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AN IMPROVED METHOD FOR THE PREPARATION OF CYCLOOCTAAMYLOSE, USING STARCHES AND THE CYCLODEXTRIN GLYCOSYLTRANSFERASE OF *Klebsiella pneumoniae* M 5 al

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ABSTRACT

Larger amounts (18.7% of total carbohydrate) of cyclooctaamylose (cyclomaltooctaose) were produced from starches by the cyclodextrin glycosyltransferase {(1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising), EC 2.4.1.19} of *Klebsiella pneumoniae* M 5 al by digestion in 200mM sodium acetate (pH 6.9) and the addition of bromobenzene after pre-incubation for 7 h. The dependence of the formation of cyclooctaamylose on the concentration of sodium acetate, initial concentration of substrate, and enzyme–substrate ratio has been studied.

A simple method for the preparation of pure cyclooctaamylose has been developed.

INTRODUCTION

Because of its large cavity and its high solubility in water, cyclooctaamylose (cyclomaltooctaose, cG_8) is of special interest. The cyclodextrin glycosyltransferases {(1→4)-α-D-glucan:[(1→4)-α-D-glucopyranosyl]transferase (cyclising) EC 2.4.1.19, CGT} from essentially seven bacterial strains, acting on starch, produce cyclohexaamylose (cG_6) and cycloheptaamylose (cG_7), but only minor amounts of cG_8. This fact may be explained by the conformational state of the starches commonly used as the substrates[1,2]. The ratios of cG_6, cG_7, and cG_8 have been reported[3] to be 2.7:1:1 (*Bacillus macerans*-CGT) and 1:2.4:1 (*Bacillus megaterium*-CGT). The product ratios can be influenced, however, by the choice of the conversion conditions[4]. The presence of suitable complexing agents can control the production of the cyclodextrins. Thus, in the presence of 1-decanol or 1-nonanol, mainly cG_6 is formed, and hexane, trichloroethylene, or toluene promote[5–7] the production of cG_7. Apart from complex procedures[8], no specific method for the economic production of cG_8 has been worked out, and this is reflected by its current market price.

Using standard conditions, the economic production of cG_8 with the CGT

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from *Klebsiella pneumoniae* M 5 al is not possible[9]. Larger amounts of cG_8 were obtained with (1→4)-α-D-glucopyranosyl chains[1] (average length, 16), or by coupling reactions with cG_6 (substrate) and very low concentrations of maltose (acceptor)[10]. Both methods are too expensive for the large-scale production of cG_8. Therefore, an attempt was made not only to increase the yield from starches by modifying the conversion conditions but also to improve the purification procedure. Several complex multistep-schemes have been recorded[11–14] for the preparation of pure cyclodextrins. Scaling-up of both production and purification of the cyclodextrins can be facilitated by the use of quantitative h.p.l.c.[9].

EXPERIMENTAL

Materials. — CGT was isolated from the culture filtrate of continuously grown *Klebsiella pneumoniae* M 5 al[15–17]. A crude (96% pure) enzyme preparation was used, the specific activity with (1→4)-α-D-glucopyranosyl chains (average length, 20) being 2.47×10^5 U/g of protein[18]. The only carbohydrase impurity was pullulanase [pullulan:(1→6)-glucanohydrolase, EC 3.2.1.41, 100 U/g of protein]. Although debranching enzymes may increase the yields of cyclodextrins from starch[19], it is questionable whether the low concentrations of pullulanase influence the conversion, because the enzyme is strongly inhibited[20] by cG_6 and cG_7. Glucoamylase [(1→4),(1→6)-α-D-glucan:glucohydrolase, EC 3.2.1.3, *Aspergillus niger*, 14 U/mg of protein] and the D-glucose test-kit GOD-Perid were purchased from Boehringer. Potato and maize starch were a gift from Henkel & Cie (Düsseldorf). All other substances were commercial materials of the highest purity available.

