrolytic activity of the enzyme was investigated. A maximum of cyclisation activity was obtained at pH 7.5, while the optimum for the hydrolytic activity was found at pH 8.0 [Fig. 3(A)]. The enzyme remained stable for 1 h between pH 6.0 and 8.0 for the cyclisation activity and between pH 6.0 and 9.0 for the hydrolytic activity [Fig. 3(B)]. The temperature optimum of the cyclisation and the hydrolytic activity was found at 50 and 60 °C, respectively [Fig. 4(A)]. Fig. 4(B) shows the residual cyclisation and hydrolysis activity in the presence and absence of Ca^{++} after incubation for 1 h at different temperatures. Both the cyclisation and the hydrolytic activity were stable up to 40 °C without Ca^{++}. In the presence of Ca^{++}, the hydrolytic activity was stable up to 60 °C whereas the cyclisation activity was stable up to 50 °C. Differences in the pH and temperature stability of the hydrolytic and the cyclisation activity and the stabilising effect of Ca^{++} on the activity of CGTases had been reported previously [24–28]. The formation of α-, β- γ- and δ-CD by the CGTase using 5% soluble starch as substrate is shown in Fig. 5. The molar ratio of α-, β- γ- and δ-CD, calculated from the initial slope of the production curve, was 0.09:1:0.25:0.14. The molar ratio between the four CD changed during incubation over a 24 h period. The amount of β-CD

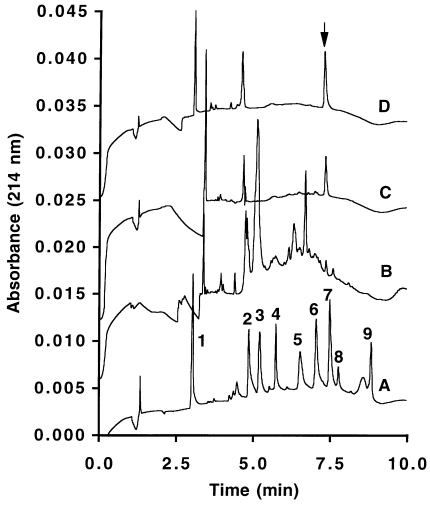

Fig. 2. SDS-CGE electropherograms of protein fractions in different purification steps. (A) Standard protein mixture: 1. Benzoic acid, 2. Lysozyme (14.4 kDa), 3. Trypsin inhibitor (21.5 kDa), 4. Carbonic anhydrase (31 kDa), 5. Ovalbumin (45 kDa), 6. Serum albumin (66.2 kDa), 7. Phosphorylase B (97 kDa), 8. β-Galactosidase (116 kDa), 9. Myosin (200 kDa). (B) Ammonium sulphate precipitated fraction (70% saturation), CGTase activity 15 mU/mL. (C) pooled fractions after the β-CD Sepharose 6B chromatography step, CGTase activity 240 mU/mL. (D) purified CGTase after gel filtration chromatography, CGTase activity 400 mU/mL. The arrow indicates the position of the CGTase peak.

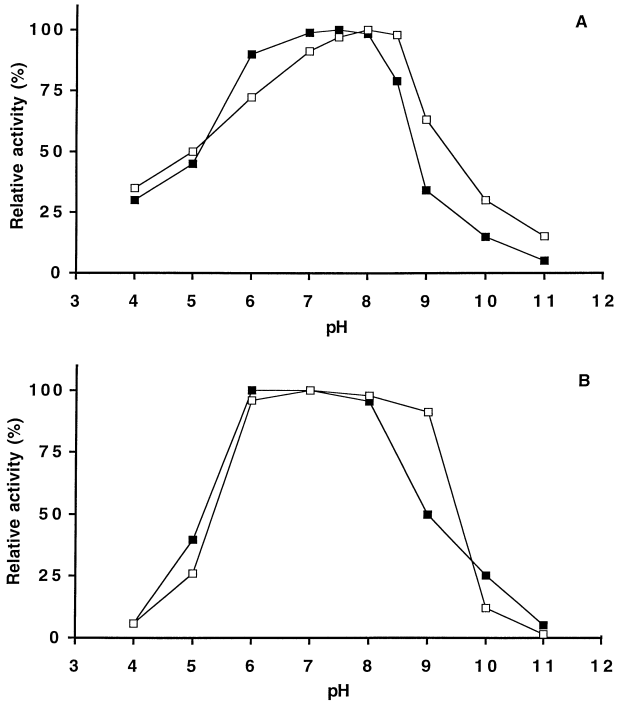

Fig. 3. Effect of pH on the activity and stability of the purified CGTase: (A) effect of pH on the activity. ■ cyclization activity, □ hydrolysis activity; (B) stability of the purified CGTase at different pH. ■ cyclisation activity, □ hydrolysis activity.

