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Enhanced Conversion of Starch to Cyclodextrins in Ethanolic Solutions by *Bacillus circulans* var *alkalophilus* Cyclomaltodextrin Glucanotransferase

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ABSTRACT

Improved formation of cyclodextrins (CDs) from starch in ethanolic solutions by *Bacillus circulans* var *alkalophilus* cyclomaltodextrin glucanotransferase was studied. The β- and γ-CD yields increased and α-CD yield gradually decreased as the ethanol concentration was raised. The ethanol concentration required for maximal CD yield depended essentially on starch concentration. The ethanol's effect was pronounced at high starch concentrations. For example, with 30% (w/v) starch, the CD yield was 2.4-fold (146.5 g/L) in the presence of 15% (v/v) ethanol. The effect of dimethylsulfoxide on the formation of CDs was similar to that of ethanol. The disintegration of β- and γ-CDs were narrowly interdependent on the formation of a α-CD and maltosugars. The amount of reducing sugars decreased from a dextrose equivalent value of roughly 7.5 to 4.5 in the presence of ethanol at starch concentrations 1-30% (w/v). The effect of ethanol on starchy materials from various sources was similar. It was concluded that ethanol retards the decomposition of β-CD by a general mechanism involving a decreased activity of water.

Index Entries: Production of cyclodextrins; effect of ethanol; cyclomaltodextrin glucanotransferase; enzymatic hydrolysis of starch.

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INTRODUCTION

Many scientific reports and patents (*1*) describe the production of alpha-, beta-, and gamma-cyclodextrins (α-, β-, and, γ-CDs) by the enzyme cyclomaltodextrin glucanotransferase (CGTase; EC 2.4.1.19). As a rule, when the starch concentration is increased, the CD yield decreases drastically. The yield can be increased by the application of those compounds which complex to CDs, e.g., toluene or trichloroethylene (*2–6*). They are not, however, entirely satisfactory because of their toxic nature. More recently, less-toxic compounds, such as decanol or limonene have been introduced (*7,8*). The decanol method is limited to the production of α-CD. Although the process utilizing limonene from mandarin peel oil seems promising, the relatively high price of this additive may rule out its use in an industrial scale.

According to literature references the equilibrium constants for binding ethyl alcohol to α-CD is 4.8–5.6 M^{-1} (*9,10*) and to β-CD 0.5–0.9 M^{-1} (*10,11*). These binding constants are of about the same order as those of certain inorganic ions (*12–14*) and, in any case, are at least two orders of magnitude smaller than those of the actual complexants used in the CD manufacturing processes. Against this background it is surprising that ethanol was reported to act as an effective CD yield-increasing additive in conversion mixtures (*15–18*).

A substantial part of scientific research on the effects of ethanol has been carried out using a system involving CGTase from *Bacillus macerans*. In the initial phase of the reaction this enzyme ("α-CGTase") produces mainly α-CD. The target of ethanol was proposed to be the inclusion complex formation with α-CD (*15,17*). Although the binding constant of β-CD is of about one order less than with α-CD (*9,10*), favorable effects of ethanol have also been found with enzymes producing mainly β-CD (*16,18*). However, with β-CGTases results employing only a starch concentration of (*18*) or below (*16*) 5% (w/v) have been described.

The purpose of the present work was to provide new aspects for the effects of solvents such as ethanol and dimethylsulfoxide (DMSO) in the synthesis of CDs by the β-CD producing enzyme from *B. circulans* var *alkalophilus* (ATCC 21783). The results suggest that organic solvents in the conversion reaction can have quite similar consequences regardless of their nature or of the source of the enzyme preparation. It is concluded that ethanol and other related solvents exclude water molecules from the active center of the enzymes and thus prevent hydrolytic reactions.