Analytical methods. — Total carbohydrate was determined with anthrone[21,22], and D-glucose with D-glucose oxidase[23]. Protein was determined by the biuret method[24]. H.p.l.c. was performed on Waters μBondapak-NH_2 columns (3.9 × 300 mm), using acetonitrile–water (65:35) at 1.3 mL/min (1,200 p.s.i., 25°) with refractometric detection. The carbohydrate contents of the cyclodextrin peaks were calculated by planimetry; the elution peaks were calibrated with pure cG_6, cG_7, and cG_8. The cyclodextrins were eluted together with G_4–G_6 (maltotetraose–maltohexaose). The amounts of linear saccharides were calculated as described in a foregoing paper[9]. For h.p.l.c. analysis, the complexing agent was removed by boiling. Non-cyclic compounds of higher molecular weight were precipitated with methanol[2] (1–1.5 vol.). After evaporation of the solvent, the samples were diluted (distilled water) to a carbohydrate content of <2%.

Digests. — Potato and maize starch (5–30%) were dissolved by heating (120°, 30 min) in 5mM $CaCl_2$ (pH 6.9) or in sodium acetate (50–400mM, pH 6.9, 5mM $CaCl_2$). The hot solutions (100 mL) were cooled to 70°, and liquefied by the addition of 1/3 of the total amount of CGT. After cooling to 40°, the remaining enzyme (total concentration, 0.1–15 mg) was added. The digests were incubated at 40° for 7 h, then bromobenzene (3 mL) was added, and the incubation was continued for a further 15–48 h with stirring. The control digests contained no complexing agent,

or the bromobenzene was added at the beginning of incubation. Digests (1 L) of potato starch (15%) in 200mM sodium acetate (pH 6.9, 5mM $CaCl_2$) with 30 mg of CGT served for the preparation of the cyclic compounds.

Purification of the cyclodextrins. — The insoluble material of the digests was collected by centrifugation (10 min, 13,000*g*) and suspended in water (1 L), and the bromobenzene was removed by boiling. Non-cyclic compounds of higher molecular weight were precipitated with methanol (1.2 vol.), and removed by centrifugation, together with other insoluble material. The clear supernatant solution was concentrated *in vacuo* to 150 mL and stored at 4° for 12 h. The crystals of cG_7 (48 g) were collected by centrifugation, washed once with ice-cold water (150 mL), and dried.

To the combined supernatant solutions from the above step (250 mL, containing 27.9 g of cG_8, and 3 g of cG_7) was added bromobenzene (10 mL). After stirring for 6 h, the insoluble clathrates were collected by centrifugation (10 min, 13,000*g*), suspended in distilled water (150 mL), and, after evaporation of the bromobenzene, freeze-dried. The dry material was suspended in pyridine (100 mL), gently heated to 60° for 1 h, and mixed with methanol (200 mL). The precipitate (26.8 g) was removed by centrifugation, washed once with ethanol, and dried. For final purification, the procedure was repeated once, yielding cG_8 (25.6 g) of 99.8% purity.

RESULTS AND DISCUSSION

Production of cG_8 by the CGT of Klebsiella pneumoniae *M 5 al from starches.* — Not more than 5% solutions of unmodified starches should be used; otherwise retrogradation of part of the substrate takes place which considerably impairs the yields of cyclodextrins[26]. Partial hydrolysis (acidic or enzymic) improves the solubility of the starch and lowers the viscosity of the solutions. Excessive hydrolysis, however, affects the yields. Thus, from starch hydrolysed to a glucose equivalent (g.e.) of 1, a 34% solution yielded[27] 45% of cyclodextrins, but only 17% from a starch of g.e. 12. The CGT from *Klebsiella pneumoniae* liquefies gels of unmodified starches very rapidly. At sufficiently high concentrations of enzyme, the insoluble (retrograded) material never exceeded 3 (potato) or 6% (maize starch) of the total carbohydrate, if the substrate was liquefied at 70° by 1/3 of the total amount of CGT. Therefore, unmodified starches were used for the present studies.