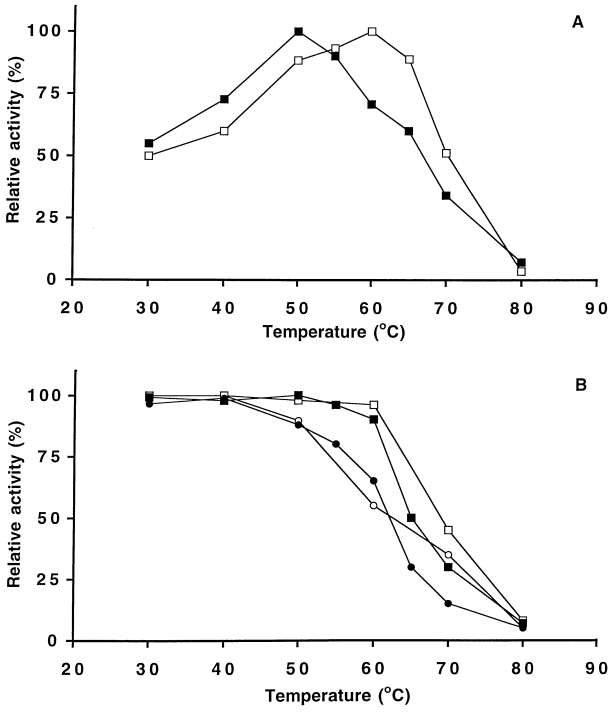

Fig. 4. Effect of temperature on the activity and stability of the purified CGTase: (A) effect of temperature on the activity. ■ cyclization activity, □ hydrolysis activity; (B) stability of the purified CGTase at different temperatures. ■ cyclisation activity with 10 mM $CaCl_2$, ● cyclisation activity with 10 mM EDTA, □ hydrolysis activity with 10 mM $CaCl_2$, ○ hydrolysis activity with 10 mM EDTA.

produced remained almost constant during the production process and showed a small increase from 67 to 70% based on the molar ratio. The amount of γ- and δ-CD decreased from 18 to 17% and 9 to 4%, respectively, while the amount of α-CD increased from 6 to 9%.

Comparison of the coupling activity of the CGTase with of α-, β-, γ- and δ-CD showed that δ-CD was a much better substrate for coupling

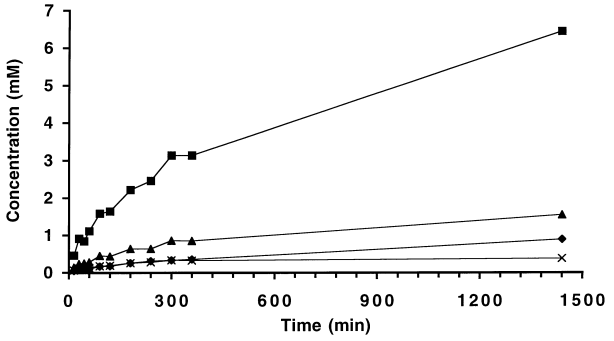

Fig. 5. Formation of α-, β-, γ-, and δ-CD by the purified CGTase (15 mU/mL) using 5% soluble starch as substrate at 50 °C and pH 7.0. ▲ α-CD, ■ β-CD, ◆ γ-CD, × δ-CD.

reactions compared to the smaller CD. The coupling rate with maltotetraose was higher than with maltose (Table 2). It is therefore likely that the rearrangement of δ-CD and to some extent γ-CD into α- and β-CD during incubation of starch with CGTase was caused by the higher coupling activity with these two CD. Which may be the result of an higher flexibility of these molecules compared to α- and β-CD.

