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Influence of Substrate and Product Concentrations on the Production of Cyclodextrins by CGTase of *Bacillus firmus*, Strain no. 37

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Abstract

The influence of substrate or product level on the initial velocity of cyclodextrin (CD) production by cyclodextringlycosyltransferase from a Brazilian isolate of *Bacillus firmus* was studied. Our results indicate that the product γ-CD is a stronger inhibitor to the reaction than β-CD. Small saccharides could also inhibit CD production, although to a lesser extent than the products, and maltose was the strongest inhibitor among small saccharides. Increasing substrate concentration resulted in greater reduction on enzyme activity for the formation of β-CD than for γ-CD. We modeled the kinetics of CD production with a set of four reversible reactions including the cyclization/coupling reaction that forms/opens CDs, and three disproportionation reactions. Our model on the initial velocity data explained well the substrate inhibition phenomenon. Kinetic parameters were determined by fitting the initial velocity data into our model.

Index Entries: Cyclodextrin; cyclodextringlycosyltransferase; inhibition; maltodextrin; kinetic parameters.

Introduction

The usual enzymatic degradation of starch is by hydrolysis leading to the formation of glucose, maltose, maltotriose, and maltooligomers with linear or branched chains, which are known as dextrins. When starch is degraded by an enzyme belonging to the class of the glycosyltransferases

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the reaction proceeds by an intramolecular chain transfer while the water molecule does not participate. The cyclodextrin glycosyltransferase enzyme (CGTase) is a special type of enzyme because its starch degradation products are cyclic; these products are known as cyclodextrins (CDs) *(1–2)*.

The most common CDs have six to eight α-1,4-D-glucopyranosyl residues, and are called α-, β- or γ-CD, respectively. Larger CDs, comprising oligosaccharides of up to 31 glucopyranosyl residues have been reported *(3–5)*. However, they are less stable than the small CDs. Smaller CDs with five or fewer glucopyranosyl residues were considered too unstable to exist owing to their excessive internal tension of the small ring. Nevertheless, the CD with five units has recently been synthesized *(6)*.

A CD molecule is shaped like a truncated cone in which the external surface is covered by glucopyranosyl residue, primary and secondary hydroxyls that give rise to hydrophilic characteristics to the CD molecule, whereas the CD internal cavity is lined with oxygen and hydrogen atoms that confer hydrophobic character to the interior of the molecule. It has been shown that the hydrophobicity of the cavity is similar to that of ethanol *(7)*. A hydrophobic molecule or hydrophobic portion of a molecule that snugly fits inside the cavity may form an inclusion complex. Generally, hydrophobic molecules or even hydrophilic ones have greater affinity for the CD cavity when they are in a water solution *(8)*.

CD inclusion complexes are stabilized by a number of intermolecular forces, such as hydrophobic, van der Waals forces, and hydrogen bonds. The molecular encapsulation can occur either in the solid state or in solution, and the physicochemical characteristics of the guest molecule can be changed. This may favor drug properties such as solubility, dissolution rate, stability, and reduction of side effects *(8)*.

Reaction Mechanism

The known reaction mechanism for the production of CDs by CGTase postulates three types of reactions: cyclization, coupling, and disproportionation *(9)*:

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

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

in which G_n is a α-1,4-D-glucopyranosyl linear chain containing n glucopyranosyl residues; G_x is the CD, and for $x = 6, 7$, and 8, it implies α-, β- and γ-CD, respectively; G_m, $G_{(n-x)}$, $G_{(n-y)}$, $G_{(m+y)}$ are defined equivalently to G_n; and $1 \leq y \leq n$ or m is the number of exchanged residues.

Cyclization proceeds by intramolecular transglycosylation and is the reaction that actually produces CDs from starch chains that must have

more than six glucopyranosyl residues and be in their helical form *(9)*. If the chains have fewer than six units but more than two, i.e., from maltotriose to maltopentose, CGTase is capable of synthesizing oligomers of higher molecular weight by the disproportionation reaction, and then produces CDs *(10)*. The coupling reaction, also known as intermolecular transglycosylation, includes the opening of a CD ring and transfer of maltooligosaccharides produced to acceptor molecules such as maltose. In the reaction medium for producing CDs, coupling occurs in the presence of CDs and certain cosubstrates, such as glucose, maltose, and maltotriose. It can compete with cyclization, which results in reducing yields *(11–12)*. For the production of CDs, raw starch is usually hydrolyzed to low dextrose equivalent (DE), but some percentage of small molecular weight saccharides is always produced; these will reduce CD yield, and the higher the DE the lower the yield *(13)*.