Digestion of a 15% solution of potato starch in 200mM sodium acetate (pH 6.9) for 24 h yielded, at an enzyme–substrate ratio of 1:5000 (g of enzyme/x g of substrate, see below), markedly more cG_8 than digests not containing sodium acetate. In the absence of complexing agents, only 34% of the substrate was converted into cyclodextrins, and the amounts of cG_8 did not exceed 5.6% of the total carbohydrate (Table I). By using clathrate-forming compounds, the reaction equilibrium can be shifted towards cyclisation[17]. A complexing agent suitable for promot-

ing the formation of cG_8 should form rather insoluble complexes. None of the commonly used guest molecules form cG_7-complexes markedly more soluble than those of cG_8 (Table II). Accordingly, cG_7, which only slowly participates in the reverse reactions[9], must be the main cyclic compound. Of the various complexing agents, bromobenzene was the most suitable (anthracene, which is specific[13] for cG_8, failed to increase the yields of cG_8). Digests containing bromobenzene at the beginning of the incubation yielded cG_6 as the main cyclic compound (Fig. 1A, Table I). Apparently, the complexing agent protects cG_6 from secondary transfer reactions. However, the proportions of cyclic products were altered drastically when bromobenzene was added after pre-incubation for 7 h (Fig. 1B); 24-h digests contained 61.4% of cyclodextrins composed of cG_6 (13.7%), cG_7 (55.8%), and cG_8 (30.5%) (Table I). On prolonged incubation, neither the total yield of cyclodextrins nor the amount of cG_8 increased markedly.

The dependence of cG_8-formation on the concentration of sodium acetate is shown in Fig. 2. With higher concentrations, the yields of total cyclodextrins de-

TABLE I

YIELDS OF CYCLODEXTRINS FROM POTATO STARCH UNDER VARIOUS CONVERSION CONDITIONS[a]

Conditions		*Yields (% of total carbohydrate)*[b]			
Sodium acetate[c]	*Bromobenzene*[d]	*Total cyclodextrins*	cG_6	cG_7	cG_8
–	++	64	16.6	42	5.4 (1:2.53:0.33)[e]
+	–	34	9.9	18 4	5.6 (1:1.86:0.56)
+	+	60	42	13.5	4.5 (1:0.32:0.11)
+	++	61.4	8.4	34.3	18.7 (1:4.1:2.23)

[a]The initial concentration of substrate was 15%, and the enzyme–substrate ratio was 1:5000. The digests (pH 6.9) were incubated at 40° for 24 h. [b]The cyclodextrins were assayed by h.p.l.c. (see Experimental, and ref. 9). [c]The concentration of sodium acetate was 200mM. [d] + and ++ indicate that bromobenzene was added at the beginning of the incubation and after pre-incubation for 7 h, respectively. [e]Ratios of cG_6, cG_7, and cG_8.

TABLE II

SOLUBILITY OF THE CYCLODEXTRIN-CLATHRATES OF SOME COMMONLY USED COMPLEXING AGENTS[a]

Complexing agent	*Solubility (g/100 mL of water at 20°)*		
	cG_6	cG_7	cG_8
Cyclohexane	0.22 (0.15)	0.13 (0.06)	1 64
Bromobenzene	1 62 (2.4)	0.01 (0 03)	1.64[b] (0.01)
p-Cymene	2.92 (3.3)	0.25 (0.04)	0.147 (0 17)
Trichloroethylene	(0 26)	(0.03)	(0.03)
Anthracene	—	—	2 76

[a]Data from ref. 26, with data from ref. 28 in brackets. [b]This value proved to be incorrect.

