Carbohydrate Research, 230 (1992) 343–359
Elsevier Science Publishers B.V., Amsterdam

Enhanced production of cyclomaltooctaose (γ-cyclodextrin) through selective complexation with C_{12} cyclic compounds *

Jacob A. Rendleman, Jr.

Biopolymer Research Unit, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Agricultural Research Service, Peoria, Illinois 61604 (USA)

(Received July 17th, 1991; accepted in revised form January 22nd, 1992)

ABSTRACT

Enhanced yields of cyclomaltooctaose (γ-cyclodextrin, γ-CD) were produced by treating, incrementally, starch or maltooligosaccharide mixtures of $\overline{dp}$ 22 with *Bacillus macerans* cyclodextrin glucanotransferase (EC 2.4.1.19) in the presence of cyclododecanone, cyclododecanol, cyclododecanemethanol, cyclododecyl methyl ether, and cyclododecane. Maximum yields achieved through use of these complexants were 50, 32, 26, 34, and 17%, respectively. Cyclododecene, *N*-cyclododecylacetamide, and C_{11} cyclic compounds favored production of β-CD. Complexants were removed from their CD complexes either by azeotropic distillation or by ether extraction from aqueous media at high pH.

INTRODUCTION

Cyclodextrins [cyclomaltooligosaccharides, cyclic (1 → 4)-α-D-glucans, CDs] are produced by the action of cyclodextrin glucanotransferase (CGTase) on starches and long-chain maltooligosaccharides. In the absence of complexants capable of forming insoluble inclusion compounds with CDs, conversion reactions normally favor noncyclic products and produce, in slightly smaller amount, cyclomaltohexaose (α-CD), cyclomaltoheptaose (β-CD), and cyclomaltooctaose (γ-CD)[1–3]. Small amounts of branched cyclodextrins are formed as minor products, but unless certain complexing agents, such as sodium dodecyl sulfate, are present in the reaction mixture, the yields are too low for practical isolation[4]. Of the three major CDs, γ-CD is generally favored the least. Its yields are usually in the range of 4–7%, based upon total glucose-unit content of the substrate. At substrate concentrations of 5% (by wt) or higher, overall yields of CD usually fall into the

Correspondence to: Dr. J.A. Rendleman, Jr., Biopolymer Research Unit, National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Agricultural Research Service, Peoria, IL 61604 USA.

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

range of 35–50%; and the relative proportions of α-CD and β-CD can vary appreciably according to reaction conditions and bacterial source of the enzyme[2]. A bacterial transferase produced by *Bacillus subtilis* converts starch into γ-CD exclusively; however, yields are very low ($\sim 5\%$)[5]. The *Bacillus* sp. AL-6 produces a transferase that, in purely aqueous media, converts starch into a mixture of β-CD and γ-CD in yields of 6 and 18%, respectively[6]. In aqueous ethanolic media, the same enzyme produces γ-CD in 35% yield, provided the substrate concentration is low ($\sim 2.5\%$ by wt). At 6% substrate concentration, γ-CD yield drops to 21%.

Although the precise mechanism for cyclization is not known, the equilibria shown in Reactions *1* and *2* are believed to be involved[3]. The symbols G_n, G_m, and G_x

$$G_n \underset{\text{coupling}}{\overset{\text{cyclization}}{\rightleftharpoons}} G_{(n-x)} + cG_x \qquad (1)$$

$$G_n + G_m \overset{\text{disproportionation}}{\rightleftharpoons} G_{(n-x)} + G_{(m+x)} \qquad (2)$$

represent straight-chain maltooligosaccharides containing n, m, and x units of (1 → 4)-α-D-glucopyranose; cG_x is a cyclodextrin composed of x units. A glucose unit of the nonreducing end of a (1 → 4)-α-D-glucopyranosyl chain appears to be transferred by way of either O-4 or C-4 to either C-1 or O-1 of another glucose unit that functions as a suitable donor. α-, β-, and γ-CD are composed of 6, 7, and 8 glucose units, respectively. Their respective cavity diameters are approximately 4.7–6, 8, and 10 Å; and their respective cavity volumes[2] are 176, 346, and 510 Å^3.

Product ratios and total CD yields can be greatly influenced by complexing agents that form insoluble or highly stable inclusion compounds with cyclodextrins. Thus, in the presence of 1-decanol, the main cyclic conversion-product is[7,8] α-CD, the yields of which are as high as 50%. Production of cyclodextrin can be enhanced by C_{1-8} aliphatic alcohols[9–11], and by C_{2-4} aliphatic ethers, esters, and ketones[9]. For example, the presence of acetone ($\sim 10\%$ by vol) led to the following yields from potato-starch substrate: 44.7 α-CD, 13.0 β-CD, and 12.4% γ-CD. Enhancement of β-CD yields has been realized by the use of toluene[12,13], trichloroethylene[14], and limonene[15]. Bender[16,17] employed a combination of bromobenzene and sodium acetate to obtain γ-CD in 18.7% yield. The same reaction also gave α- and β-CD in 8.4 and 34.3% yield, respectively. A combination of butanone and 1-naphthol has also been used to enhance production of γ-CD[18]. Pentacyclic and tetracyclic terpenoids (such as glycyrrhizic acid and stevioside) have been employed to produce γ-CD in 40% yield[19].

Concurrent with these studies of the enhancing effect of cyclic complexants on γ-CD production, Schmid et al.[20] reported their success in using a variety of cyclic compounds to achieve similar results. Patents based upon this work were subsequently applied for and obtained by Schmid and Eberle[21]. Their claims were limited to cyclic complexants having 13–24 ring atoms. Yields of γ-CD were

34–45%. Their experiments indicated that cyclic complexants of smaller ring size were incapable of enhancing γ-CD formation. With cyclododecanone (12-atom ring) as complexant, their yield of γ-CD was only 1.5%.