Because starch is cheap, the industrial production of CDs is made preferably with concentrated substrate solutions that allow the use of a smaller reaction vessel and reduce process costs at the downstream steps of separation and recovery of products. However, CD yield is also reduced by substrate and product inhibition of CGTase. Higher substrate concentration increases substrate and product inhibition of CGTase, and the intermolecular transglycosylation reactions. Consequently, the optimal starch concentration is a compromise that depends on the kinetic characteristics of the enzyme, reactor, and downstream costs *(13–14)*.

In this article, we present results for the influence of substrate concentration on the initial velocity of β- and γ-CD production by the CGTase from *Bacillus firmus*, strain no. 37, isolated from Brazilian soil. The kinetic parameters K_m, V_{max}, and K_s were obtained from these data. The influence of the smaller maltooligosaccharides, glucose, maltose and maltotriose and the products β- and γ-CD on the initial velocity of β-CD production was also investigated. A new model for the initial velocity of reaction was developed and fit to the experimental data.

Materials and Methods

Substrate

The substrate solutions were made with maltodextrin (DE 10) (article 31410, average mol wt of 1.672 kD; Fluka Chemie AG, Buchs, Switzerland). This dextrin was made by enzymatic hydrolysis of starch and contains <4% (w/v) of glucose plus maltose, 16% maltooligosaccharides with three to five glucopyranosyl residues, and the remaining 80% being hexasaccharides or larger oligosaccharides.

Enzyme

CGTase was obtained from the alkalophilic microorganism (*B. firmus*, strain no. 37) isolated from Brazilian soil. The isolate was cultivated in 250 mL of a liquid medium, pH 10.0, with the following composition:

1.0% (w/v) maltodextrin, 0.5% (w/v) polypeptone, 035% (w/v) yeast extract, 0.1% (w/v) K_2PO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 1.0% (w/v) Na_2CO_3. Cultivation proceeded at 37°C for 6 d with stirring at 150 rpm. After this period, the cells were removed by centrifugation. The proteins from the cell-free supernatant were precipitated by salting out with ammonium sulfate (80% of saturation) at 4°C for 48 h. A second centrifugation was carried out, and the precipitated CGTase was further purified by biospecific affinity chromatography using β-CD as ligand. The purified CGTase was concentrated by ultrafiltration with a 30-kD mol wt cutoff *(15–16)*. This CGTase enzyme produced essentially β- and γ-CD from maltodextrin and negligible amounts of α-CD.

Enzyme Activity

One unit of activity corresponds to the amount of CGTase that produces 1 µmol of CD/min under the defined reaction conditions (β- or γ-CD). Activity assays were carried out at 50°C in a maltodextrin solution with 50 m*M* Tris-HCl buffer (pH 8.0) and 5 m*M* $CaCl_2$. The concentrations of the substrate solution and buffer-diluted enzyme are specified in each different type of test that follows. Substrate and enzyme solutions were warmed separately for 5 min to reach the reaction temperature. One mililiter of diluted enzyme and 1 mL of substrate solution were mixed and incubated for 5–30 min with 5-min intervals. CGTase was then inactivated by boiling the test tube for at least 5 min *(15–16)*. The enzyme concentration and other conditions were selected to obtain a linear relationship between the concentration of CD and time, in order to reduce the effects of longer reaction times according to the criteria published by Dixon and Webb *(17)*. The control was a reaction time of 0 min, and in this case, the CGTase was deactivated first by boiling, and then the substrate solution was added.

The initial velocity of production of β- and γ-CD (i.e., the enzymatic activity of the CGTase for each product) was determined from the linear regression of data on CD concentration (m*M*) vs time (min).

CD and Protein Assays

The reaction products β-CD or γ-CD were determined by colorimetric methods. In the case of β-CD, the dye used was phenolphthalein which loses its redness after complexing with β-CD, and the assay was carried out at 550 nm. Bromocresol green was the dye used for assaying γ-CD; in this case, light absorption increases on complexation with γ-CD, and it was measured at 620 nm *(18)*. Protein concentration was determined by Bradford's method using bovine serum albumin as the standard *(19)*.

Influence of Substrate Concentration on CD Production

The influence of substrate concentration was measured in relation to the production of β- or γ-CD, and this allowed the determination of substrate inhibition. The test was carried out at 50°C and pH 8.0. The substrate

solution for the γ-CD assay was prepared in the following concentrations: 0.1, 0.25, 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, and 20.0% (w/v). The substrate solution for the β-CD assay was prepared in the following concentration: 0.05, 0.1, 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0, 25.0, and 30.0% (w/v). Given the higher initial rate of β-CD production, higher enzyme dilution was used in this case, 1:400, whereas in the case of γ-CD assay, the dilution was 1:20. The purified enzyme stock solution was 0.118 mg/mL. The tests were carried out in a similar manner as described under Enzymatic Activity.

