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Directed enzymatic synthesis of linear and branched gluco-oligosaccharides, using cyclodextrin-glucanosyltransferase

Dirk Vetter, Werner Thorn,
Institut für Biochemie und Lebensmittelchemie der Universität Hamburg, Martin-Luther-King-Platz 6, D-2000 Hamburg 13 (F.R.G.)

Heiko Brunner, and Wilfried A. König
Institut für Organische Chemie der Universität Hamburg, Martin-Luther-King-Platz 6, D-2000 Hamburg 13 (F.R.G.)

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ABSTRACT

Cyclodextrin-glucanosyltransferase, in a kinetically controlled reaction, transfers one maltohexaosyl residue from cyclomaltohexaose (αCD) to HO-4 of an acceptor to form a linear or a branched gluco-oligosaccharide. The primary transfer product can be isolated in yields up to 45% and in high purity, if the reaction is stopped at an early stage. With increasing time of incubation, secondary and tertiary transfer products are formed by stepwise addition of maltohexaosyl units. At equilibrium, a mixture with almost equal proportions of oligosaccharides is obtained. Glucose and malto-oligosaccharides of any chain length carrying a free 4-hydroxyl group and with HO-1 free or substituted, and regardless of the configuration at C-1, may serve as acceptors. Substrates with *galacto* or *manno* configuration were not utilised by the enzyme. The selectivity of the enzyme with respect to the site of chain elongation in branched acceptor molecules has been investigated. The technique described here may be applied to prepare linear gluco-oligosaccharides of any chain length or branched oligosaccharides of the amylopectin type.

INTRODUCTION

Malto-oligosaccharides with a d.p. up to 7 can be isolated from "glucose syrup" or by hydrolysis of cyclomalto-hexaose (αCD) or -heptaose βCD. Problems arise if oligosaccharides with d.p. >7 need to be isolated by gel-permeation chromatography. In addition, branched oligosaccharides that originate from the amylopectin component of starch are present in considerable proportions and they are difficult to separate from other higher oligosaccharides.

Enzymes may also be used to synthesise higher gluco-oligosaccharides. The reaction of maltotetraose with D-glucose 1-phosphate under the action of potato phosphorylase[1] yields a mixture of oligosaccharides with a maximum yield at d.p. ~ 20. However, separation of the components of this complex mixture is tedious.

Higher gluco-oligosaccharides can be prepared by utilising the disproportionating properties of D-enzyme[2]. In a model reaction, the maximum yield was found at d.p. ~ 3 with decreasing proportions of the higher oligosaccharides, and fractionation of the mixture was necessary. French *et al.*[3] reported that fragments of αCD can be coupled to

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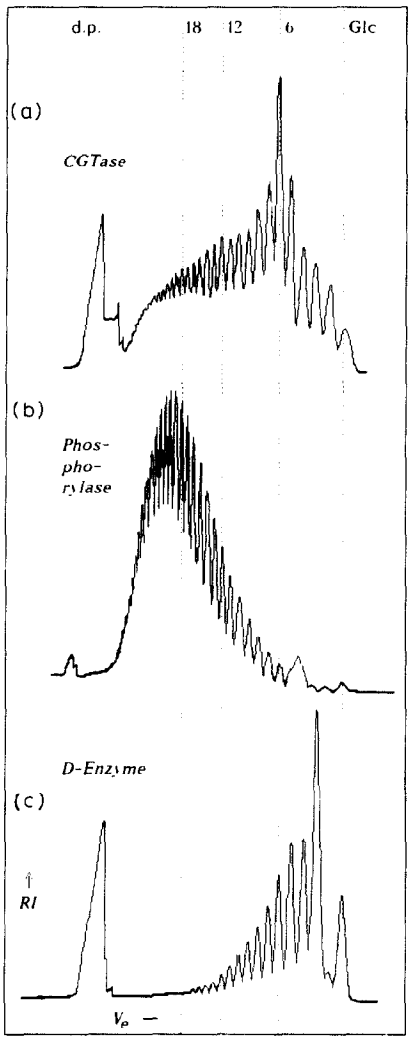