This report describes how cyclic complexants having 12 ring atoms can be used effectively to enhance production of γ-CD from starch or maltooligosaccharide mixtures ($\overline{dp}$ 22) and how a high pH can effect the rapid release of complexant from a CD complex and thus provide a means for recovering complexants from reaction mixtures or for dissociating a complex for the purpose of isolating the cyclodextrin.

EXPERIMENTAL

Materials.—CGTase (EC 2.4.1.19) from *Bacillus macerans* was obtained as an aqueous solution [> 600 units/mL according to the method of Tilden and Hudson[22]] from Amano International Enzyme Co., Inc. In 30 min at pH 6.0 and 60°, a single 5-μL application of the CGTase to a maltooligosaccharide mixture of dp 22.1 (0.3 g in 3 mL of water) produced 16.5 mg (0.85 μmol) of combined CDs (2.9 α, 1.8 β, and 0.8% γ, by wt). Thus, if one unit of activity is defined as the quantity of enzyme that produces 1 μmol of combined CDs per min from soluble starch at pH 6 and 60°, then each mL of CGTase possesses ~5.6 units of activity. Amyloglucosidase [(1 → 4)-α-D-glucan glucohydrolase, EC 3.2.1.3, from *Aspergillus niger*, 37 units/mg of solid or 42 units/mg of protein] was obtained from Sigma Chemical Company. The potato starch (12.0% H_2O), waxy maize corn starch (16.1% H_2O), and maltodextrin M-050 (7.76% H_2O) were from Avebe (Veendam, Netherlands), National Starch and Chemical Co. (Bridgewater, NJ), and Grain Processing Corporation (Muscatine, IA), respectively. All other substances were commercial materials of the highest purity. The cyclodextrins were hydrated and contained the following percentages of water at 31% relative humidity (25°): α-CD, 8.75; β-CD, 13.7; and γ-CD, 9.0%. Water of hydration was determined by weight loss from heating samples to constant weight (2 h was sufficient) at 100° under vacuum. Because the degree of hydration of starches and dextrins varies with relative humidity, all such substances were stored at 25° under conditions of 31% relative humidity. The maltooligosaccharide mixture had a dextrose equivalent of 5 and $\overline{dp}$ of 22.1. Water was distilled and deionized.

The CGTase used in these studies was described in data provided by the manufacturer as being most stable above pH 7, with stability decreasing rapidly below pH 6. However, activity decreases rapidly with increase in pH above 7 and decrease in pH below 5. Thermostability is very high at 50° (~99% of the activity remaining after a 10 min treatment), but only moderate at 60° (~93% remaining after 10 min); at 65°, only ~60% remains after 10 min. On the basis of the thermostability data, one can calculate that about 3% of the activity remains at the end of 8 h at 60°.

Analytical methods. —Total carbohydrate was determined either by HPLC or by the phenol–H_2SO_4 method [23] where absorbance was measured at 488 nm. HPLC

was performed on a DuPont Zorbax NH_2 column (4.6 × 250 mm) at 40° with 13:7 acetonitrile–water at 1.0 mL/min; detection was by means of a Waters 410 differential refractometer; reference standard for comparison with elution peaks was a mixture of pure α-, β-, and γ-CD (0.40 mg of each anhydrous CD per mL of aqueous solution); all solutions were filtered with Millipore HV filter-units (0.45-μm pore size) prior to injection. Prior to HPLC analysis, cyclodextrin solutions containing maltooligosaccharides were treated with amyloglucosidase to eliminate maltooligosaccharides that would interfere with CD determinations.

Conversion procedures. —Individual "solutions" of starch were prepared by first gelatinizing, on a steam bath, mixtures of starch and water in 50-mL culture tubes (25 × 150 mm) equipped with Teflon-lined screw caps. This was followed by cooling to 25° and adding, to each tube, an increment of CGTase equal to 5 μL per 3 mL of mixture. Liquification was sufficiently complete in 1 h to allow for pH adjustment or for buffering, if desired. Measured amounts of complexant (if used) and small Teflon-coated bars (to facilitate mixing) were then introduced. Reactions were conducted in a constant-temperature shaker bath with periodic adjustment of pH. With reactions involving incremental addition of CGTase, additional applications of CGTase were added periodically at a rate determined by the particular study; and the final increment was followed by a reaction period of 24 h prior to inactivation of the enzyme at 100°. The conversion procedure for maltodextrin M-050 was similar to that for starch, but did not require the gelatinization and liquification steps. For conversion reactions involving only a single application of enzyme, a reaction period of at least 48 h was allowed.

Maltooligosaccharide solutions were usually prepared in large amounts by heating, on a steam bath, appropriate amounts of water and M-050. At 25°, a buffering agent was added (where desired) and the pH was adjusted to the desired level. Aliquots (3 mL each) were placed in individual screw-capped culture tubes (with or without complexant) and an appropriate amount of CGTase was added to each.

Removal of guest from reaction mixtures and inclusion complexes.—*(A) Azeotropic distillation.* Reaction mixtures or mixtures of inclusion complex and water were heated to ~95° while N_2 was introduced at a controlled, moderate rate beneath the liquid surface by means of a capillary tube; length of the operation varied according to the stability of the complex and the volatility of the complexant (cyclododecanone and cyclotridecanone rarely required more than 3 h for complete removal; cyclopentadecanone, ~6 h; 8-cyclohexadecen-1-one, 8 h or longer); frequent replenishment of water was necessary during the operations. The complexant-free solutions were then diluted appropriately and filtered with Millipore HV 0.45-μm filters prior to HPLC analysis.