Determination of Kinetic Parameters

From the data on enzymatic activity as a function of substrate concentration, the kinetic parameters K_m, V_{max}, and K_s were obtained. When substrate concentration is low enough to neglect substrate inhibition, Michaelis-Menten kinetics are adequate to fit the data and K_m and V_{max} are obtained *(20)*. For higher substrate concentrations, the data were used to initially fit to the standard model of inhibition by substrate *(20)*:

$$V = V_{max}/(1 + K_m/S + S/K_s) \quad (3)$$

in which V is the initial velocity for CD production, V_{max} is the maximal reaction velocity, K_m is the Michaelis-Menten parameter, S is the substrate concentration, and K_s is the substrate inhibition constant. The values already obtained for K_m and V_{max} with the lower substrate concentration data were kept while fitting Eq. 3 to the higher concentration data, and, consequently, K_s was the single adjusting parameter.

A computer program was written for fitting the Michaelis-Menten equation to the low substrate concentration data by either the Lineweaver-Burk method ($1/V \times 1/S$) or the Hanes method ($S/V \times S$), and drawing the corresponding graphics. In this work, the Hanes method gave a better fit to the data. The computer program also used Eq. 3 transformed to Eq. 4 and calculated K_s by minimizing the sum of the squares of the relative errors *(15)*:

$$(S/V) = (K_m/V_{max}) + (1/V_{max})\,S + [1/(K_s V_{max})]\,S^2 \quad (4)$$

Inhibition by Low Molecular Weight Saccharides

The influence of the low molecular weight maltooligosaccharides on CGTase activity was determined in nine tests in which a 2% (w/v) maltodextrin solution was prepared with one of the three different concentrations (2, 4, and 10% [w/v]) of glucose, maltose, or maltotriose. The purified CGTase in this test was 0.209 mg/mL of protein and it was diluted 1:400. CGTase activity was measured as described before. Mixing the substrate and enzyme solutions in these tests gave maltodextrin concentrations of 1% (w/v) and 1, 2, or 5% (w/v) for the exogenous low molecular weight saccharides.

Inhibition by the Products β- and γ-CD

To test for inhibition of reaction by the products β- and γ-CD, a protocol similar to that described in the previous section was used. However, in this case, the added saccharides were substituted for β- and γ-CD. The CD concentrations at the beginning of these tests were 0.125, 0.25, and 0.5% w/v.

Results and Discussion

Influence of Substrate Concentration on CD Production

Figure 1 illustrates the initial rate of reaction (*V*) as a function of the substrate concentration for β-CD production (Fig. 1A) and for γ-CD production (Fig. 1B). In both cases, substrate inhibition is clearly seen, because activity initially increases with substrate concentration, but it reaches a maximum and then decreases with a further increase in substrate concentration. The point at which the maximum occurs is different for each CD, and substrate concentrations at these points are about 0.02 and 0.03 *M* for β- and γ-CD, respectively. Substrate inhibition reduces the initial rate of CD production by about 10% at the substrate concentrations of 0.0075 and 0.023 *M* for the production of β- and γ-CD, respectively. These results show that CGTase from *B. firmus* is inhibited by substrate to a greater degree for β-CD production. The observed reduction in the initial rate of CD production is accentuated at higher substrate concentrations. According to Dixon and Webb *(17)*, this type of enzymatic kinetic behavior for different substrate concentrations is characteristic of reactions that show inhibition at high substrate concentration.

The cyclization reaction, Eq. 1, implies an increase in the number of moles. Consequently, according to thermodynamics *(21)*, the conversion of substrate to CDs should decrease with higher substrate concentration. This was experimentally observed by Horikoshi et al. *(13)*. They used CGTase from *B.* alkalophilic no. 38-2 and 1–30% potato starch solutions hydrolyzed by CGTase to DE 2. They observed that β-CD yield increased from 10 to 50% when the initial starch concentration decreased from 25 to 2%.

Bender *(9)* has shown that the relative production of CDs in relation to the total carbohydrates decreases strongly with an increase in the initial concentration of the substrate. For 1% (w/v) substrate, more than 85% amylose, approx 65% amylopectin, and 55% glycogen are converted to CD, whereas 5% starch produces only 35% CD with the CGTase from *Klebsiella pneumoniae* and 50% with *B.* sp. alkalophilic.