Analysis of the reaction products by high performance anion exchange chromatography (HPAEC) showed that CD were the main products formed by the *Paenibacillus* CGTase using soluble starch as the substarte, while only a small amount of linear compounds were produced (Fig. 6). The retention times of the different cyclic and linear oligo-saccharides is shown in Table 3. A small increase in the amount of reducing sugars detected during the enzyme reaction also indicated that only a few non-cyclic compounds had been produced.

CGTases from different microorganisms have been described previously (Table 4). These include *Thermoanaerobacterium* [25], *Thermoanaerobacter* [29], *Clostridium* [30], *Micrococcus* [27], *Klebsiella* [31], *Brevibacterium* [28] and several *Bacillus* species [14–16]. Although most of these CGTases have been characterised with respect to their catalytic activity towards the formation of α-, β-, and γ-CD, quantitative data on the production of large CD have not been reported. The ratio of the production of α-, β-, and γ-CD by *Paenibacillus* sp. F8 is similar to the values found in the literature for other CGTases [14–16]. Less accurate analysis and determination methods used for the determination of the CD production ratio made a detailed comparison with previously reported results on CD production by CGTases difficult. All CGTases previously investigated produced more than one type of CD. Although the dominating end product (α-, β-, or γ-CD) may vary, β-CD was generally found to be the major product followed by α- and γ-CD.

The *Paenibacillus* sp. F8 CGTase also showed comparable properties with other CGTases

Table 2
Coupling activity of the purified CGTase (U/mg)

	Maltose	Maltotetraose
α-CD	0.4	0.2
β-CD	0.3	0.4
γ-CD	1.0	1.0
δ-CD	4.8	8.2

regarding the apparent molecular weight, the temperature optimum, the stabilising effect of Ca^{++} and the pH optimum and stability.

3. Experimental

Materials.—Benzoic acid, maltose, maltoheptaose, carboxymethylcellulose and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St Louis, MO, USA. Phenolphthalein (3,3-bis(4-hydroxyphenyl)-1(3*H*)-isobenzofuranone, 3,3-bis(4-hydroxyphenyl) phthalide), ethylenedinitrilo tetraacetic acid disodium salt (EDTA), glucose and soluble starch were obtained from E. Merck, Darmstadt, Germany. Oxoid yeast extract was obtained from Unipath Ltd., Basingstoke, Hampshire, UK. Bacto tryptone was obtained from DIFCO lab., Detroit, MI, USA. Brown rice from Neue Allgemeine Reisgesellschaft mbH, Hamburg, Germany was used. Meriwax waxy maize starch was obtained from Amylum, Aalst, Belgium. Pharmaceutical grade α-, β-, and γ-CD were a gift from Wacker Chemie Danmark ApS, Glostrup, Denmark. δ-CD was provided by Dr H. Ueda, Hoshi University, Tokyo. All chemicals were, unless stated otherwise, of analytical grade.

Isolation of Paenibacillus *sp. F8*.—Strain F8 was isolated from a wheat sample collected at Drabækmølle, Lunderskov, Denmark near a heater aggregate as described in Larsen et al. (1998) [22].

Production of CGTase.—*Paenibacillus* sp. F8 was grown in a medium consisting of 2% w/v whole rice, 0.5% w/v yeast extract, 0.5% w/v tryptone, 0.2% w/v K_2HPO_4, 0.2% w/v Na_2HPO_4, 0.02% w/v $MgSO_4.7H_2O$, 0.02% w/v $CaCl_2.2H_2O$ and 0.1% w/v $(NH_4)_2SO_4$ for 24 h at 37 °C in a shaking incubator at 200 rpm.