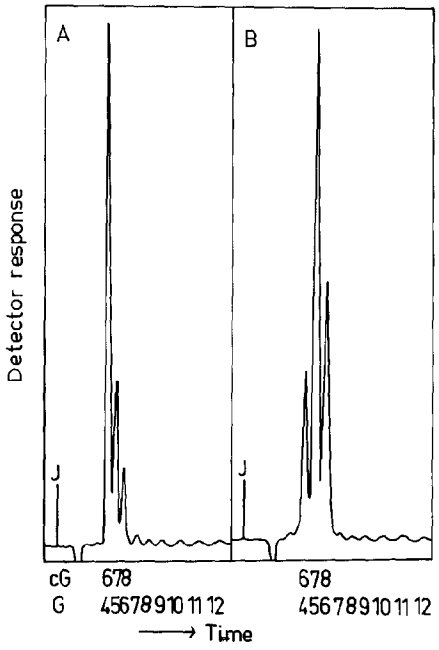

Fig. 1. H.p.l.c. of the transfer products of low molecular weight obtained after incubation for 24 h (40°) of potato starch (15%) in 200mM sodium acetate (pH 6.9) with the CGT of *Klebsiella pneumoniae* M 5 al (enzyme–substrate ratio, 1:5000). A, Bromobenzene (3 mL/100 mL) was added at the beginning of incubation [0.6 mg of carbohydrate (20 μL) was injected]; B, bromobenzene was added after pre-incubation for 7 h [0.66 mg of carbohydrate (20 μL) was injected]. For preparation of the samples, and the conditions of h.p.l.c., see Experimental.

creased, but the amounts of cG_8 increased up to 200mM of sodium acetate. The cyclisation reaction is thought to depend on the helical conformation of the substrate[2,29,30]. One turn of the amylose helix is made up of 6–7 D-glucopyranosyl residues[31], thus facilitating the formation of cG_6 and/or cG_7. However, cG_8 must be produced either from non-helical regions, or by distortion of helices during binding of the substrate, thus explaining the low rate of formation. The sodium acetate modifies the conformation of the substrate in such a way as to favour the formation of cG_8. This supposition is strengthened by the facts that the total yield of cyclodextrins decreased with increase in concentration of sodium acetate, and that maize starch, which contains more helical regions than potato starch[2], is an excellent substrate for the formation of cG_6, but a poor substrate for the production of cG_8, even in the presence of 200mM sodium acetate.

In contrast to most other enzymes, there exists a significant correlation[6,26] between the attainable degree of conversion into cyclodextrins and the concentration of CGT. The yields of total cyclodextrins and cG_8 increased up to an enzyme–substrate ratio of 1:5000 (Fig. 3). The increase was time-independent.

At an enzyme–substrate ratio of 1:5000, the yields of cyclodextrins decreased

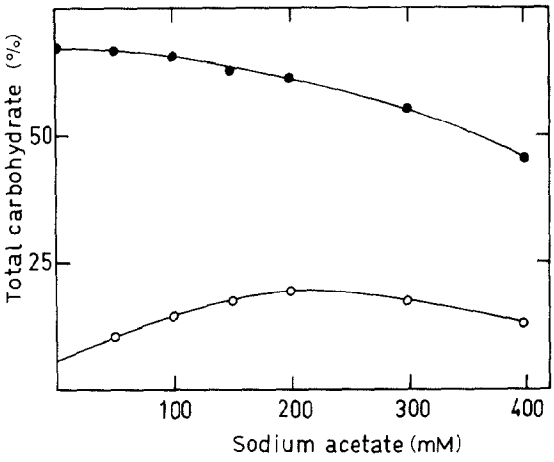

Fig. 2. Dependence of the production of total cyclodextrins (—●—) and of cG_8 (—○—) from potato starch (15%) by the CGT from *Klebsiella pneumoniae* M 5 al (enzyme–substrate ratio, 1:5000) on the concentration of sodium acetate (pH 6.9). Bromobenzene (3 mL/100 mL) was added to digests (24 h, 40°) after pre-incubation for 7 h (see Experimental).