Hokse et al. *(22)* patented the production of β-CD from soluble starch with concentrations from 1 to 10% (w/v) and the CGTase from *B. circulans* RIV no. 11115. The total yield of CDs was 60% for 1% starch and a 5:50 ratio of milligrams of enzyme/milliliter of substrate solution. However, even when they used the highest ratio of enzyme to substrate, they obtained only 30% CDs for 10% starch.

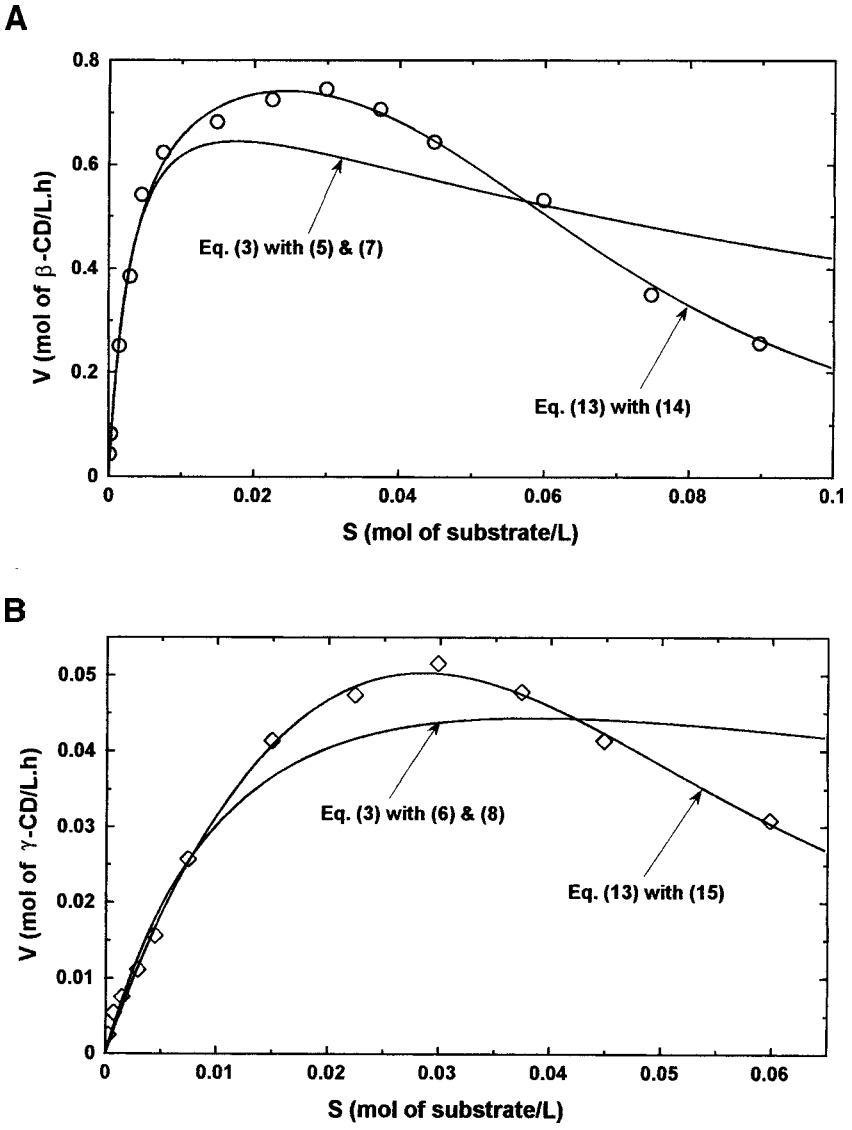

Fig. 1. Initial velocity of CD production (V) by the CGTase of *B. firmus* as a function of substrate concentration (S), and model comparison. Conditions were pH 8.0, 50°C, 0.05 *M* Tris-HCl buffer, 5 m*M* $CaCl_2$, and the substrate was maltodextrin (DE 10). **(A)** β-CD production ; **(B)** γ-CD production.

Kinetic Parameters

Figure 2 show the Hanes plots used with the low substrate concentration points to determine V_{max} and K_m. The adjusted straight lines gave the following values for V_{max} and K_m:

For β-CD:

$$V_{max} = 0.8878 \text{ mol of β-CD/(L·h)}; K_m = 0.0033 \text{ mol/L}; r = 0.9607 \quad (5)$$