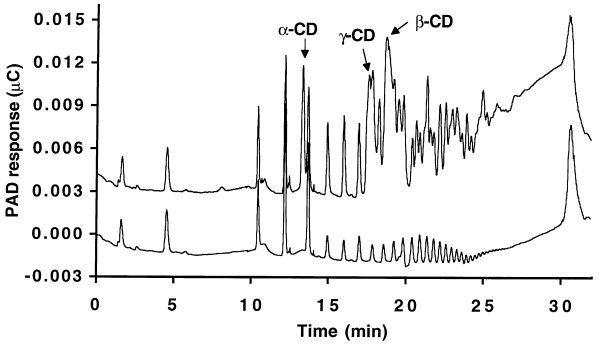

Fig. 6. HPAEC chromatograms of (bottom) 5% soluble starch and (top) after 24 h of incubation with 15 mU/mL CGTase. Twenty-five μl sample diluted 1:5 with water was loaded onto the column. The retention times of the compounds are shown in Table 3. It should be noted that linear oligosaccharides gives an approximately 11 times higher detector response (based on peak area) on a molar basis compared to the cyclic oligosaccharides when using HPAEC-PAD.

Purification of CGTase.—Cells and insoluble material were removed from the fermentation broth by centrifugation. The CGTase was concentrated by ammonium sulphate precipitation (70% saturation). Precipitated material was dissolved in 50 mM phosphate, pH 7.0 and 2 mM $CaCl_2$. The CGTase was further purified using an affinity gel prepared by covalently binding β-CD to an epoxy-activated Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden) [33]. Three g of freeze-dried epoxy activated sepharose 6B was washed on a sintered glass filter with 600 mL of water. The gel was mixed with 0.9 g β-CD in 20 mL 0.1 M NaOH at 45 °C for 24 h. Unreacted epoxy groups were blocked in 25 mL 1 M ethanolamine for 24 h at 45 °C and washed with 600 mL of each 0.1 M acetate buffer pH 4.0, 0.1 M NaCl; 0.1 M NaOH, 0.5 M NaCl and 0.01 M phosphate buffer pH 9.0.

Table 3
Retention times of cyclic and linear oligosaccharides separated by HPAEC (Fig. 6)

DP[a]	Retention time (min)	n[b]
1	4.54 ± 0.02	22
2	10.44 ± 0.02	24
3	12.18 ± 0.01	24
6 (α-CD)	13.26 ± 0.10	13
4	13.69 ± 0.04	24
5	14.93 ± 0.02	24
6	15.99 ± 0.02	24
7	16.98 ± 0.03	24
8 (γ-CD)	17.64 ± 0.15	4
8	17.84 ± 0.01	14
9	18.56 ± 0.01	12
10	19.22 ± 0.01	13
9 (δ-CD)	19.31 ± 0.20	2
7 (β-CD)	19.37 ± 0.20	11
11	19.81 ± 0.01	13
12	20.36 ± 0.01	11
13	20.87 ± 0.01	11
14	21.34 ± 0.01	11
15	21.77 ± 0.01	11
16	22.19 ± 0.01	11
17	22.57 ± 0.02	11
18	22.94 ± 0.02	11
19	23.28 ± 0.02	11
20	23.60 ± 0.02	6
21	23.90 ± 0.02	6
22	24.19 ± 0.02	6
23	24.46 ± 0.01	6
24	24.76 ± 0.10	6
25	25.00 ± 0.10	6

[a] DP, degree of polymerisation.
[b] n, denotes the number of measurements.

Table 4
Comparison of CGTases from various microorganisms

Origin	Molecular weight (Da) SDS-PAGE	Calculated[a]	Optimum Temperature (°C)	Heat stability −Ca	Heat stability +Ca	Optimum pH	pH stability	Major product	References
Paenibacillus sp. F8	72,000	—	50	40	50	7.5	6.0–8.0	β-CD	This work
Brevibacterium sp. no. 9605	75,000	—	45	30	40	8.0–9.0	6.0–8.0	γ-CD	[28,39]
Thermoanaerobacter sp. ATCC 53627	103,000 110,000 117,000	75,291	90–95	80		5.8	5.0–6.7	β-CD	[29,30,40]
Thermoanaerobacterium thermosulfurigenes EMI	68,000	75,100	80–85	70[b]	—	4.5–7.0	—	β/α-CD	[25]
Micrococcus varians M 849	85,000[c]	—	55–65	45	50	5.0–8.0	5.0–9.0	α/β-CD	[27]
Bacillus circulans E 192	78,000	—	60	—	45	5.5–5.8	7.0–8.0	β-CD	[41]
Bacillus macerans IFO 3490	75,000	74,008	55	55	—	5.2–5.7	8.0–10.0	α-CD	[42–44]
Alkalophilic *Bacillus* sp. 38-2 Neutral CGTase	88,000	—	50	60	70	7.0	6.0–8.0	β-CD	[26,46]
Alkalophilic *Bacillus* sp. 38-2 Acidic CGTase	88,000	—	45	65	65	4.5–4.7	6.0–10.0	β-CD	[45,46]
Klebsiella oxytoca M5Al	—	69029	—	—	45	6.0–7.5	5.0–7.5	α/β-CD	[31,47,48]