Fig. 1. Gel-permeation chromatography of (a) the products of the reaction of D-glucose with αCD and CGTase, (b) the products of the reaction of D-glucose 1-phosphate with maltotetraose and phosphorylase (sample by courtesy of C. Niemann, Technische Universität Berlin), and (c) the disproportionation of maltotriose by D-enzyme.

gluco-oligosaccharides by cyclodextrin-glucanosyltransferase (CGTase). Bender[4] also used this reaction to produce glucans. However, no detailed kinetic investigations of the coupling reaction have been reported. A comparison of the distribution of products in the different enzymatic syntheses is given in Fig. 1.

Higher linear or branched gluco-oligosaccharides are of interest as model compounds for mass spectrometry and for studies of supramolecular properties. The methods now described can yield pure linear or branched (1→4)-linked gluco-oligosaccharides of defined chain length in good yields.

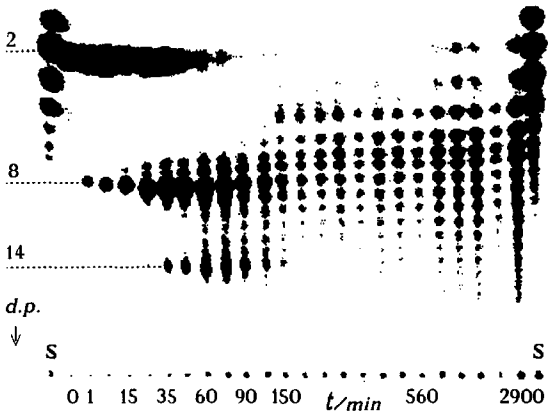

Fig. 2. T.l.c. of the products of the reaction of αCD and maltose in the presence of CGTase; S is a mixture of standards.

RESULTS AND DISCUSSION

Directed synthesis of linear glucans. — Mainly the two types of reaction shown in Eqs. *1* and *2*[4] are catalysed by CGTase[5].

$$\text{D-Glc}_{(n+6)} \xrightleftharpoons[\text{Coupling}]{\text{Cyclisation}} \text{D-Glc}_n + \alpha\text{CD} \qquad (1)$$

$$2\ \text{D-Glc}_n \xrightarrow{\text{Disproportionation}} \text{D-Glc}_{(n+a)} + \text{D-Glc}_{(n-a)}\ (1<a<n) \qquad (2)$$

The reaction in Eq. *1* is predominant only at the beginning of the reaction (see Fig. 2).

Using maltose as an acceptor, the coupling reaction with αCD under the action of CGTase yielded malto-octaose almost exclusively during the first 15 min. Subsequently, products of d.p. 14 and 20 gradually appeared. The maximum yields were observed after 40 min for malto-octaose (45%), after 90 min for the product with d.p. 14 (15%), and after 375 min for the product with d.p. 20 (7%) (160 K.U. of CGTase, 2.3 mmol of maltose, and a 3-fold excess of αCD; Fig. 3).

The yields of products depended on the concentration of αCD and increased up to a 15-fold excess of αCD. A larger excess of αCD caused solubility and isolation problems. Variations of other reaction parameters, although affecting the rates of reaction, do not alter the selectivity of the transfer reactions. After the consumption of maltose, the disproportionating activity of the enzyme according to Eq. *2* takes over and finally a homogeneous distribution of chain lengths in the products is reached. Hence, high yields of uniform products can be obtained if the reaction is interrupted at an early stage. This situation is also demonstrated in Fig. 4, which shows the CGTase-catalysed coupling reaction of maltose with αCD and the distribution of products as a function of the time of incubation. The primary coupling product (d.p. 8) can be isolated in highest