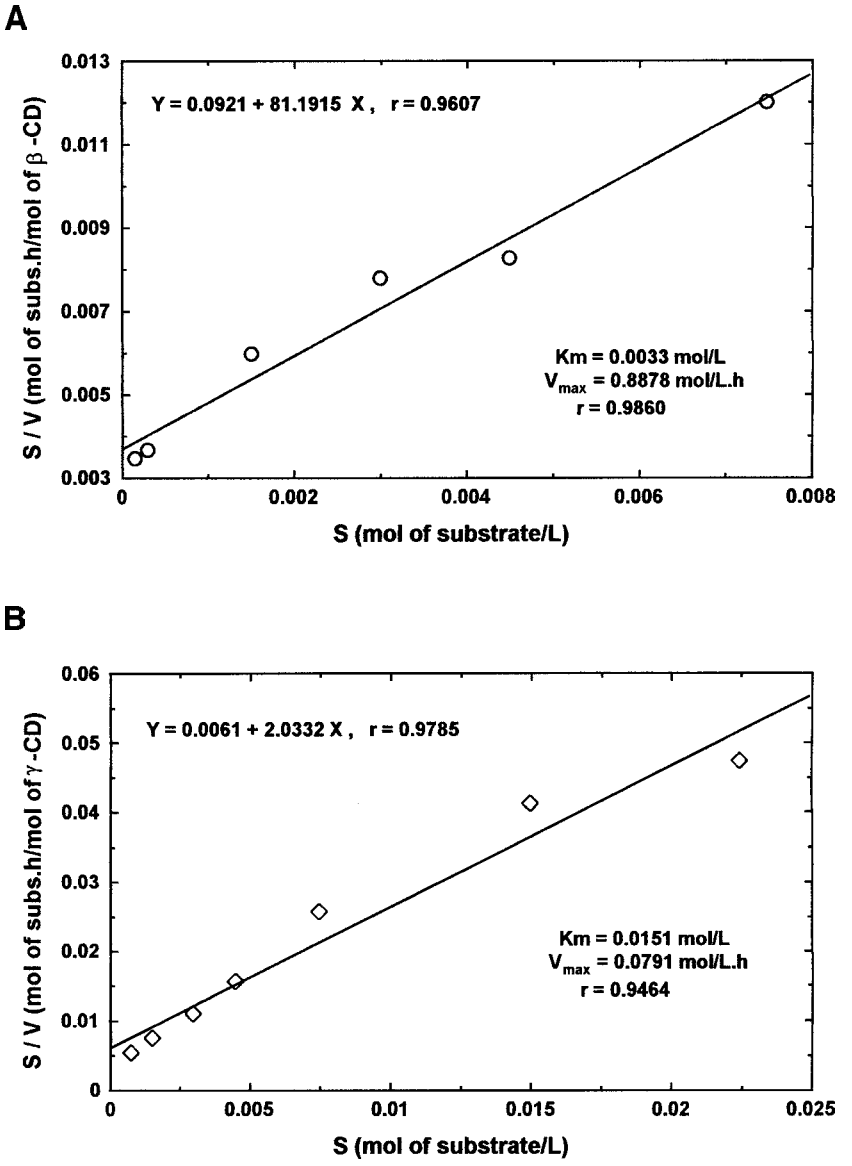

Fig. 2. Determination of K_m and V_{max} by the Hanes method for the CGTase of *B. firmus*. Only the points shown in Fig. 1 with low substrate concentration were used. **(A)** β-CD production; **(B)** γ-CD production. Experimental conditions were as in Fig. 1.

For γ-CD:

$$V_{max} = 0.0791 \text{ mol of } \gamma\text{-CD}/(\text{L}\cdot\text{h}); K_m = 0.0151 \text{ mol/L}; r = 0.9785 \quad (6)$$

In Fig. 1, the initial velocity data for CD production is compared with the standard substrate inhibition model, Eq. 3, adjusted to the data as indicated in Materials and Methods. As Fig. 1 shows, this model does not follow the data trend very well at high substrate concentration and the fit is not entirely satisfactory. The adjusted equations and K_s values are as follows:

For β-CD:

$$(S/V) = 0.0037 + 1.126\ S + 12.14\ S^2 \quad \text{and}$$
$$K_s = 0.0928\ \text{mol/L};\ r = 0.8210 \tag{7}$$

For γ-CD:

$$(S/V) = 0.1912 + 12.64\ S + 128.35\ S^2 \ \text{and}$$
$$K_s = 0.0985\ \text{mol/L};\ r = 0.9415 \tag{8}$$

The results for V_{max} and K_m, Eqs. 5 and 6, allow calculation of the ratio of initial rates, i.e., $[(V_{max}/K_m)_{\beta\text{-CD}}/(V_{max}/K_m)_{\gamma\text{-CD}}]$, and conclude that the initial rate of β-CD production is 51.4 times that of γ-CD. The value of K_m for β-CD is 4.6 times smaller than for γ-CD, indicating that, as substrate concentration is increased, the highest initial rate of CD production will be attained earlier in the case of β-CD production. In addition, it should be stressed that the same enzyme gave different K_m values for each product and closer values for K_s. These results can be explained with the new kinetic model for the initial rate of CD production which is introduced next.