pH and temperature optimum and stability refer to the cyclisation reaction.
[a] Derived from the amino acid sequence of the enzyme.
[b] In the presence of starch.
[c] Determined by ultracentrifugation.

β-CD Sepharose 6B gel (3 g) was mixed with the sample for 1 h at room temperature in 50 mL centrifuge tubes under continuous stirring. The supernatant was decanted after centrifugation. Unbound protein was removed by mixing the gel with 30 mL 50 mM phosphate, pH 7.0 and 2 mM $CaCl_2$ for 10 min under continous stirring followed by centrifugation and removal of the supernatant. This procedure was repeated eight times. Bound CGTase was eluted with 5×10 mL 20 mM α-CD in 50 mM phosphate, pH 7.0, 2 mM $CaCl_2$, each for 20 min under continous stirring. The pooled elution fractions were concentrated 18 fold in a Servapor dialysis tube (Serva, Heidelberg, Germany) with a molecular weight cut off of 12–14 kDa placed in carboxymethylcellulose. Gel filtration chromatography was performed using a FPLC system (Pharmacia Biotech, Uppsala, Sweden). Aliquots of 50 μl were loaded onto a a prepacked Sephadex 75 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) and eluted with 50 mM acetate buffer, pH 6.0, 2 mM $CaCl_2$.

The protein content was estimated by the method of Bradford, 1976 [23] using the BIO-RAD Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as standard.

CGTase assays.—For the determination of the temperature optimum, the purified CGTase was incubated in 2% soluble starch, 10 mM $CaCl_2$, 100 mM phosphate buffer, pH 8.0 at 30, 40, 50, 55, 60, 65, 70 and 80 °C. Determination of temperature stability was performed by incubating the purified CGTase in 100 mM phosphate buffer, pH 8.0 for 1 h at 30, 40, 50, 55, 60, 65, 70 and 80 °C in the presence of 10 mM $CaCl_2$ or 10 mM EDTA. Residual activity was determined by incubation with 2% soluble starch, 100 mM phosphate, pH 8.0 with 10 mM $CaCl_2$ or 10 mM EDTA. The pH optimum was determined in 2% soluble starch, 10 mM $CaCl_2$ solubilised in 100 mM acetate buffer (pH 4 and 5), 100 mM phosphate buffer (pH 6–8) and 100 mM borate buffer (pH 9–11). The pH stability was determined by incubation of purified CGTase in 10 mM actual buffer without starch (see above) at 50 °C for 1 h prior to the determination of residual activity by incubation in 2% soluble starch, 100 mM phosphate and 10 mM $CaCl_2$.

The reactions were stopped by boiling for 5 min. Cyclisation activity was determined by CE [21] and hydrolysis activity was determined using the $CuSO_4$/bicinchonate reducing sugar assay [34]. One unit of cyclisation activity corresponds to the amount of enzyme that produces 1 μmole CD/min at 50 °C and pH 7.0.

The production of CD by CGTase was determined by incubating 5% w/v soluble starch in 50 mM phosphate buffer, pH 7.0, 2 mM $CaCl_2$ with

aliquots of the purified CGTase preparation for varying lengths of time at 50 °C. For CE analysis, the enzyme reactions were stopped by the addition of three volumes of cold methanol and placed at 4 °C for 30 min. After the precipitated material was removed by centrifugation, the CD containing supernatant was decanted and dried in a heating block at 100 °C. The dried CD fractions were dissolved in double distilled water prior to analysis. Samples for HPLC analysis were stopped by boiling for 10 min.