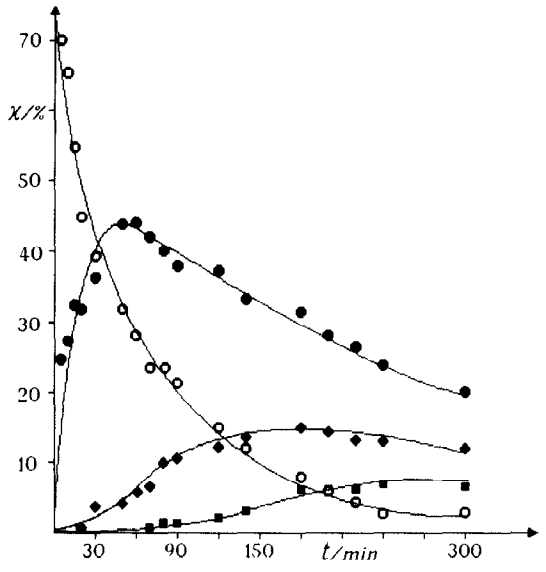

Fig. 3. Kinetics of the coupling reaction of αCD with maltose under CGTase catalysis: acceptor (○), and the products with d.p. 8 (●), 14 (◆), and 20 (■).

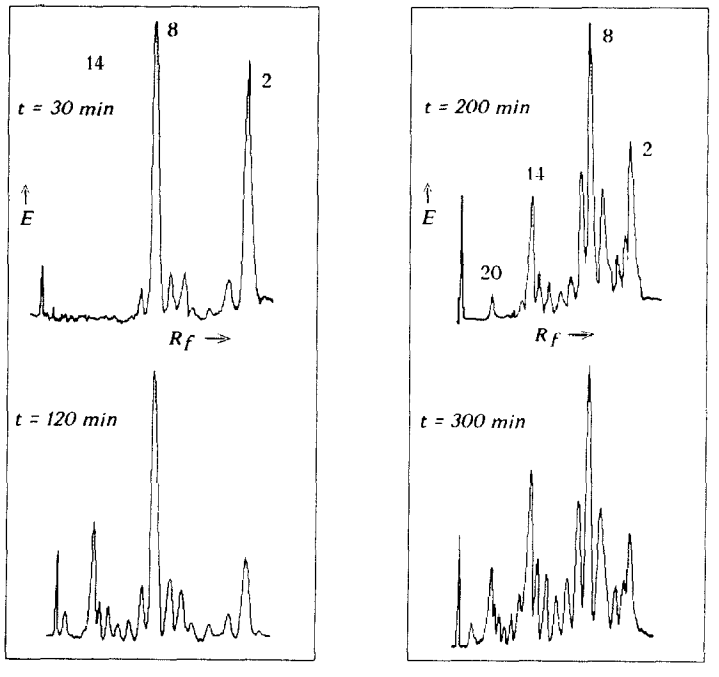

Fig. 4. T.l.c.-densitograms (see Experimental) of the products of the coupling reaction of maltose and αCD under CGTase catalysis: d.p. indicated by the numbers on the peaks.

yield and purity. At longer times of reaction, the disproportionating property of the enzyme produces a greater variety of compounds. Thus, from maltotriose (1 g), products of d.p. 9 (586 mg), 15 (71 mg), and 21 (3 mg) could be obtained (20 K.U. of CGTase; 19.3 g of αCD; reaction time, 14 h).

In order to investigate the relationship between chain length and acceptor quality, D-glucose, maltose, and malto-oligosaccharides of d.p. 3–13 were submitted to the coupling reaction with αCD. Maltose was by far the best acceptor, as also observed by Bender[4]. The preference of methyl α-D-glucopyranoside over D-glucose also suggests that the enzyme recognises the configuration at the anomeric centre and generally prefers the α configuration, which is present to a minor degree in glucose. A notable drop in acceptor quality was observed for maltohexaose (possibly due to a competitive inhibition of the hexaosyl-transfer by the enzyme) and for maltododecaose, where an inversion of the transfer reaction may result in the formation of the product with d.p. 6.

The sensitivity of the enzyme towards changes in the structure of the (1→4)-α-D-glucan structure was also investigated. The results in Table I indicate high selectivity towards HO-4 and sensitivity to the configuration at C-2 and C-3.

The only variable tolerated was the substituent at the anomeric centre, although there are exceptions. α-D-Glucopyranose 1-phosphate and trehalose are not acceptors for the enzyme, and sucrose is a rather poor acceptor.