(B) Extraction with ethyl ether at high pH. The pH of an aqueous mixture containing a complex was adjusted to 12.5–13.5 with NaOH (a pH of less than 12.5 might not be effective in dissociating the complex) and an equal volume of ether was added. For example, a 3-mL reaction mixture in a 50-mL culture tube was

diluted with water (17 mL), pH was adjusted to 13.0, and ethyl ether (20 mL) was added. The vessel was capped and magnetic stirring was begun at a rate that would bring released guest particles into rapid contact with the ether surface. Stirring was not so rapid that objectionable "sudsing" would occur at the water–ether interface. After 45 min, the ether layer was removed (by suction) and replaced with fresh ether. Stirring was continued for an additional 45 min. The operation of adding ether, stirring, and finally removing the ether layer was repeated three times, leaving a clear, transparent, complexant-free, aqueous layer containing liberated cyclodextrin. The pH of this layer was then lowered to 7.0 by cautiously adding HCl; and the dissolved salts were removed by mixed-bed ion-exchange resin.

Determination of CD solubility in the presence of an equimolar amount of complexant. —A mixture of 8.33 mM CD solution (3 mL) and complexant (0.025 mmol) was stirred magnetically for 1–2 weeks at 25° in 25-mL (20 × 120 mm) culture tubes equipped with Teflon-lined screw caps. Where the complexant was a solid at 25°, the mixture was first shaken at 60° for 24 h prior to being stirred at 25°. Occasionally, gentle end-over-end shaking was necessary because of the tendency for many volatile complexants to condense in the upper region of the tubes. Reaction mixtures were centrifuged at 25° and filtered through Millipore HV 0.45-μm filter units. Filtrates were then diluted appropriately with water and analyzed either by HPLC or by the phenol–H_2SO_4 method to determine the concentration of CD remaining in the aqueous phase of each reaction mixture.

Reactions involving a higher molar ratio of host-to-guest (3:1) were also conducted with certain complexants to determine whether combining ratios greater than 1:1 are possible. The initial concentration of complexant was the same (0.025 mmol per 3 mL) in each of these systems.

Selectivity of complexant for CDs as determined by competitive interaction. —Complexant (0.025 mmol) was stirred rapidly for a minimum of 11 days at 25° in an aqueous solution (6.0 mL) containing 0.025 mmol each of β-CD and γ-CD. The procedure was similar to that just given for the determination of CD solubility in the presence of an equimolar amount of complexant. Reaction mixtures were centrifuged and filtered; filtrates were diluted appropriately with water for determination of CDs by HPLC.

RESULTS AND DISCUSSION

Knowledge of the solubility of CD in the presence of guest compounds and knowledge of complexant selectivity provide information useful in choosing a complexant for enhancing production of CD in enzymic conversions of maltooligosaccharides and starch. Observations in this laboratory have suggested that, in general, a close relationship exists between the stability of a CD complex (as determined qualitatively by studying CPK molecular models and noting closeness of fit of a nonpolar guest molecule within the CD cavity) and the solubility of that

complex. The greater the stability, the lower is the solubility. This relationship applies only to complexes of nonionic, nonpolar (or weakly polar) molecules. Anionic forms of certain organic acids can form relatively strong, soluble CD complexes. For example, sodium dodecyl sulfate forms a highly soluble complex with β-CD. Unionized glycyrrhizic acid forms a soluble complex with γ-CD.

Table I presents results of solubility studies at 25° with selected complexants and Table II presents results of selectivity studies where equimolar amounts of β-CD, γ-CD, and complexant were combined and allowed to reach equilibrium in aqueous media at 25°. Only the cyclic complexants cyclododecane, cyclododecanol, cyclododecanone, cyclododecanemethanol, cyclododecyl methyl ether, cyclotridecanone, cyclopentadecanone, and 8-cyclohexadecen-1-one were found to have solubility and selectivity characteristics that might serve to enhance γ-CD production in enzymic reactions.

Solubility data from studies where initial host-to-guest ratios were varied indicated that the stoichiometry of complex formation between CDs and *cyclic* complexants is 1 : 1. With long, straight-chain complexants, such as dodecane and hexadecane, each guest molecule can bind two or more molecules of either α-CD or β-CD; γ-CD is less capable of multiple binding. In designing procedures for enzymic conversions of starch and maltooligosaccharides in the presence of cyclic complexants, it was assumed that the combining ratio of CD-to-guest is 1 : 1.

Within the temperature range of 48–70°, the use of a single application of CGTase to starch or maltooligosaccharide of high $\overline{dp}$ (~ 22) led only to low yields of γ-CD when cyclododecanone was present and reaction periods were 48 h (Table III). At 48° with cyclododecanone, the lowest yields (~ 2%) occurred at or above 10 μL of CGTase per mL of reaction mixture. Lengthening the reaction period from 48 to 148 h had little or no effect on γ-CD yield. Schmid and Eberle[21] reported a similarly low yield (1.5%) from starch conversion conducted at 50° and pH 7 with cyclododecanone and a single application of CGTase. In contrast, under similar reaction conditions, cyclic complexants of ring size larger than that of cyclododecanone can greatly enhance yields of γ-CD. Table III presents data from single-application conversions of M-050 under the influence of larger cyclic complexants. The enhancing effect of cyclotridecanone and 8-cyclohexadecen-1-one on γ-CD yield was likewise observed by Schmid and Eberle[21], who reported yields of 42 and 46%, respectively, for single-application conversions involving these two compounds. Their reported yield of 46% from use of 8-cyclohexadecen-1-one was considerably higher than any realized in my studies with the same complexant. Cyclic complexants smaller than cyclododecanone have not been found to enhance γ-CD production in single-application studies; however, they may enhance β-CD production.