The coupling activity of the CGTase was measured by incubating 5 mM α-, β-, and γ-CD with 5 mM maltose or maltotetraose and 1 mM δ-CD with 1 mM maltose or maltotetraose with purified CGTase (15 mU/mL) for various lengths of time. The amount of residual CD were determined by CE and the coupling activity was calculated using the slopes of the CD degradation curves. The coupling activity is defined as the amount of enzyme degrading 1 μmol of CD/min. at pH 7.0 and 50 °C in the presence of a given acceptor.

Maltotetraose was prepared using maltotetraohydrolase (EC 3.2.1.60) from *Pseudomonas stutzeri* [35]. Meriwax waxy maize starch (50 mg/mL) was gelatinised at 100 °C for 15 min and cooled to room temperature under continuous stirring. Maltotetraohydrolase (7.8 U/g starch) was added to the gelatinised starch and incubated at 40 °C under continuous stirring for 120 min. One unit of maltotetraohydrolase activity corresponds to the amount of enzyme that hydrolyses one μmole of glycosidic bonds/min at 37 °C and pH 7.0. The reaction was stopped by the addition of two volumes of ethanol [36] and the precipitate was removed by centrifugation at 10,000 *g* for 15 min. Ethanol was removed from the supernatant by evaporation at 50 °C at reduced pressure prior to lyophilisation. The lyophilised products were desalted using a molecular grade AG 501-X8(D) mixed bed resin (Bio-Rad Laboratories, Hercules, CA, USA) (6 g/g of lyophilisate). The maltotetraose preparation had approximately 85% purity determined by HPAEC-PAD. Maltotriose was the main contaminant.

Analysis of CD.—The analysis of CD was performed as described in Larsen and Zimmermann, 1998 [21]. Capillary electrophoretic analysis of δ-CD was performed according to Larsen et al. (1997) [32] using a range of aromatic anions as BGE in the concentration range of 50 to 100 mM in 2 mM phosphate buffer, pH 7.0. The effective mobility of the δ-CD/aromatic anion complex in the CGTase assays was equal to the effective mobility of a standard δ-CD aromatic anion complex in all BGE solutions used.

Analysis of linear oligosaccharides.—HPAEC-PAD was performed using a CarboPac PA-1 column on a Dionex DX-300 system (Dionex Corporation, Sunnyvale, CA, USA). The starch degradation products formed by the action of CGTase were separated in 0.1 M NaOH with a gradient of 1.0 M Na-acetate from 0 to 43% from 5 to 30 min after injection. Residual bound material was eluted by raising the Na-acetate concentration from 43 to 90% over 1 min. The starch hydrolysis products were identified using α-, β-, γ- and δ-CD, glucose, maltose and maltoheptaose as standards. Since the retention times of homologous linear oligosaccharides increases with the degree of polymerization, linear oligosaccharides of intermediate D.P. could be easily identified [37,38].

SDS-CGE.—Prior to SDS-CGE analysis, the samples were concentrated and concomitantly desalted by use of 10 kDa Microsep centrifuge filters (Filtron, Northborough, MA, USA). This step also removed residual α-CD, which could otherwise interfere with the binding of sodium dodecyl sulphate to the proteins. Samples for SDS-CGE were prepared by mixing 5 μl sample, 5 μl Bio-Rad CE-SDS protein kit sample buffer (Bio-Rad Laboratories, CA, USA) and 1 μl 1 mg/mL benzoic acid. After thorough mixing, the samples were boiled for 10 min and placed in an icebath for 3 min. The supernatant was applied to the capillary after centrifugation at 14,000 rpm for 5 min.

SDS-CGE was performed on a Beckman P/ACE System 5010 equipped with a P/ACE UV absorbance detector. Capillaries were 27 cm (20 cm to detector) 50 μm i.d. fused silica capillaries obtained from Composite Metal, USA. Separations were performed using the Bio-Rad CE-SDS protein kit run buffer (Bio-Rad Laboratories, CA, USA). Prior to analysis, the capillary was rinsed with 1 M NaOH for 3 min, followed by water for 30 s. and subsequently filled with Bio-Rad CE-SDS protein kit run buffer for 4 min, all by use of a high pressure purge (20 psi). Before the sample was loaded onto the capillary, excess gel on the outer surface of the cathodic side of the capillary was washed off in two vials containing water. Sample loading was performed by a low pressure purge (0.5 psi) for 90 s at the cathodic side of the capillary. Separation was carried out at 16.80 kV constant voltage for 10 min

with a Bio-Rad CE-SDS protein kit run buffer at both cathode and anode. Absorbance was recorded at 214 nm and capillary temperature was maintained at 25 °C.