New Kinetic Model for Initial Velocity of CD Production

The model is based on the following assumptions:

1. Because the data were obtained for the initial velocity of CD production, the composition of the substrate will be considered practically constant; consequently, products and new substrates formed will not affect the velocity of reaction. This includes the oligomers generated by the disproportionation reactions.
2. Three disproportionation reactions in series were included in the model. The criterion to choose how many reactions to include was based on the goodness of the fit of model and data using the smallest number of equations.
3. CDs are formed by similar sequences of reactions but each CD follows its own sequence. The linear chains of maltooligosaccharides can bind to the active site of CGTase in more than one position and form different activated complexes that produce one CD each.

Reaction Mechanism

Cyclization, i.e., CD forming reaction is as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ES) \xrightarrow{k_3} CD + P_1 + E \tag{9}$$

Disproportionation, i.e., intermolecular transglycosylation reactions are as follows:

$$(ES) + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (ES_2) \xrightarrow{k_6} P_2 + P_3 + E \tag{10}$$

$$(ES_2) + S \underset{k_8}{\overset{k_7}{\rightleftarrows}} (ES_3) \xrightarrow{k_9} P_4 + P_5 + E \quad (11)$$

$$(ES_3) + S \underset{k_{11}}{\overset{k_{10}}{\rightleftarrows}} (ES_4) \xrightarrow{k_{12}} P_6 + P_7 + E \quad (12)$$

in which k_1 to k_{12} are kinetic constants and P_1 to P_7 are glucose and maltooligosaccharide chains.

The initial rate of CD formation (V) is given by Eq. 13:

$$V = \frac{V_{max} S}{K_m + S + \frac{S^2}{K_{S1}} + \frac{S^3}{K_{S2}} + \frac{S^4}{K_{S3}}} \quad (13)$$

in which

$$V_{max} = \frac{k_1 k_3 (k_5 + k_6) E_t}{k_1 (k_5 + k_6) + k_4 k_6}$$

is a maximal velocity for CD formation; E_t is the total enzyme concentration;

$$K_m = \frac{(k_2 + k_3)(k_5 + k_6)}{k_1 (k_5 + k_6) + k_4 k_6}$$

is a Michaelis-Menten type of constant for the formation of CD; and K_{S1}, K_{S2}, and K_{S3} are substrate inhibition constants that originate from disproportionation, and are given by the following relations:

$$K_{S1} = \frac{k_1 (k_5 + k_6) + k_4 k_6}{k_1 k_4}$$

$$K_{S2} = \frac{[k_1 (k_5 + k_6) + k_4 k_6] (k_8 + k_9)}{k_1 k_4 k_7}$$

$$K_{S3} = \frac{[k_1 (k_5 + k_6) + k_4 k_6] (k_8 + k_9)(k_{11} + k_{12})}{k_1 k_4 k_7 k_{10}}$$

Kinetic Parameters for the New Kinetic Model

Equation 13 was adjusted to data by nonlinear fitting using the Statistica Program, and the results were excellent (Fig. 1):

For β-CD:

$$V_{max} = 0.8793 \text{ mol of β-CD/(L·h)}; K_m = 0.003397 \text{ mol/L; and}$$
$$K_{S1} = 3.659 \times 10^8 \text{ mol/L}; K_{S2} = 3.447 \times 10^5 \text{ (mol/L)}^2;$$
$$K_{S3} = 3.143 \times 10^{-4} \text{ (mol/L)}^3; \text{ r} = 0.9962 \quad (14)$$

For γ-CD:

$$V_{max} = 0.1203 \text{ mol of γ-CD/(L·h)}; K_m = 0.02772 \text{ mol/L; and}$$

$$K_{S1} = 1.281 \times 10^{10} \text{ mol/L}; K_{S2} = 0.002847 \text{ (mol/L)}^2;$$
$$K_{S3} = 0.0001738 \text{ (mol/L)}^3; r = 0.9982 \quad (15)$$

The new model conforms very well to data and links the high substrate inhibition observed on CD production to the disproportionation reactions. In addition, the values obtained for K_{S1}, K_{S2}, and K_{S3} implicate that the highest inhibition comes from the term in S^4 at Eq. 13, which results from the third disproportionation reaction. The ratio of formation/consumption of the complex (ES^4), given by K_{S2}/K_{S3}, is very high for β-CD production, giving 1.1×10^9, whereas for γ-CD it is 16.4. Interestingly, depending on reaction conditions, disproportionation reactions *(23)* and coupling *(24)* may lead to *de novo* synthesis of amylose starch chains from CDs given sufficient reaction time.