Earlier investigators are not known to have used procedures where multiple increments of CGTase are applied at intervals over an extended period of time. Such a procedure is now described, where the incremental method of conversion with CGTase EC 2.4.1.19 can lead to high yields of γ-CD when cyclododecanone is

TABLE I

Precipitability of CDs from aqueous solution at different molar ratios [a] of host-to-guest at 25°

Guest	Guest properties			CD Precipitated (%)					
	mp (°)	bp (°)	Solubility (g/100 g H_2O)	1:1 Ratio			3:1 Ratio		
				α	β	γ	α	β	γ
Cyclic:									
Benzene	5	80	0.06^{25}; 0.26^{60}	0	15	0			
Bibenzyl	50–53	284	$< 0.01^{25}$		98		4	35	24
Bromobenzene	−31	156	0.04^{30}	0	44	29			
Cyclodecane	9–10	201	–	2	97	62			
Cyclodecanone	24	106/12 mm	0.08^{25}	5	91	70			
Cyclododecane	59–61	–	$< 0.01^{25}$	3	95	76		37	23
Cyclododecanemethanol	30–32	90–94/0.1 mm	$< 0.03^{25}$		85	94			
Cyclododecanol	76–79	–	$< 0.03^{25}$	1	88	78		36	23
Cyclododecanone	59–61	85/1 mm	$< 0.03^{25}$	2	95	80			38
Cyclododecene (*cis* + *trans*)	–	232–245	$< 0.01^{25}$	0.4	96	68		35	25
N-Cyclododecylacetamide	143–144	–	$< 0.03^{25}$		63	72			
Cyclododecyl methyl ether	–	89–92/3 mm	$< 0.03^{25}$		86	96			
8-Cyclohexadecen-1-one	–	193–195/19 mm	$< 0.03^{25}$	0.4	2	100			33
Cyclohexane	6.5	81	0.01^{20}	32	56	0			
Cyclohexanone	−47	155	9^{25}	2	3	0			
Cyclooctane	10–13	151/40 mm	$< 0.02^{25}$	46	81	63			
Cyclooctanone	39–41	195–197	1.5^{25}	18	17	18			
Cyclopentadecanone	63	120/0.3 mm	$< 0.03^{25}$	1	2	99			36
Cyclotridecanone	32	138/12 mm	$< 0.03^{25}$		91	95			
Cycloundecanone	–	130–132/15 mm	$< 0.03^{25}$		90	75			
Pentamethylbenzene	50–52	231	$< 0.01^{25}$	2	14	67			20
Toluene	−95	111	0.06^{30}	0	45	23			
Noncyclic:									
Dodecane	−12	216	0.00002^{25}	93	77	16	75	59	43
1-Dodecanol	26	260–262	$< 0.03^{25}$	78	74	46		50	33
Hexadecane	18	283–286	$< 0.03^{25}$	90	89	17	92	72	51
Nonadecane	32	330	$< 0.03^{25}$	91	71	24		78	
Octacosane	61–63	278/15 mm	$< 0.03^{25}$	64	26	22		53	
1-Octanol	−15	196	0.06^{25}	30	61	54	43	32	32
1,1,2,2-Tetrachloroethane	−43	147	0.02^{20}	75	55	38			

[a] Each mixture was initially 8.33 mM in guest and contained only one CD host. The 3:1 mixtures were 25 mM in CD.

TABLE II

Selectivity of guest for CDs in aqueous mixtures equimolar (8.33 mM) in β-CD, γ-CD, and guest compound at 25°

Guest	pH	CD precipitated (%)		Ratio (γ/β)
		β	γ	
Bibenzyl	ND [a]	88	7	0.08
Cyclodecane	ND	65	20	0.31
Cyclododecane	4	4	62	16
	6	4	64	16
	7	3	75	25
	8	8	82	10
Cyclododecanemethanol	ND	13	72	5.5
Cyclododecanol	4	7	82	12
	5	8	87	11
	6	4	78	20
	7	0	73	∞
Cyclododecanone	4	8	75	9
	5	4	70	18
	7	8	72	9
	8	13	77	5.9
Cyclododecene (*cis* + *trans*)	ND	48	43	0.90
N-Cyclododecylacetamide	ND	51	44	0.86
Cyclododecyl methyl ether	ND	6	84	14
Cyclooctanone	ND	0	0	–
Cyclopentadecanone	ND	2	95	48
Cyclotridecanone	ND	3	87	29
Cycloundecanone	ND	48	36	0.75

[a] ND: not determined.

present as complexant. At the point of maximum γ-CD yield, yields of α- and β-CD are very low, provided optimum reaction conditions are used. Fig. 1 illustrates a typical reaction of this type. During the initial stages of reaction, formation of β-CD is very rapid compared to that of γ-CD; and much of the β-CD accumulates as an insoluble cyclododecanone–β-CD complex. With continued incremental addition of enzyme, the yield of β-CD diminishes and the yield of γ-CD increases, which behavior suggests that, in the later stages of reaction, γ-CD is formed at the expense of β-CD. γ-CD accumulates as an insoluble cyclododecanone–γ-CD complex. In the absence of complexant, incremental addition of CGTase leads to CD yields that are similar to those attained by methods employing only a single application of enzyme (Fig. 2).

Table IV describes the conversion of potato starch where 5-μL increments of CGTase are applied at pH 7.2 and 60° to 3-mL systems differing in complexant content and buffer content. The variation in complexant content had relatively little influence on yields. Tris buffer (0.02 M) had a small retarding effect on the rate of attaining maximum CD yield. Moderately high concentrations of either Tris or sodium phosphate buffer (~ 0.1 M) can have a severely limiting effect on γ-CD yield, producing a near-equilibrium situation where high yields of β-CD and

TABLE III

Effect of size of single CGTase application on yields of CDs from 10% M-050 [a] after 48 h at pH 7.2 in the presence of complexant