MATERIALS AND METHODS

Materials

Glucose, maltose, bovine serum albumin (fraction V), CDs, amylose, and amylopectin from potato and starch from potato, corn, wheat, and rice were purchased from the Sigma Chemical Co. (St. Louis, MO). Barley starch was the product of Suomen Nestesokeri Co. (Jokioinen, Finland). Linear malto-oligo-saccharides were from Boehringer-Mannheim (FRG). Methylorange and DMSO were products of E. Merck A. G. (Darmstadt, FRG), while 3,5-dinitrosalicyclic acid (DNS) was from the Aldrich Chemical Co. (Milwaukee, WI). Other chemicals used were of reagent grade.

Methods

Enzyme CGTase

CGTase was produced by growing *Bacillus circulans* var *alkalophilus* (ATCC 21783) under conditions described elsewhere (*19*). The enzyme was recovered and purified by affinity chromatography (*20*). CGTase activity was measured using the maltotriose-methylorange method (*21*). One enzyme U was defined as the amount of enzyme producing 1 μmol of CDs per min and L.

Protein Concentration

Proteins were determined according to the method of Lowry et al. (*22*) using bovine serum albumin as the standard.

Conversion Experiments

Conversion of starch (1%; w/v) to CDs was performed as follows: Native starch was suspended in water and gelatinized by immersing the reaction mixture in boiling water for 5 min. The suspension was then diluted with buffer stock solution (1*M* imidazole-HCl, pH 6.8, containing 50 m*M* $CaCl_2$) and with ethanol or DMSO to obtain 1% (w/v) starch solution with a buffer strength of 25 m*M*. The reaction was usually started with 100 U of the enzyme per g of starch. The reaction was allowed to take place at 60°C with a reaction vol of 10 mL.

The experiments with higher starch concentrations (up to 30%; w/v) were carried out enzymatically in two steps. Calculated amounts of starch, buffer (see preceding discussion), and ethanol or DMSO were mixed in order to obtain appropriate concentrations of the solutes. The mixture was liquified with CGTase in tightly capped reaction bottles placed on a one-dimensional shaking water bath (250 rpm) at 70°C for 1 h (unless otherwise indicated). The reaction was then allowed to proceed in an incubator at 60°C. The percentage concentrations of additive solvents (ethanol or DMSO) or starch are expressed throughout this paper as v/v and w/v, respectively.

Sugar Analyses

Maltooligosaccharides and CDs were analyzed with HPLC (*23,24*). Reducing sugars were assayed with the dinitrosalisylate reaction against the glucose standard (*25*). The accuracy of the dilution processes was controlled by measuring the total carbohydrates using the Morris method (*26*).

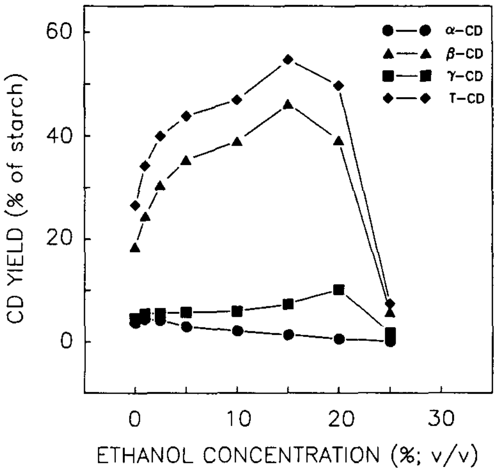

Fig. 1. Effect of ethanol on yield (% of starch) of α-, β-, and γ-CDs and the sum of these CDs (T-CD). Reaction conditions were as follows: Initial starch concentration, 15%; enzyme, 100 U/g of starch; reaction time, 24 h; temperature, 60°C; 25 m*M* imidazole-HCl buffer, pH 6.8, supplemented with 1.25 m*M* $CaCl_2$.

RESULTS

Conversion of 15% Starch to CDs in the Presence of Ethanol or DMSO

The formation of CDs from a fixed 15% concentration of soluble potato starch by CGTase at different ethanol concentrations (1–25%) was investigated (Fig. 1). The optimal ethanol concentration for maximal CD yield depended on the starch concentration. At a starch concentration of 15% the maximal conversion to CDs (55% of starch) was achieved with 15% ethanol. The resulting increase in total CD yield by ethanol was about twofold.