Acknowledgements

Kim Lambertsen Larsen was supported by grant no. T.130-9 from the Danish Research Academy. Dr. H. Ueda, Hoshi University, Tokyo is acknowleged for a kind gift of δ-CD. Wacker Chemie Danmark Aps, Glostrup, Denmark are acknowledged for their donation of α-, β-, and γ-CD.

References

[1] J. Szejtli, *Cyclodextrin Technology*, Kluwer Academic, Dordrecht, The Netherlands, 1988.
[2] K.-H. Frömming and J. Szejtli, *Cyclodextrins in Pharmacy*, Kluwer Academic, Dordrecht, The Netherlands, 1994.
[3] J. Szejtli, *Med. Res. Rev.*, 14 (1994) 353–386.
[4] E. Albers and B.W. Müller, *CRC Crit. Rev. Ther. Drug Carrier Syst.*, 12 (1995) 311–337.
[5] D.O. Thompson, *CRC Crit. Rev. Ther. Drug Carrier Syst.*, 14 (1997) 1–104.
[6] W.L. Hinze, *Sep. Purif. Methods*, 10 (1981) 159–237.
[7] C.J. Easton and S.F. Lincoln, *Chem. Soc. Rev.*, (1996) 163–170.
[8] T. Fujiwara, N. Tanaka, and S. Kobayashi, *Chem. Lett.*, (1990) 739–742.
[9] T. Endo, H. Ueda, S. Kobayashi, and T. Nagai, *Carbohydr. Res.*, 269 (1995) 369–373.
[10] T. Endo, H. Nagase, H. Ueda, S. Kobayashi, and T. Nagai, *Chem. Pharm. Bull.*, 45 (1997) 532–536.
[11] T. Endo, H. Nagase, H. Ueda, A. Shigihara, S. Kobayashi, and T. Nagai, *Chem. Pharm. Bull.*, 45 (1997) 1856–1859.
[12] Y. Terada, M. Yanase, H. Takata, T. Takaha, and S. Okada, *J. Biol. Chem.*, 272 (1997) 15729–15733.
[13] T. Takaha, M. Yanase, H. Takata, S. Okada, and S.M. Smith, *J. Biol. Chem.*, 271 (1996) 2902–2908.
[14] M. Vihinen and P. Mäntsälä, *Crit. Rev. Biochem. Mol. Biol.*, 24 (1989) 329–417.
[15] S. Kobayashi, *Cyclodextrin Producing Enzyme (CGTase)*, in K.H. Park, J.F. Robyt, and Y.-D. Choi (Eds.), *Enzymes for Carbohydrate Engineering*, Elsevier Science, Amsterdam, 1996, pp 23–41.
[16] S. Kitahata, *Cyclomaltodextrin Glucanotransferase*, in The Amylase Research Society of Japan (Eds.) *Handbook of Amylases and Related Enzymes*, Pergamon Press, Oxford, 1988, pp 154–164.
[17] D. French, *The Schardinger dextrins*, in M.L. Wolfrom, and R.S. Tipson (Eds.) *Advances in Carbohydrate Chemistry*, Vol. 12, Academic Press, New York, 1957, pp 189–260.
[18] D. French, A.O. Pulley, J.A. Effenberger, M.A. Rougvie and M. Abdullah, *Arch. Biochem. Biophys.*, 111 (1965) 153–160.
[19] I. Miyazawa, H. Ueda, H. Nagase, T. Endo, S. Kobayashi, and T. Nagai, *Eur. J. Pharm. Sciences*, 3 (1995) 153–162.
[20] A. Wakamiya, T. Endo, H. Nagase, H. Ueda, S. Kobayashi, and T. Nagai, *Yakuzaigaku*, 57 (1997) 220–223.
[21] K.L. Larsen and W. Zimmermann, *J. Chromatogr. A*, 811: 193–199.
[22] K.L. Larsen, H.J.S. Christensen, F. Mathiesen, L.H. Pedersen, and W. Zimmermann, *Appl. Microbiol. Biotechnol.*, in press.
[23] M. Bradford, *Anal. Chem.*, 72 (1976) 248–254.
[24] M. Nomoto, C.-C. Chen, and D.-C. Sheu, *Agric. Biol. Chem.*, 50 (1986) 2701–2707.
[25] R. Wind, W. Liebl, R.M. Buitelaar, D. Penninga, A. Spreinat, L. Dijkhuizen, and H. Bahl, *Appl. Environ. Microbiol.*, 61 (1995) 1257–1265.
[26] N. Nakamura and K. Horikoshi, *Agric. Biol. Chem.*, 40 (1976) 1785–1791.
[27] Y. Yagi, K. Kouno, and T. Inui, US Patent 4 317 881 (1980).
[28] S. Mori, S. Hirose, T. Oya, and S. Kitahata, *Biosci. Biotech, Biochem.*, 58 (1994) 1968–1972.
[29] B.E. Norman and S.T. Jørgensen, *Denpun Kagaku*, 39 (1992) 101–108.
[30] R.L. Starnes, P.C. Trackman, and D.M. Katkocin, US Patent 5 501 968 (1996).
[31] H. Bender, *Arch. Microbiol.*, 111 (1977) 271–282.
[32] K.L. Larsen, F. Mathiesen, and W. Zimmermann, *Carbohydr. Res.*, 298 (1997) 59–63.
[33] E. Laszlo, K. Banky, G. Seress, G., and J. Szejtli, J., *Starch*, 33 (1981) 281–283.
[34] L.H. Pedersen, H.J.S. Christensen, F. Mathiesen, K.L. Larsen, and W. Zimmermann, *Starch*, 49 (1997) 250–253.
[35] J.F. Robyt and R.J. Ackerman, *Arch. Biochem. Biophys.*, 145 (1971) 105–114.
[36] J.F. Robyt and R.J. Ackerman, *Methods Enzymol.*, 28 (1972) 925–930.
[37] Y.C. Lee, *J. Chromatogr. A*, 720 (1996) 137–149.
[38] R.N. Ammerall, G.A. Delgado, F.L. Tenbarge, and R.B. Friedman, *Carbohydr. Res.*, 215 (1991) 179–192.
[39] S. Mori, T. Mase, and T. Ohya, Eur. Patent Appl. 0 614 971 A2 (1994).
[40] R.L. Starnes, K. Amemiya, P.L. Jorgensen, and S. Jorgensen, *Cloning and Expression of the Thermoanaerobacter s.p. Cyclodextrin Glycosyl*