CGT Vol (μL)	Complexant	Concentration (mmol)	Buffer [b]	CD yield (%)			
				α	β	γ	Combined
48°							
1	Cyclododecanone	0.18	Tris	5.8	30.4	10.2	46.4
1		0.18	None	10.9	19.1	6.3	36.3
5		0.18	Tris	2.4	45.4	12.6	60.4
5		0.18	None	3.2	40.1	10.8	54.1
10		0.18	Tris	0.8	50.8	11.9	63.5
10		0.18	None	1.6	48.4	11.7	61.7
30		0.18	Tris	4.3	52.0	2.3	58.6
60		0.18	Tris	1.3	54.4	2.4	58.1
60		0.18	None	1.5	57.2	3.6	62.3
50°							
10	Cyclododecanone	0.54	Tris + Ca	1.5	49.6	15.4	66.5
10		0.54	None	1.6	50.7	17.4	69.7
40		0.18	Tris	0	42.5	10.2	52.7
40		0.18	Phosphate	1.6	52.9	16.6	71.1
40	Cyclotridecanone	0.18	Tris	0	5.9	51.9	57.8
40		0.18	Phosphate	0	6.0	49.8	55.8
40		0.18	None	2.7	4.9	39.9	47.5
10	Cyclopentadecanone	0.18	Tris + Ca	6.8	14.0	20.6	41.4
10		0.18	None	4.8	10.1	15.1	30.0
40		0.18	Tris	4.1	8.5	37.6	50.2
40		0.18	Phosphate	3.1	8.2	42.2	53.5
40		0.18	None	4.1	8.5	27.6	40.2
10	8-Cyclohexadecen-1-one	0.18	Tris + Ca	9.6	17.6	31.5	58.7
10		0.18	None	5.5	9.4	13.4	28.3
40		0.18	Tris	4.3	7.4	25.1	36.8
40		0.18	Phosphate	4.6	8.5	18.8	31.8
40		0.18	None	4.8	7.8	28.0	40.6
40	No complexant	0.18	Phosphate	10.9	18.2	2.27	31.4
60°							
1	Cyclododecanone	0.18	None	10.9	19.1	6.3	36.3
5		0.18	None	3.2	40.1	10.8	54.1
5		0.54	None	1.2	42.4	14.6	58.2
10		0.18	None	1.6	48.4	11.7	61.7
10		0.54	None	1.1	44.7	17.9	63.7
30		0.18	None	1.3	52.5	7.1	60.9
50		0.54	None	1.2	35.7	18.3	55.2
60		0.18	None	1.5	57.2	3.6	62.3
100		0.54	None	1.7	28.2	21.7	51.6
200		0.54	None	3.3	26.5	17.9	47.7

TABLE III (continued)

CGT Vol (μL)	Complexant	Concentration (mmol)	Buffer [b]	CD yield (%)			
				α	β	γ	Combined
70°							
10	Cyclododecanone	0.18	Tris	9.2	7.7	3.9	20.8
25		0.18	Tris	13.3	12.6	5.6	31.5
50		0.18	Tris	12.2	23.9	8.8	44.9
100		0.18	Tris	5.6	35.3	11.1	52.0
200		0.18	Tris	3.4	40.1	8.3	51.8
400		0.18	Tris	3.4	46.8	3.3	53.5

[a] Volume of substrate solution, 3.00 mL; 1.85 mmol of glucose residues. [b] 0.02 M Tris or 0.05 M phosphate. Where used, Ca^{2+} is 5.0 mM.

relatively low yields of γ-CD prevail. For example, at pH 7.2 and 60°, the addition of 5-μL increments of enzyme to 10% M-050 (3 mL) containing cyclododecanone (0.18 mmol) and Tris (0.1 M) led to the following CD yields at the point of maximum γ-CD production: 2.0 α, 37 β, and 24% γ. With 0.05 M phosphate in place of Tris yields were 1 α, 43 β, and 13% γ. Ionic strength (μ) might be at least a partial explanation for this effect. In the absence of buffer, μ varied from 0.01 to 0.03 during the course of a conversion reaction held at or about pH 7.2 by periodic adjustments with NaOH. Ionic strength levels in the 0.1 M Tris and 0.05 M phosphate solutions were close to 0.10 and 0.13, respectively, if it is assumed that the preponderant phosphate species at pH 7.2 is HPO_4^{2-}. Unbuffered 10% M-050 (3 mL), containing 0.18 mmol of cyclododecanone, and with $\mu = 0.13$ (adjusted to this level by addition of NaCl), gave the following CD yields when treated at pH 7.2 and 60° with 5-μL increments of CGTase: after 25 increments, 2 α, 20 β, and

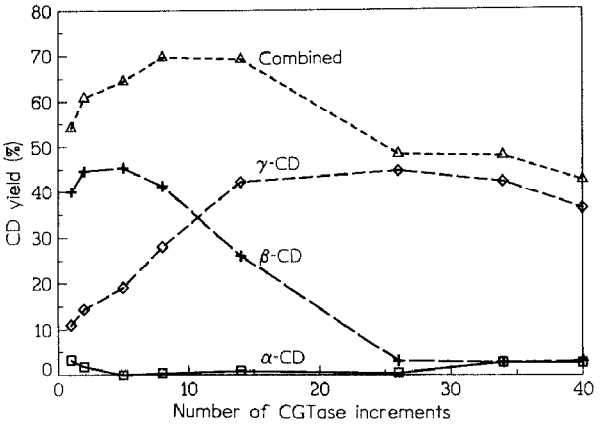

Fig. 1. Enzymic conversion of M-050 (unbuffered 10% solution) into cyclodextrins at pH 7.2 and 60° with cyclododecanone as complexant. Volume of reaction mixture, 3.00 mL; wt of complexant, 0.066 g (0.36 mmol). CGTase is added in 5-μL increments at a frequency of 2 increments per day (separated by intervals of at least 8 h).

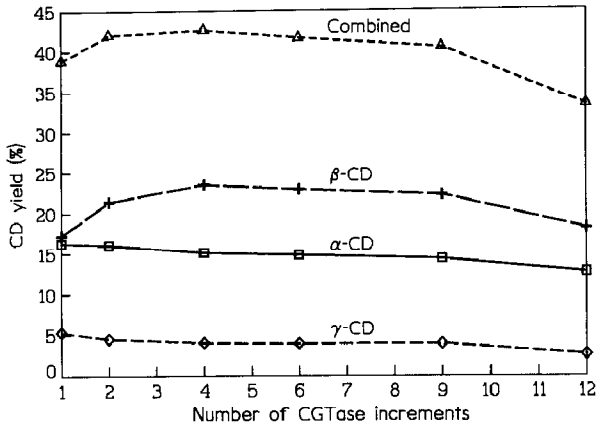

Fig. 2. Enzymic conversion of M-050 (unbuffered 5% solution) into cyclodextrins at pH 6.0 and 60° without complexant. Volume of reaction mixture, 3.00 mL. CGTase is added in 5-μL increments at a frequency of 2 increments per day (separated by intervals of at least 8 h).