Ethanol promoted mainly the formation of β-CD (Fig. 1). The content of β-CD increased from 19% to 46% of starch as the ethanol concentration reached its optimum. Interestingly, the formation of γ-CD also nearly doubled (with 20% of ethanol the γ-CD yield was 10% starch). In contrast, at the same time the portion of α-CD decreased. It should be noticed that, after the partial crystallization of β-CD from the conversion mixture, it contained substantial amounts of γ-CD. The parallel appearance and disappearance of β- and γ-CDs suggests that they are synthesized with an equal mechanism by the present enzyme. At above 20% ethanol, CD yields decreased abruptly, possibly because of the denaturation of the enzyme.

DMSO is an aprotic solvent with a high dielectric constant. Starch granules can be completely solubilized in DMSO without any change in the primary structure of the polysaccharide (*27*). The effects of DMSO on CD formation from starch were studied under the same reaction conditions as those described in the context of ethanol. As with ethanol, DMSO

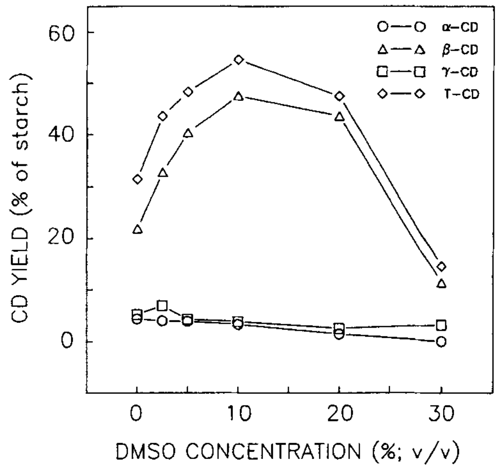

Fig. 2. Effect of DMSO on CD yield (% of starch). The reaction was carried out as in Fig. 1 with the exception that the reaction time was 25 h.

increased the formation of CDs and also favored β-CD (Fig. 2). The maximal yield of total CDs under the reaction conditions studied was 55% (of which 47 percentage U consisted of β-CD). The maximal yield was the same as with ethanol but the optimum solvent concentration was 5 percentage U lower with DMSO. The formation of α-CD decreased as the DMSO concentration increased and finally ceased at a DMSO concentration of 30%, whereas the amount of γ-CD remained stable. The amount of CDs decreased as the DMSO concentration rose above 20%. However, the decrease was not as steep as with ethanol. Sodium chloride up to a concentration of 1*M* did not affect the conversion reaction.

Both ethanol and DMSO greatly increased the relative proportion of β-CD (Fig. 3), which increased from 69% to 84% with ethanol (15%) and from 70% to 87% with DMSO (10%). Among CDs the relative content of β-CD was at maximum at the same ethanol concentration (15%) as the maximal total CD concentration, but with DMSO the relative β-CD content did not reach maximum until a DMSO concentration of 20%, where it was as high as 92%. At high solvent concentrations (ethanol above 15%; DMSO above 20%) there was a significant increase in the relative amount of γ-CD, whereas the amount of α-CD was negligible.

Dependence of CD Yields on Ratios of Ethanol, Starch, and CGTase

The effect of enzyme activity on CD concentration under a constant starch concentration (10%) and a constant reaction time (4 h) with and without ethanol (10%) was studied (Fig. 4). In aqueous starch solution β-CD increased, until at a CGTase activity of 100 U/g of starch, it leveled