Kinetic parameters found in the literature do not coincide *(25–27)*. Our experimental values for the kinetic parameters also diverge from those published. This is to be expected since the parameters values are influenced by the source of enzyme, test conditions, and kinetic model used to derive these parameters.

Sato et al. *(25)* compared the production of α- and β-CD by the CGTase from *Bacillus obhensis* and verified that this enzyme is indicated for the production of β-CD because it has shown a higher affinity and higher reaction rate in the case of β-CD production. They attributed this result to the lower K_m value and higher V_{max} obtained for β-CD, as was the case in the present work in relation to β- and γ-CD. Oguma et al. *(27)* observed that the K_m value that they obtained for the CGTase from *Bacillus sphaericus* was smaller than those published, and this implied that their CGTase when compared with the other enzymes had a greater affinity in the case of β-CD production. On the same line of reasoning, it can be concluded that the value of K_m, 8.2 times lower for β-CD production, and V_{max}, 7.3 times greater (new model, Eqs. 14 and 15), is a consequence of the CGTase from *B. firmus* having a greater affinity and higher reaction rate in the case of β-CD production. The same kind of behavior was observed with the CGTase studied by Hamon and de Moraes *(18)*. With the new model the ratio of initial velocity of CD production by the CGTase of *B. firmus* is slightly higher: $[(V_{max}/K_m)_{\beta\text{-CD}} / (V_{max}/K_m)_{\gamma\text{-CD}}] = 59.8$.

Inhibition by Low Molecular Weight Saccharides

Figure 3 show that the low molecular weight saccharides glucose, maltose, and maltotriose inhibit the production of β-CD by *B. firmus* CGTase. It can be seen that the greater the concentration of the exogenous saccharide the higher inhibition was, and the disaccharide maltose caused the greatest inhibition while the effects of glucose and maltotriose were almost equivalent. Rendleman *(28)* studied the influence of various saccharides as inhibitors of CD production by the CGTase from *Bacillus macerans* using 10% (w/v) maltodextrin (DE 5) as substrate and 0.2 *M* exogenous saccharide. He also found that maltose had a strong inhibitory effect while

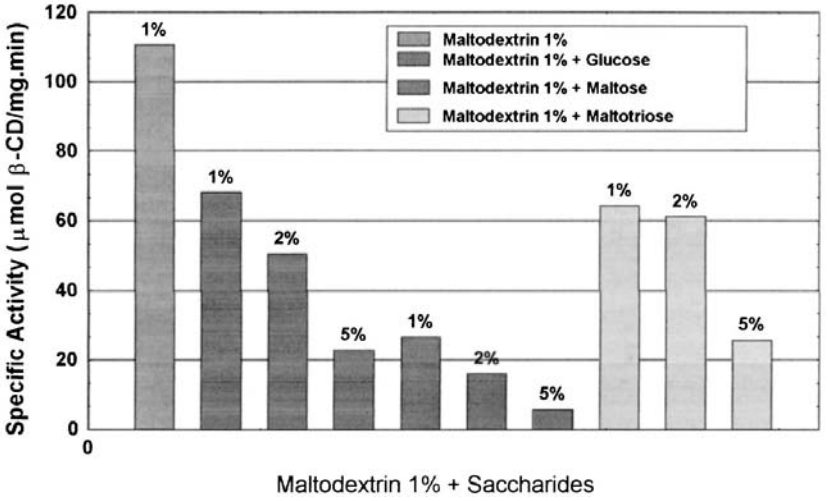

Fig. 3. Influence of the low molecular weight saccharides, glucose, maltose, and maltotriose on the specific activity of *B. firmus* CGTase for the production of β-CD. Conditions were: pH 8.0, 50°C, 0.05 *M* Tris-HCl buffer, 5 m*M* $CaCl_2$, and the substrate was maltodextrin 1% (w/v) (DE 10). The exogenous saccharide concentration is given over the bars.

larger maltooligosaccharides either had a smaller influence (maltotriose), negligible influence (maltopentaose), or increased CD production (maltotetraose, maltohexaose). Low molecular weight saccharides can participate in the coupling reaction and disproportionation, reducing either the initial velocity of CD formation or their yield for long reaction times *(28)*. According to Szejtli *(14)*, in the process of CD production, the total quantity of glucose plus maltose should amount to <5% of starch weight in which case CD yield may vary from 30 to 70% depending on starch concentration. If the total quantity of glucose plus maltose is >20% of starch weight, then CD yield is reduced to about 15%. Note that in the pure maltodextrin control test (Fig. 3), endogenous glucose plus maltose accounted for only 4% of the maltodextrin used. These results highlight the importance of using low DE hydrolyzed starch for producing CDs, because the higher the DE the greater will be the amount of glucose plus maltose in the hydrolyzed starch. Horikoshi et al. *(13)* have shown that increasing the initial DE from 1 to 25% has decreased β-CD yield from 25 to 10%.