32% γ; after 35 increments, 2 α, 18 β, and 32% γ. These results would suggest that μ and/or buffer concentrations be kept as low as possible to avoid undesirably low yields of γ-CD in reactions involving cyclododecanone as complexant. Study of the influence of μ and buffer on incremental-type reactions involving complexants other than cyclododecanone has not yet been made.

The ability of C_{12} cyclic complexants other than cyclododecanone to enhance γ-CD production by the incremental method was investigated (Table V). Cyclodo-

TABLE IV

Conversion of 5% potato starch [a] by multiple addition of CGTase at pH 7.2 and 60° in the presence of cyclododecanone

No. of 5-μL increments [b]	Complexant (mmol)	Buffer [c]	CD yield (%)			
			α	β	γ	Combined
10	0.18	None	2.5	33.9	24.7	61.1
15			2.3	21.4	35.8	59.5
20			1.8	5.7	40.9	48.4
25			1.8	5.0	44.7	51.5
30			1.4	5.0	38.2	44.6
10	0.18	Tris [c]	1.5	41.2	21.3	64.0
15			2.4	42.1	21.4	65.9
20			2.2	26.2	26.9	55.3
25			2.0	20.3	38.7	61.0
30			1.8	12.7	44.3	58.8
10	0.54	None	2.0	38.0	23.8	63.8
15			2.1	24.1	31.3	57.5
20			2.1	10.8	41.5	54.4
25			1.4	4.6	45.9	51.8
30			1.2	4.2	43.7	49.1

[a] Volume of potato starch solution, 3.00 mL; 0.925 mmol of glucose residues. [b] Frequency of addition is 2 per day (separated by an interval of at least 8 h). [c] 0.02 M Tris.

TABLE V

Effect of C_{12} cyclic complexants other than cyclododecanone on conversion of 10% M-050 [a] by multiple addition of CGTase at pH 7.2 and 60°

Complexant	Concentration (mmol)	Buffer [b]	No. of 5-μL increments [c]	CD yield (%)			
				α	β	γ	Combined
Cyclododecane	0.18	None	1	2.7	48.2	8.0	58.9
			15	3.5	22.6	15.4	41.5
			20	7.5	12.0	14.7	34.2
			25	5.9	5.3	16.7	27.9
Cyclododecene (*cis* + *trans*)	0.18	Tris	10	2.1	46.3	1.2	49.6
			20	2.2	55.5	0.2	57.9
			30	1.9	44.2	1.8	47.9
Cyclododecanol	0.18	None	1	2.3	38.0	19.2	59.5
			15	2.4	4.1	41.0	47.5
			20	2.6	4.4	31.5	38.5
			25	3.3	5.1	32.4	40.8
Cyclododecanemethanol	0.18	Tris	10	3.1	28.3	21.2	52.6
			15	3.2	19.2	23.4	45.8
			24	4.0	12.0	25.6	41.6
			35	3.0	10.5	19.6	33.1
Cyclododecyl methyl ether	0.18	Tris	10	3.9	8.7	33.8	46.4
			20	2.6	7.1	28.9	38.6
			25	4.3	8.3	22.5	35.1
			35	4.2	6.2	16.5	26.9
N-Cyclododecylacetamide [d]	0.18	None	10	9.2	18.9	5.4	33.5
			20	9.0	10.4	1.4	20.8
			30	2.3	3.0	0	5.3

[a] Volume of 10% M-050, 3.00 mL; 1.85 mmol of glucose residues. [b] 0.02 M Tris. [c] CGTase is added at a frequency of 2 increments per day (separated by an interval of at least 8 h). [d] Because the low volatility of *N*-cyclododecylacetamide precluded use of azeotropic distillation to remove this complexant from reaction mixtures, removal was accomplished by ether extraction at pH 13.

decene and *N*-cyclododecylacetamide exhibited no ability to enhance γ-CD production; however, the former was outstanding in enhancing β-CD yield. The inability of *N*-cyclododecylacetamide to enhance yields can best be explained by the relatively low stabilities of its complexes with β-CD and γ-CD at the reaction temperature of 60°. Among the remaining complexants in Table V, only cyclododecanol and cyclododecyl methyl ether show promise as enhancers, and neither of these two is as good as cyclododecanone.

Cyclic complexants having fewer than twelve ring atoms do not appear to favor γ-CD production. Cyclodecanone, cycloundecanone, and cycloundecanemethanol all produce γ-CD in very low yield, but give β-CD in very high yield (Table VI).

Table VII presents data from multiple-increment runs at 48, 60, and 65° and illustrates the influence of temperature on the conversion of M-050 in systems containing cyclododecanone. Low temperature (48°) resulted in a relatively low

TABLE VI

Effect of C_{10} and C_{11} cyclic complexants on conversion by multiple addition of CGTase at pH 7.2 and 60°

Complexant	Concentration (mmol)	Substrate [a]	Buffer [b]	No. of 5-μL increments [c]	CD yield (%)			
					α	β	γ	Combined
Cyclodecanone	0.54	5% potato starch [d]	None	10	0.8	46.4	1.0	48.2
				15	0	45.4	0.9	46.3
				20	1.0	43.6	1.3	45.9
Cycloundecanone	0.18	10% M-050 [e]	None	1	3.2	52.4	4.8	60.4
				15	2.1	56.3	0.6	59.0
				20	2.0	53.9	0.5	56.4
				25	2.1	52.4	0.5	55.0
Cycloundecane-methanol	0.18	10% M-050 [e]	Tris	8	2.2	51.4	5.1	58.7
				15	1.6	50.8	1.0	53.4