An interesting example of a directed synthesis is the coupling reaction of αCD to cellopentaose and the subsequent treatment of the undecasaccharide with amyloglucosidase. Thus, five glucose residues from the non-reducing end were cleaved quite rapidly, whereas that of the last glucose residue was retarded by a factor of 60. This effect allowed the isolation of 4^5-α-D-glucosyl-cellopentaose. The time dependence of the cleavage reaction, monitored by t.l.c.[6], is shown in Fig. 5.

TABLE I

Compounds tested as acceptors and yields of hexaosyl transfer products[a]

Acceptor[b]	*Relative yield*
Sophorose	>0
Nigerose	>0
Sucrose	0.28
D-Glucono-1,5-lactone	0.39
6^3-D-Glucosyl-maltotriose	0.88
Maltulose	0.92
Palatinose	1.03
p-Nitrophenyl maltoside	1.04
Cellobiose	1.08
6^2-Maltosyl-maltose	1.10
Methyl α-D-glucopyranoside	1.20
Maltose	1.35

[a]All transfer reactions involved equivalent concentrations of αCD and acceptor, reaction time, and temperature. [b] D-Ribose, D-fructose, D-galactose, D-mannose, D-allose, D-quinovose, D-xylose, D-glucuronic acid, α-D-glucose 1-phosphate, D-glucose 6-phosphate, 2-deoxy-D-*arabino*-hexose, 2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, *myo*-inositol, D-glucitol, and trehalose were not acceptors.

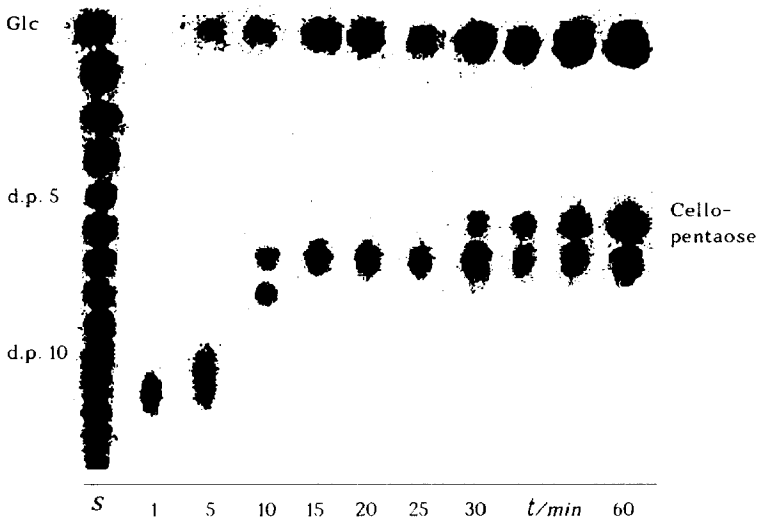

Fig. 5. T.l.c. of the products obtained by degradation of maltohexaosyl-cellopentaose with amyloglucosidase: 4^5-α-D-glucosyl-cellopentaose was formed after 15–25 min.

The coupling reaction of αCD to methyl α-D-glucopyranoside was used to prepare a series of methyl glucosides which are useful model compounds for studying fragmentation pathways in f.a.b.-mass spectrometry[7].

The isolation of pure gluco-oligosaccharides was possible only after optimisation of the gel-permeation chromatography on Biogel P4 (see Experimental).

Synthesis of branched glucans. — Summers and French[8] described the synthesis of branched gluco-oligosaccharides by CGTase-induced coupling of acceptors of the pullulan and dextran type. Isomaltose and panose yielded homologous series which were isolated and characterised up to d.p. 7. The same method was applied by Kainuma *et al.*[9,10] but no procedure for the separation of the isomeric products was given. Uniform and well-defined coupling products can only be obtained if the reaction is kinetically controlled and disproportionation is prevented. As shown in Table II for isomaltose, panose, and other branched gluco-oligosaccharides that contain (1→6) linkages, the coupling reaction involved HO-4 of the glucose residue with HO-6 unsubstituted. With 6^3-D-glucosyl-maltotetraose, only HO-4 of the main chain was coupled, whereas each HO-4 in 6^3-maltosyl-maltopentaose or in 6^3-maltotriosyl-maltotetraose served as acceptors. The coupling products were isolated by gel filtration and characterised by t.l.c. and f.a.b.-mass spectrometry. The sites of chain elongation could further be analysed by investigating the products of digestion with pullulanase. The results are summarised in Table II.