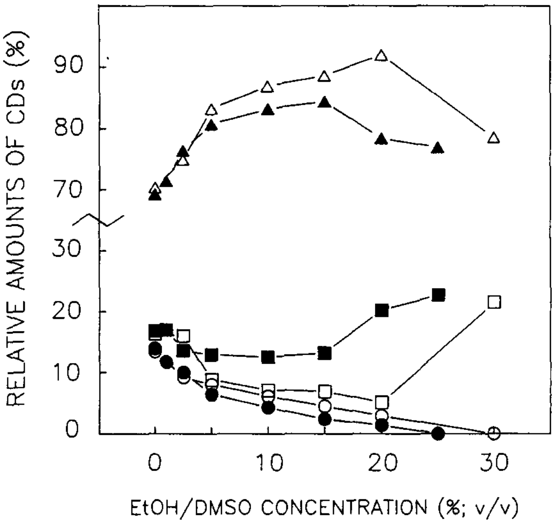

Fig. 3. Relative amounts of CDs (% of total) as measured in different concentrations of ethanol or DMSO. For reaction conditions and symbols, *see* Figs. 1 and 2.

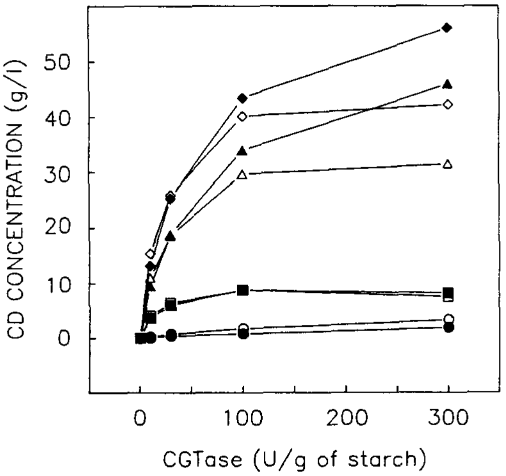

Fig. 4. Relationship between CD concentration and the amount of enzyme with (filled symbols) and without (open symbols) 10% ethanol. Reaction conditions were as in Fig. 1 with the exception that reaction time was 4 h and initial starch concentration being 10%. The symbols are as in Fig. 1.

off. In the presence of 10% ethanol, β-CD continued to increase to at least 300 U/g of starch. Thus, in ethanolic solutions the CD formation can be accelerated by high enzyme activity. At low enzyme activity (10 U/g of starch) ethanol slightly retarded CD formation.

In addition to enzyme/starch ratios, the ethanol/starch ratios should also be taken into consideration when seeking improved reaction conditions. This is illustrated by the β-CD formation with 1, 5, and 15% ethanol

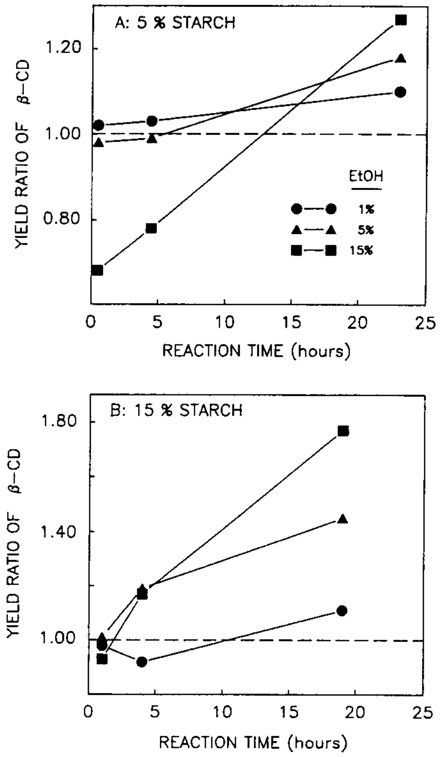

Fig. 5. Time course of yield ratio of β-CDs formed with 1%, 5%, and 15% ethanol to β-CDs formed without ethanol at starch concentrations (A) 5% and (B) 15%. The reaction was performed as in Fig. 1.

as compared with the formation of β-CDs without ethanol (Fig. 5). Starch concentrations were 5% and 15%, whereas the level of enzyme was constant (Fig. 5, A and B). At 5% starch (Fig. 5A) the increase of ethanol from 1% to 15% retarded the β-CD formation at the beginning of the reaction. However, when the reaction proceeded the effect of ethanol gradually changed to favor the β-CD formation. At a starch concentration of 15%, the retarding effect of ethanol was less significant (Fig. 5B). Figure 5 (A and B) indicates that the positive effect of ethanol is especially evident at high starch concentrations.