[a] Volume of substrate solution, 3.00 mL. [b] 0.02 M Tris. [c] Frequency of addition is 2 increments per day (separated by an interval of at least 8 h). [d] 0.925 mmol of glucose residues. [e] 1.85 mmol of glucose residues.

yield (32%) of γ-CD. At 60 and 65°, much higher yields (46.0 and 49.5%, respectively) were achieved; however, only over the 48–60° range was there a rapid decrease in β-CD concentration during the course of reaction. The marked difference in yield between reactions at 48° and reactions at 60–65° suggests that the complexant itself participated directly in temperature-sensitive enzymic processes that determine both rate of γ-CD formation and positions of reaction equilibria. However, variation in reaction behavior with variation in temperature might also have arisen from variation in rate of enzyme inactivation. For example, the need for twice as many GCTase increments at 65 as at 60° was probably caused by a much lower enzyme stability at the higher temperature. And at 48°, where stability is relatively high, low γ-CD yields were possibly caused, at least partly, by a gradual increase in active enzyme concentration as increments of CGTase were periodically added to the system. The detrimental effect of high concentrations of enzyme will be discussed later.

Low pH can also be detrimental to γ-CD production. A pH close to 7 should be maintained for best results at 60° with cyclododecanone as complexant and Amano CGTase EC 2.1.4.19 as the transferase enzyme. At pH 4.0 and 5.2, maximum γ-CD yields at 60° were 24 and 25%, respectively.

High yields of γ-CD, with accompanying low yields of α- and β-CD, can be achieved at 60° by incremental addition of CGTase over a wide range of increment size. Table VIII presents data for experiments in which the size of each CGTase application was varied from 1 to 25 μL. Where increment size was varied from 1 to 15 μL, maximum γ-CD yields were not only very similar but were also reached at roughly the same volumes of total CGTase. However, in reactions involving increments larger than 10 μL, it was necessary to increase the time interval

TABLE VII

Influence of temperature on conversions of unbuffered 10% M-050 [a] by incremental addition of CGTase at pH 7.2 in the presence of cyclododecanone

No. of 5-μL increments [b]	Complexant (mmol)	CD yield (%)			
		α	β	γ	Combined
48°					
10	0.18	0.7	44.1	13.7	58.5
15	0.18	1.9	32.3	21.3	55.5
20	0.18	0	13.8	22.9	36.7
25	0.18	0	4.3	32.3	36.6
60°					
8	0.36	0.4	41.3	28.0	69.7
14	0.36	0.9	26.1	42.2	69.2
26	0.36	0.5	3.2	44.7	48.4
34	0.36	2.9	2.9	42.2	48.0
40	0.36	2.8	3.3	36.3	42.4
8	0.54	0.8	36.6	33.7	71.1
14	0.54	1.0	21.5	44.8	67.3
26	0.54	1.5	2.4	46.0	49.9
34	0.54	0.8	1.0	42.7	44.5
40	0.54	1.7	3.4	41.1	46.2
65°					
8	0.36	1.1	51.8	21.5	74.4
15	0.36	1.2	48.7	25.3	75.2
20	0.36	1.0	42.4	34.2	77.6
25	0.36	1.3	30.7	43.9	75.9
30	0.36	0.9	24.7	47.4	73.0
40	0.36	1.3	11.7	49.5	62.5

[a] Volume of 10% M-050, 3.00 mL; 1.85 mmol of glucose residues. [b] The frequency of addition of CGTase at 48, 60, and 65° is 1 increment per day, 2 increments per day (separated by an interval of at least 8 h) and 3 increments per day (separated by intervals of at least 4 h), respectively.

between applications in order to achieve highest yield. Even with increased time intervals, yields of γ-CD suffered slightly when increment size was 25 μL or greater. With 50-μL increments applied at 2-day intervals, maximum γ-CD yield was reached after 5 applications: 2 α, 2 β, and 37% γ-CD. These results indicate that the thermostability of CGTase at 60° is not low enough to permit the use of large increments and frequent application. A low concentration of enzyme is essential for maximum γ-CD yield. Conceivably, the process of incremental addition could be obviated by the use of a single application of a *thermally stable* CGTase.

Comparison of HPLC analysis of total reaction product mixture with that of precipitated complex. —Two identical reactions were conducted where mixtures of cyclododecanone (0.18 mmol) and 10% M-050 (3.00 mL) were treated incrementally with 25 5-μL amounts of CGTase at pH 7.2 and 60°. One of the product mixtures was azeotroped to remove the complexant; HPLC analysis showed the

TABLE VIII

Influence of size of CGTase increment on CD yields in multiple addition of enzyme to substrate at pH 7.2 and 60° with cyclododecanone as complexant

No. of increments [a]	Substrate [b]	Complexant (mmol)	Buffer [c]	CD yield (%)			
				α	β	γ	Combined
1-μL increments							
5	5% potato starch [d]	0.18	Tris	9.0	40.9	15.6	65.5
10				4.1	53.0	18.0	75.1
20				2.0	45.4	16.6	64.0
40				2.0	49.1	23.0	74.1
180				1.3	4.0	49.5	54.8
10-μL increments							
5	10% M-050 [e]	0.18	None	1.2	41.0	21.3	63.5
10				1.0	35.5	28.2	64.7
15				0	21.0	31.4	52.4
20				0.8	5.0	43.0	48.8
15-μL increments							
3	10% M-050 [e]	0.18	None	1.5	43.5	18.1	63.1
5				1.1	36.8	23.2	61.1
7				1.2	26.5	35.9	63.6
10				1.3	9.2	48.4	58.9
15				0	2.7	46.2	48.9
25-μL increments							
4	10% M-050 [e]	0.18	None	1.0	31.8	21.5	54.3
6				2.8	11.3	34.3	48.4
7				2.6	5.4	37.3	45.3
8				3.1	5.4	34.2	42.7
9				2.6	4.5	37.7	44.8