EXPERIMENTAL

T.l.c. — 1–5% Solutions of glucans in water (0.1 μL) were applied to heat-activated h.p.t.l.c. plates (Si 50000 Merck, Darmstadt) with a syringe (Desaga, Heidel-

TABLE II

CGTase-catalysed coupling of maltohexaosyl groups to (1→4/1→6)-α-D-glucans

Multiple acceptor	*Primary, secondary, tertiary transfer products (d.p.)*	*Products of pullulanase-catalysed hydrolysis (d.p.)*	*Accepting non-reducing ends*[a]
Isomaltose	8	–	→O┐
	14	–	Φ
	20	–	
Panose	9	2, 7	→O┐
	15	2, 13	O-Φ
	21	2, 19	
6^3-Maltotriosyl-maltotriose	12	3, 9	→O-O-O┐
	18	3, 15	O-O-Φ
	24	3, 21	
6^3-Maltosyl-maltotriose	11	3, 8	→O-O┐
	17	3, 14	O-O-Φ
	23	3, 20	
Isomaltotriose	9	–	→O┐
	15	–	O┐
	21	–	Φ
6^3-Maltotriosy-(6^3-maltotriosyl)-maltotriose	15	2, 9	→O-O-O┐
	21	3, 15	O-O-O┐
	27	3, 21	O-O-Φ
6^3-D-Glucosyl-maltotetraose	11	–	O┐
	17	–	→O-O-O-Φ
6^3-Maltosyl-maltopentaose	13	2, 5, 8, 11	→O-O┐
	19	2, 5, 8, 11, 14, 17	→O-O-O-O-Φ
6^3-Maltotriosyl-maltotetraose	13	3, 4, 9, 10	→O-O-O┐
	19	3, 4, 9, 10, 15, 16	→O-O-O-Φ

[a] Φ, Reducing end; –, (1→4) linkage; ┐, (1→6) linkage; →, receptor residue.

berg). Mixtures of d.p. 8–30 were developed 3–4 times with 1-butanol–pyridine–water[6] (6:5:4), and mixtures of d.p. 1–20 2–3 times with 1-propanol–ethyl acetate–water (6:1:3). Detection was effected with the diphenylamine–aniline reagent at 100° for 15 min. The blue spots were quantified at 610 nm with a densitometer (CD 50, Desaga).

Gel-permeation chromatography. — Polyacrylamide gel (1000 g, Biogel P4, extra fine, − 400 mesh, Bio-Rad) was diluted to twice its volume with water and poured onto a sieve in an ultrasonic bath. Sieves of mesh widths of 15, 25, and 32 μm were used. The particle sizes (23.9 ± 4.8, 33.6 ± 5.4, and 39.7 ± 7.9 μm) were determined by a comput-

er-assisted evaluation procedure of light microscopic photographs (Digital Device). For every batch of gel, the size of 200 of the spherical particles was measured and calculated statistically (we thank Dipl.-Ing. K. Meyer, Universität Hamburg-Harburg for this work).

After packing the columns with the standardised gels, 2 mL (semi-preparative runs) or 10 mL (preparative runs) of 10–20% aqueous solutions of the gluco-oligosaccharides were applied onto the columns, using a three-way valve and a pressure driven adaptor. The columns (two of 2.6 × 95 cm, LKB, in series for analytical or semi-preparative runs, and one of 5 × 100 cm for preparative runs) were kept at 60° by a thermostat (2209 Multitemp, LKB). The eluates passed refractometers (R-401 or R-403, Waters–Millipore) which were kept at 30° by a thermostat (G-D 3, Haake).