Relationship Between α-CD and Disintegration of β-CD

In the reaction of α-CGTase, β-CD eventually accumulated at the expense of α-CD is deduced to be due to a transformation of α-CD to β-CD (*28*). Figure 6 shows that with β-CGTase the disintegration of β-CD and appearance of α-CD were narrowly interdependent. Disintegrations of both β- and γ-CD occurred in a related way, even though γ-CD seemed to disintegrate earlier. In prolonged reactions, small maltosugars increased markedly. Thus, with the present β-CGTase the relative amount of α-CD

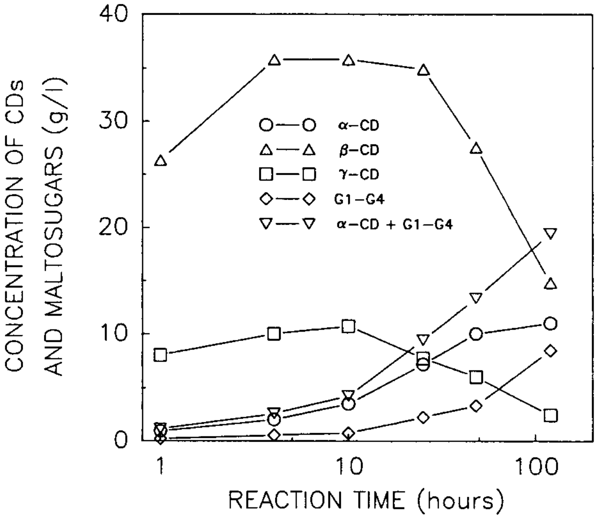

Fig. 6. Time course of CD and maltosugar (G1-G4) concentration (g/L). The reaction conditions were as in Fig. 1.

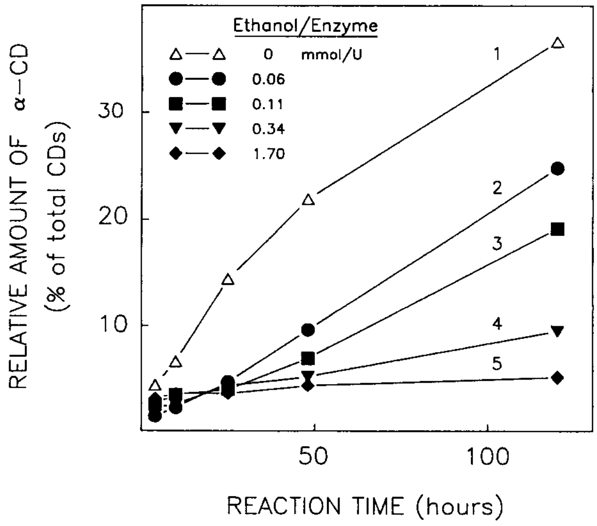

Fig. 7. Time course of the relative amount of α-CD (% of total CDs) as measured with different ethanol/enzyme ratios (mmol/U). The reaction was carried out with constant enzyme (100 U/g of starch) and ethanol (10%) concentrations. Control experiment was performed at a starch concentration of 15%.

(or α-CD + G1-G4) among β-CD correlated well with the progress of the reaction and with the decomposition of the main product.

Results shown in Fig. 6 and curves 1 and 3 in Fig. 7 are directly comparable. Ethanol decreased significantly the relative portion of α-CD in conditions suitable for β-CD production (reaction time 10–40 h; Fig. 7, curves 1 and 3), whereas the relative amount of α-CD increased linearly throughout the reaction time. Even with prolonged reaction times ethanol clearly retarded the conversion of β- to α-CDs.