[a] Increments were added at a frequency of 3, 2, 1, and 1 per day, respectively, in the 1-, 10-, 15- and 25-μL studies. Intervals between additions for the 1- and 10-μL runs are no shorter than 4 h and 8 h, respectively. [b] Volume of substrate solution, 3.00 mL. [c] 0.02 M Tris. [d] 0.925 mmol of glucose residues. [e] 1.85 mmol of glucose residues.

following CD yields: 1.4 α, 3.5 β, and 48.6% γ. The other reaction-product mixture was centrifuged to isolate the cyclododecanone complex (mixed with a small mount of free complexant). After the precipitate had been washed with a few mL of water, it was subjected to azeotropic distillation to remove the complexant; HPLC analysis revealed only one peak (γ-CD). Calculations showed a yield of 46.7% γ-CD, with or without amyloglucosidase treatment. The supernatant from the centrifugation contained small amounts of all three CDs; HPLC analysis showed that the contribution of the supernatant to total CD yield was 1.4 α, 2.7 β, and 0.7% γ. The conclusion reached from this study was that the precipitate formed in the conversion reaction contained, at the point of maximum conversion to γ-CD, virtually all of the γ-CD and was essentially free of such contaminants as maltooligosaccharides and other CDs.

Removal of complexant from a γ-CD-cyclododecanone complex by ether extraction at 25° and elevated pH. —Using a procedure similar to that described in the Experimental section for extraction with ethyl ether at high pH, equal weights of 1 : 1 γ-CD–cyclododecanone (each sample containing 0.0702 mmol of γ-CD) were ether extracted, each at a different level of alkalinity. Periods of stirring with ether were 3 days at pH levels of 11.0 and 12.0, 8 h at pH 12.5, and 1 h at pH 13.0 and 13.5. After neutralization of the aqueous layers and removal of inorganic salt from these layers by mixed-bed ion-exchange resin, HPLC analyses of the aqueous solutions showed the following recoveries of γ-CD. pH/% γ-CD liberated: 11.0/80; 12.0/85; 12.5/98; 13.0/100; and 13.5/102.

These experiments revealed that, at pH 13–13.5, rapid dissociation of complex occurs and that removal of the liberated complexant by ether is easy and rapid. At pH 12.5, dissociation of complex was slow; however, it was complete in 3 h, as evidenced by the disappearance of turbidity caused by suspended particles of complex. Use of high-pH extraction is particularly useful where azeotropic distillation is not a practical method because of low volatility of the complexant and/or high stability of the CD complex.

ACKNOWLEDGMENTS

The author is indebted to Kevin L. Loeffelmann, who conducted many of the reactions and most of the analyses, and to Nancy A. Oliver, who participated in many related investigations that facilitated the present study.

REFERENCES

1 D. French, M.L. Levine, J.H. Pazur, and E. Norberg, *J. Am. Chem. Soc.*, 71 (1949) 353–356.
2 J. Szejtli (Ed.), *Proc. Int. Symp. Cyclodextrins*, Vol. 1, Akademiai Klado, Budapest, 1982.
3 H. Bender, in A. Mizrahi (Ed.), *Advances in Biotechnological Processes*, Vol. 6, Liss, New York, 1986, pp. 31–71.
4 S. Kobayashi, N. Shibuya, B.M. Young, and D. French, *Carbohydr. Res.*, 126 (1984) 215–224.
5 T. Kato and K. Horikoshi, *J. Jpn. Soc. Starch Sci.*, 33 (1986) 137–143.
6 K. Tomita, T. Tanaka, Y. Fujita, and K. Nakanishi, *J. Ferment. Bioeng.*, 70 (1990) 190–192.
7 F.C. Armbruster and W.A. Jacaway, Jr., U.S. Pat. 3 640 847, February 8, 1972.
8 E. Flaschel, J.P. Landert, D. Spiesser, and A. Renken, *Ann. N.Y. Acad. Sci.*, 434 (1984) 70–77.
9 Toyo Jozo Co., Ltd., *Jpn. Kokai Tokkyo Koho*, JP 60 120 998, June 28, 1985.
10 F. Shiraishi, K. Kawakami, H. Marushima, and K. Kusunoki, *Staerke*, 41 (1989) 417–420.
11 Y. Lee and H. Kim, *Enzyme Microb. Technol.*, 13 (1991) 499–503.
12 F. Cramer and D. Steinle, *Justus Liebigs Anal. der Chemie*, 595 (1955) 81–100.
13 H. Vakaliu, M. Miskolci-Torok, J. Szejtli, M. Jarai, and G. Seres, Hung. Teljes 16, 098, February 28, 1979.
14 W.S. McClenahan, E.B. Tilden, and C.S. Hudson, *J. Am. Chem. Soc.*, 64 (1942) 2139–2144.
15 R.N. Ammeraal, U.S. Pat. 4 738 923, April 19, 1988.
16 H. Bender, *Carbohydr. Res.*, 124 (1983) 225–233.
17 H. Bender, Ger. Offen DE 3 317 064, November 15, 1984.
18 G. Seres, M. Jaria, S. Piukovich, M.S. Gabanyi, and J. Szejtli, Ger. Offen DE 3 446 080, July 4, 1985.
19 M. Sato and Y. Yagi, *ACS Symp. Ser.*, 458 (1991) 125–137.

20 G. Schmid, O. Huber, and H.J. Eberle, in O. Huber and J. Szejtli (Eds.), *Proc. Int. Symp. Cyclodextrins*, 4th 1988, pp. 87–92.
21 G. Schmid and H.J. Eberle, Eur. Pat. Appl. EP 0 291 067 A2, November 17, 1988; G. Schmid and H.J. Eberle, U.S. Pat. 4 822 874, April 18, 1989.
22 E.B. Tilden and C.S. Hudson, *J. Bacteriol.*, 43 (1942) 527–544.
23 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.