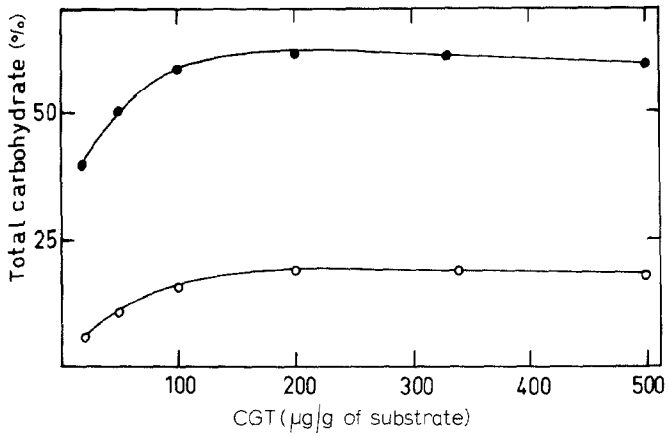

Fig. 3. Dependence of the production of total cyclodextrins (—●—) and of cG_8 (—○—) from potato starch (15%) in 200mM sodium acetate (pH 6.9) on the concentration of the CGT from *Klebsiella pneumoniae* M 5 al. Bromobenzene (3 mL/100 mL) was added to digests (24 h, 40°) after pre-incubation for 7 h (see Experimental).

with increase in substrate concentration, but the proportion of cG_8 was a maximum with 15% of potato starch in 200mM sodium acetate (pH 6.9) (Fig. 4). It is not clear, at present, whether the formation of cyclodextrins is impaired other than by retrogradation. Since alteration of the enzyme–substrate ratio did not affect the yields of cyclodextrins, the degree of conversion into cyclodextrins with higher concentrations of pre-hydrolysed starches merits further investigation.

Purification of cG_8. — In addition to the cyclodextrins, the digests contained

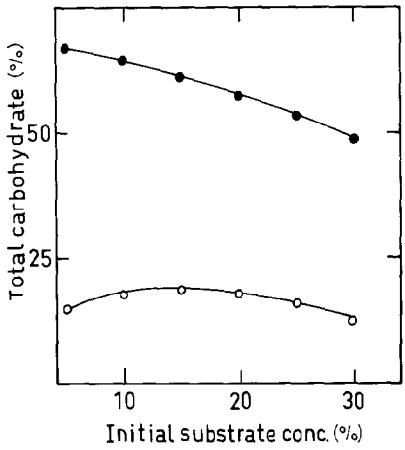

Fig. 4. Dependence of the production of total cyclodextrins (—●—) and cG_8 (—○—) by the CGT from *Klebsiella pneumoniae* M 5 al (enzyme–substrate ratio, 1:5000) on the initial concentration of potato starch in 200mM sodium acetate (pH 6.9). Bromobenzene (3 mL/100 mL) was added to digests (24 h, 40°) after pre-incubation for 7 h (see Experimental).

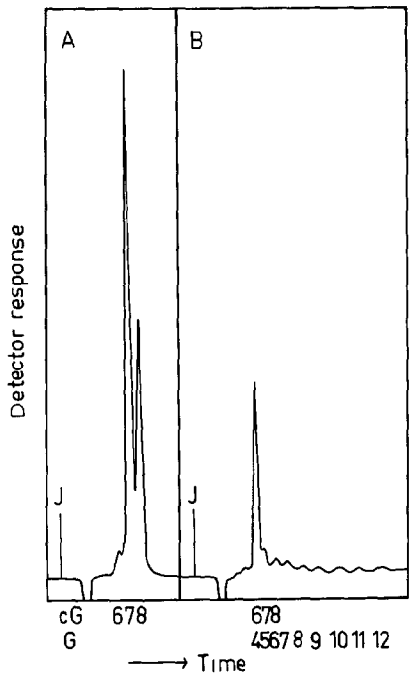

Fig. 5. H.p.l.c. of the bromobenzene-clathrates [A, methanol-supernatant; 0.4 mg of carbohydrate (20 μL) was injected] and the bromobenzene-supernatant [B, 0.5 mg of carbohydrate (20 μL) was injected] from digests (24 h, 40°) of potato starch (15%) in 200mM sodium acetate with the CGT from *Klebsiella pneumoniae* M 5 al (enzyme–substrate ratio, 1:5000). Bromobenzene (30 mL/L) was added after pre-incubation for 7 h.