Enzymatic syntheses. — CGTase (EC 2.4.1.19) from *Bacillus macerans*, obtained from Lucerna-Chem AG (Luzern), had an activity of 200 Kitahata units (K.U.)/mg. Enzymatic reactions were performed at 25° in aqueous solutions at pH 6.4 that contained 2nM $CaCl_2$ and 10mM sodium citrate. The reactions were stopped by heating to 100° for 30 min.

αCD, obtained from Consortium für Elektrochemische Industrie (München), was freed from glucose by gel filtration. The excess of αCD (in enzymatic reactions) was precipitated by adding 1,1,2,2-tetrachloroethane (1 mL/g of αCD).

Substrates. — Maltotriose, pullulan, isomaltose, isomaltotriose, nigerose, sophorose, and maltose were commercial samples. The other linear and branched glucose oligomers were prepared enzymatically and purified to ~ 95% by chromatography, or were available in the laboratory.

Preparation and use of D-enzyme. — A suspension of activated charcoal (30 g) in water (600 mL) was heated to the boiling point, then cooled to room temperature, and the juice (400 mL) from washed and peeled potatoes (1000 g) was added with stirring. After repeated filtration and centrifugation of the mixture, a slightly brownish solution was obtained which was cooled to −1°, then stirred, and ethanol (82 mL) was added during 20 min. On cooling the solution to −4°, a white precipitate formed, which was removed by centrifugation at −5°. More ethanol (102 mL) was added and cooling at −5° was continued to give a fine white precipitate that was collected by centrifugation and suspended in 10mM citrate buffer (100 mL, pH 7.0).

To a solution of maltotriose (1 g, 2 mmol) in water (11 mL) were added 0.2M sodium citrate buffer (4 mL, pH 7.0) and the suspension of D-enzyme (9 mL). The mixture was stirred for 18 h at 37°, the enzyme was deactivated by raising the temperature to the boiling point, and the solution was then filtered and lyophilised.

Analytical hexaosyl transfer reactions. — A 3.5mM solution of the acceptor in 0.2mM citrate buffer (pH 7.0) was incubated with a 15-fold excess of purified αCD and 108 K.U. of CGTase/mmol of acceptor. The reaction was monitored by t.l.c. as shown in Fig. 2. For comparison of the acceptors (D-glucose–maltotriadecaose), the reaction was stopped after 160 min by heating and the αCD was removed by precipitation.

6^3-Maltotriosyl-maltotriose. — Solubilised pullulanase (EC 3.2.1.41) was donated by Cerestar (Vilvoorde, Belgium). The hydrolysis of pullulan was performed in 20mM

sodium acetate (pH 4.8). The reaction products (1.2 g) were subjected to gel-permeation chromatography and the fraction with d.p. 6 was isolated (191 mg).

Preparative and semi-preparative hexaosyl transfer reactions. — A 2.7mM solution of the multiple acceptor in 0.2mM citrate buffer (pH 7.0) was reacted with a 38-fold excess of αCD in the presence of CGTase (275 K.U./mmol of acceptor). After 2.5 h, the reaction was stopped by heating and the αCD was removed. Under these conditions, also cellopentaose was coupled to a hexaosyl residue.

Maltotriose (1 g, 2.0 mmol was added to a solution of αCD (19.3 g) in 0.2mM citrate buffer (100 mL, pH 7.0). The reaction was started by adding 20 K.U. of CGTase. After 14 h, the enzyme was deactivated by heating, and the αCD was precipitated and removed by filtration. Gel-permeation chromatography of the lyophilised sample yielded maltotriose (489 mg), maltononaose (586 mg), maltopentadecaose (71 mg), and maltohenicosaose (3 mg).

F.a.b.-mass spectrometry. — Positive and negative ion spectra were recorded with a VG 70-250 S instrument, using xenon as the collision gas and glycerol/1-thioglycerol (for positive-ion mass spectra) or triethanolamine–tetramethylurea (for negative-ion mass spectra) as matrices. Cesium iodide was employed for mass calibration.

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