Inhibition by the Products β- and γ-CD

The inhibition of *B. firmus* CGTase by its products β- and γ-CD is shown in Fig. 4. Comparison of Figs. 3 and 4 reveals that the CDs are stronger inhibitors than the low molecular weight saccharides and that γ-CD inhibits more than β-CD. These results indicate that the reaction given by Eq. 1 is highly reversible and that the added CD participates in the coupling reaction. The extension of inhibition caused by each CD varies with the

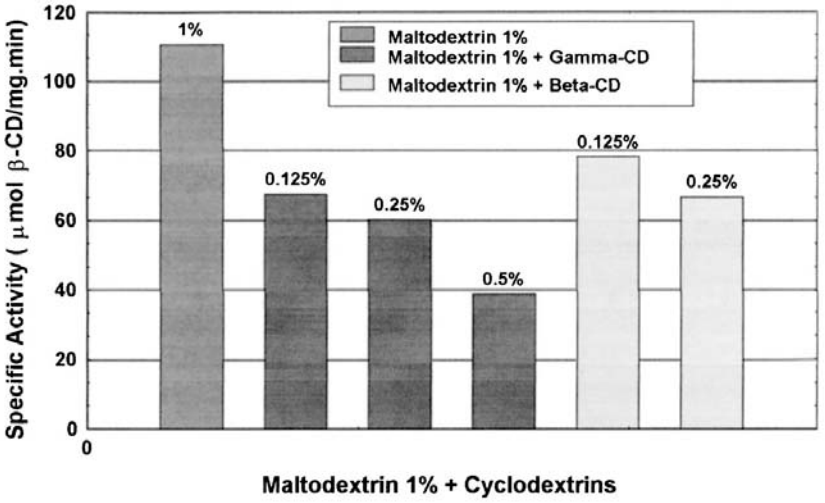

Fig. 4. Influence of products β- CD and γ-CD on the specific activity of *B. firmus* CGTase for production of β-CD. Conditions were: pH 8.0, 50°C, 0.05 *M* Tris-HCl buffer, 5 m*M* $CaCl_2$, and the substrate was maltodextrin 1% (w/v) (DE 10). The exogenous CD concentration is given over the bars.

source of enzyme because Bergsma et al. *(29)* found that in their case β-CD was a more intense inhibitor. Building a complete model for CD production from starch that can be applied to short or long reaction times should include the reversibility of reactions, inhibition by substrate, product, and small saccharides. The result would be a very complex model because of the complex reaction mixture formed by different CDs and linear saccharides of various chain lengths.

Conclusion

The enzymatic activity of *B. firmus* CGTase is inhibited by the substrate maltodextrin and the products β- and γ-CD, the latter being stronger inhibitors. Maltose is a stronger inhibitor than glucose and maltotriose, and γ-CD is the strongest of all. The substrate inhibition for the production of β-CD is greater than for the production of γ-CD. The initial velocity of reaction for producing β- and γ-CD from maltodextrin is well modeled by a cyclization reaction that produces CD followed by three disproportionation reactions. These later reactions lead to intense substrate inhibition at high substrate concentration because of the term in S^4 that appears at the denominator of the equation for the initial velocity of CD production.

The kinetic parameters obtained with initial velocity data for *B. firmus* CGTase are as follows:

For β-CD production;

$$V_{max} = 0.8793 \text{ mol of β-CD/(L·h)}; K_m = 0.003397 \text{ mol/L}; \text{ and}$$

$$K_{S1} = 3.659 \times 10^8 \text{ mol/L}; K_{S2} = 3.447 \times 10^5 \text{ (mol/L)}^2; K_{S3} = 3.143 \times 10^{-4} \text{ (mol/L)}^3;$$

γ-CD production: V_{max} = 0.1203 mol of γ-CD/(L·h); K_m = 0.02772 mol/L; and $K_{S1} = 1.281 \times 10^{10}$ mol/L; K_{S2} = 0.002847 (mol/L)^2; K_{S3} = 0.0001738 (mol/L)^3.

A model for CD production that would be valid up to long reaction times should require the inclusion of all reactions as reversible because the CDs produced participate in the coupling reaction that was shown to be of great importance as CD concentration rises. The resulting model would necessarily be complex because of the many reactants and products including the various CDs, glucose, and a great number of maltooligosaccharides that should be considered.

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