Transferase Gene in Escherichia coli *and* Bacillus subtilis, in A. R. Hedges (Ed.), *Minutes of the Sixth International Symposium on Cyclodextrins*, Editions de sante, Paris, 1992, pp 46–52.
[41] L.J. Bovetto, D.P. Backer, J.R. Villette, P.J. Sicard, and S.J.-L. Bouquelet, *Biotech. Appl. Biochem.*, 15 (1992) 48–58.
[42] S. Kitahata and S. Okada, *J. Jap. Soc. Starch Sci.*, 29 (1982) 7–12.
[43] S. Kitahata and S. Okada, *J. Jap. Soc. Starch Sci.*, 29 (1982) 13–18.
[44] S. Fujiwara, H. Kakihara, K.B. Woo, A. Lejeune, M. Kanemoto, K. Sakaguchi, and T. Imanaka, *Appl. Environ. Microbiol.*, 58 (1992) 4016–4025.
[45] N. Nakamura and K. Horikoshi, *Agric. Biol. Chem.*, 40 (1976) 935–941.
[46] K. Horikoshi, *Enzymology and Molecular Genetics of CD-forming Enzymes*, in O. Huber and J. Szejtli (Eds.), *Proceedings of the Fourth International Symposium on Cyclodextrins*, Kluwer Academic, Dordrecht, The Netherlands, 1988, pp 7–17.
[47] H. Bender, *Carbohydr. Res.*, 124 (1983) 225–233.
[48] F. Binder, O. Huber, and A. Böck, *Gene*, 47 (1986) 269–277.