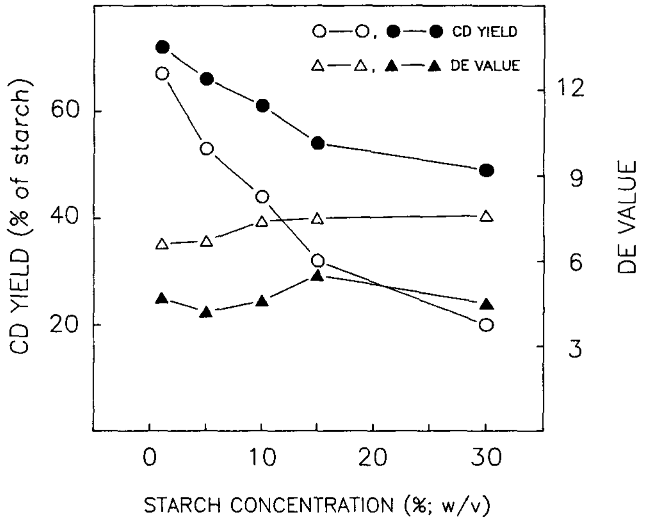

Fig. 8. Effect of starch concentration on the amount of CDs and reducing sugars with and without ethanol. The relationship between ethanol and starch concentrations was optimized in order to obtain maximum CD yield (ethanol/starch, %/%; 5/1; 10/5; 10/10; 15/30). The reaction conditions were as in Fig. 1. with the exception that the reaction time was 21 h. Filled symbols refer to reactions carried out with ethanol.

Since the enzyme/starch ratio affected the relative amount of α-CD in the reaction mixture (*see*, e.g., Fig. 4), it was difficult to design an exact experiment showing the effect of ethanol on the α/β-CD ratio in the product. The ethanol/enzyme ratio was changed, while employing a constant enzyme/starch ratio (Fig. 7, curves 2–5). The relative proportion of α-CD clearly decreased as the ethanol/enzyme ratio was raised.

Optimal Starch Concentration

The concentrations of ethanol applied at certain starch concentrations were optimized in order to achieve maximal CD yield. In the absence of ethanol the total CD yield decreased from 67 to 20% when the starch concentration was raised from 1 to 30%. Although ethanol increased the CD yield 1.1-fold with 1% starch, the increase with 30% starch was as high as 2.4-fold (Fig. 8). The maximal CD concentration, 146.5 g/L was attained at a starch concentration of 30% with 15% ethanol. The reducing sugars decreased from a DE value of roughly 7.5 to 4.5, regardless of the starch concentration (Fig. 8).

Starch Source

The effect of ethanol on the formation of CDs from various starches, amylose and amylopectin, is presented in Table 1. In the presence of 5% ethanol the formation of CDs from potato, corn, wheat, and barley starches was 1.7–1.9 times that without ethanol. The degree of conversion increased

Table 1
Conversion Degree (% of Initial Starch) of Total CDs
from Various Starchy Materials with and without 5% Ethanol

Starchy material	Conversion to cyclodextrins (%)	
	No ethanol	5% ethanol
Potato	37.8	66.7
Corn	37.3	63.6
Wheat	32.4	60.2
Rice	13.2	27.7
Barley	35.2	62.6
Amylose	30.9	46.8
Amylopectin	26.7	44.7

Initial starch concentration was 10% and the CGTase activity was 100 U/g of starch. The liquefaction reaction was carried out for 0.5 h at 70°C followed by a 24-h reaction at 60°C.

from 32–38% to 60–67% with ethanol. The starch material did not affect the product ratio. Rice starch appeared to be a poor substrate. However, the yield of CDs, from this as with other starches, doubled with ethanol. The degree of conversion of amylose to CDs was only 47% in the presence of ethanol. Under the same conditions, an almost identical conversion (45%) was attained with amylopectin.