insoluble material together with branched and linear transfer-products[2]. The soluble non-cyclic compounds may be digested with glucoamylase, thus facilitating the isolation of the cyclodextrins[32,33]. Since cG_8 is hydrolysed by fungal glucoamylase (or alpha-amylase impurities of the commercial preparations)[25], this method is unsuited for the preparation of cG_8. The purification of cG_8 from digests of 15% solutions of potato starch could be performed in a few simple steps.

Due to the low solubility of their bromobenzene-clathrates in water (see Table II), 99.7% of cG_7 and 99.6% of cG_8 were recovered with the insoluble material (Fig. 5A), together with some insoluble starch compounds (3% of total carbohydrate) and clusters of larger sizes (8% of total carbohydrate). After evaporation of the bromobenzene and removal of the non-cyclic products by precipitation with methanol, 92% of pure cG_7 was obtained by crystallisation (Fig. 6A). In order to obtain cG_8 completely free of non-cyclic compounds (which interfere with the final purification), the cyclodextrins of the cG_7-supernatant solutions were precipitated once more by complexing with bromobenzene (Fig. 6B).

The final purification of cG_8 was based on the fact that cG_7 is easily soluble in pyridine (60°), whereas cG_8 is not[34], and 91.3% of the cG_8 originally present was isolated pure (Fig. 6C). The cG_7 remained in the pyridine–methanol supernatant solutions (Fig. 6D).

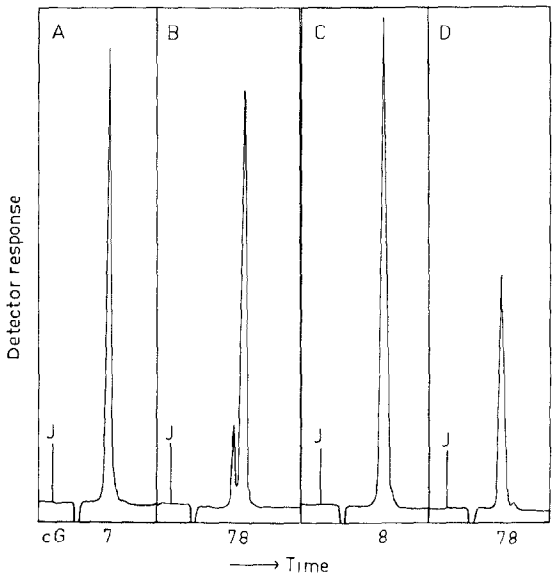

Fig. 6. H.p.l.c. of the fraction obtained during purification of cG_8. A, cG_7 crystallised (4°) from the concentrated solution of the bromobenzene-clathrates [0.29 mg of carbohydrate (20 μL) was injected]; B, 4°-supernatant solution of cG_7 [0.31 mg of carbohydrate (20 μL) was injected], C, cG_8 methanol–pyridine insoluble material [0.36 mg of carbohydrate (20 μL) was injected]; D, methanol–pyridine soluble material. The solvents were evaporated, and the dry matter was re-dissolved in distilled water [0.18 mg of carbohydrate (20 μL) was injected]. For the conditions of h.p.l.c., see Experimental.

On the strength of the markedly higher solubility of its bromobenzene-clathrate, only 7.3% of the cG_6 was found in the insoluble material. The bulk of the cG_6 was present in the supernatant solutions of the digests which, in addition, contained the main part of the non-cyclic products (Fig. 5B). It may be isolated by precipitation with 1-decanol or cyclohexane. The purification of the very low concentrations of cG_6 is not economic.

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