DISCUSSION

There are at least four mechanisms by which ethanol can promote the enzymatic conversion of starch to CDs: (a) ethanol affects the structure and availability of starch, (b) it decreases the effective concentration of cyclic products by precipitating out, or complexing to them, (c) ethanol changes the inherent properties of the enzyme itself, or (d) it modifies the general properties of the reaction medium. Some of these possibilities can be excluded with presently available data.

It is conceivable that ethanol or DMSO could open the complex structure of gelatinized starch and increase effective concentration of a beneficial starting material. Because both starchy compounds of various lengths and the CDs serve as the substrates, the preceding possibility cannot be readily excluded. However, as ethanol also enhances synthesis of CDs from starchy hydrolysis products at different concentrations *(21)*, the increase in availability of starch is not a feasible target.

Ethanol in the conversion mixture affected most distinctly the yield of β-CD. The increase in the main product (α-CD) was also reported in the case of CGTase from *B. macerans* *(17)*. These two results tend to exclude the possibility of ethanol changing the steric structure of starch molecules so as to promote the formation of a specific CD form as proposed earlier *(17)*.

As suggested in the Introduction, the binding constants of ethanol with α- or β-CDs do not correlate with the experimental results on promotion of CD production by ethanol. Its effect should only be seen in rather high concentrations and be especially useful in the production of α- although not in β-CD synthesis. Ethanol does not improve the precipitation of the conversion products over the concentration range employed but rather facilitates solubility (*1*). If the phenomenon is based either on complexation or precipitation, various solvents should show at least some basic differences, this being not in harmony with the available experimental data.

Although DMSO has a completely different chemical nature from ethanol, they promote the formation of β-CD almost similarly. Furthermore, it has been reported that in the presence of moderate concentrations of several other solutes, such as acetone, methyl-, 2-propyl-, *iso*-butyl, *sec*-, and *tert*-butyl alcohols, the formation of β-CD by CGTase from *B. circulans* is enhanced (*16*). Most probably the formation of CDs promoted by these employs identical mechanisms.

A change in the conformation of CGTase may take place adding ethanol, thus producing the observed results. Alternatively, the molecules may bind to specific sites on the enzyme and inhibit the hydrolytic reactions. It has been found that the binding of CGTase to starch is considerably increased by adding 10–20% ethanol (unpublished data). This can be due to a change of Km, general solubility effects, or the enhanced surface area on starch. The binding of CGTase to starchy materials is stronger at low temperatures and, in addition, the reaction is directed to cyclic products by low temperatures (*29*). It is evident that conformational changes slightly affect, e.g., the product composition. However, insensitivity of the enzymes to various solvents ranging from 1*M* salt solution, DMSO, to acetone and alcohols suggest other mechanisms.

We tentatively propose that the enhanced conversion of starch to CDs by α- and β-CGTases in the presence of hydrophilic, weakly complexing solvents results from a decreased activity of water in the reaction mixture. This decreases specifically the hydrolytic reactions of the CGTase. The hydrolysis may be an occasional side event on the active site that is made less probable by the presence of a substantial molar amount of any other solvent than water. Low reaction temperature could improve accuracy of the catalysis through the entropic advantage of the main reactions.

While the CD yield improvement through product complexation is straightforward, knowledge of the mechanism of action by solvents can be utilized alone, or in combination with product complexation, to improve the CD manufacturing processes.

According to the present and previous studies (*15–18*) ethanol seems to be an especially powerful tool in the production of CDs. It is practically a harmless, evaporable additive that can be readily distilled and reused. Its application overcomes microbial contamination risks in the manufacturing process. As with other solvents, the yield of a single CD-species is significantly increased especially at high substrate concentrations. Direction of the reaction toward one species facilitates the purification processes. Although the CGTase reaction rates can be slightly decreased by the addition of solvents, this drawback is greatly compensated by the possibility of accelerating the reaction by the addition of a greater amount of the enzyme.

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