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Kinetics of Cyclization Reaction Catalysed by the Enzyme Cyclomaltodextringlucanotransferase

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In this work a kinetic model that takes into account the reversibility of the cyclization reaction, the simultaneous production of β– and γ–CD and the inhibitory influence of the substrate and products (CDs), on the enzymatic activity of the CGTase from *Bacillus* sp alkalophilic, cloned in *E. coli*, is developed. The model is compared to experimental data and it was verified that it showed flexibility to closely follow experimental data up to the time limit of the experimental test of 24 hours.

1. Introduction

CDs have many important industrial applications mainly in the food, pharmaceutical and cosmetic industries (Lante and Zocca, 2010; Loftsson and Duchêne, 2007; Martin and Valle, 2004), motivating many studies on CDs production. Over the years the interest in the industrial use of cyclodextrins have greatly grown and encouraged a large number of research projects on the processes of obtaining these maltooligosaccharides (Gaston *et al.*, 2009 Mazzer *et al.*, 2008; Szerman *et al.*, 2007). The kinetics of reactions involved in the production of CDs are represented schematically by the following reaction

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

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

Where G_n, G_m and G_x are oligosaccharides with n, m and x glucose units, respectively. cG_x is the α–CD for x = 6, β–CD for x = 7, and γ–CD for x = 8.

The enzyme CGTase catalyses the reactions of cyclization, which form the closed rings of CDs, coupling (reverse reaction of cyclization that opens the rings) and disproportionation, in which two molecules of linear dextrins exchange segments of their chains and are converted into two other dextrins of different sizes (Szejtli, 1988; van der Veen *et al.*, 2000). The hydrolysis of starch to produce linear dextrins occurs at a much lesser extent.

The disproportionation reactions are generally much faster than the other reactions, leading rapidly to a state of quasi-equilibrium with respect to the concentration of linear dextrins that will be cyclized by the enzyme. The disproportionation also allows producing larger chains (which undergo cyclization) from small linear chains, such as maltotriose. For this reason the production of CDs from maltotriose is possible, but the CD production from glucose has not been reported in the literature. The production of CDs from maltotriose has been proposed as a method of determination of CGTase activity (Mäkelä and Korpela, 1988; Brunei *et al.*, 1998).

Literature shows that different kinetic models have already been proposed for the CD production, however, these models did not take into account the reversibility of the cyclization reaction and many side reactions

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that occur at same time. Hamon and Moraes (1990) studied and characterized the enzyme CGTase from alkalophilic *Bacillus* sp cloned in *E. Coli*, produced by the WACKER company, and proposed a kinetic model for the cyclization catalysed by this enzyme, whose main products are β–CD and γ–CD, while the α–CD is produced in insignificant amounts. The model proposed by the authors assumes a single substrate and product and irreversible reaction, taking into account that the enzyme undergoes inhibition by the substrate and by the product. Comparing the theoretical curve with the experimental data, Hamon and Moraes concluded that their proposed kinetic model is only able to adequately describe the data for a range of reaction time up to 60 minutes. Desouza *et al*. (2002) proposed a kinetic model that considers the reversibility of the cyclization reaction and the influence of substrate concentrations and CDs on the enzyme CGTase activity, achieving with this model representation of the experimental data for long reaction times. Nevertheless, Desouza *et al*. (2002) considered only the formation of β–CD, although it was known that the cyclization reaction catalysed by the enzyme provided by the Wacker company also produced γ–CD simultaneously. The aim of this paper is to propose a new kinetic model that takes into account the production of γ–CD, resulting in a model that satisfactorily represent the simultaneous production of two CDs, for reaction times greater than 60 minutes.

2. Kinetic model

The proposed model was developed based on the following assumptions:

- Simultaneous production of β– and γ–CD: this model considers that the way in which the substrate binds to the enzyme (ES_β or ES_γ) determines what will be the product formed;
- Reversible reaction: the cyclodextrins produced bind again to the enzyme for producing linear dextrins (coupling reaction, which is thus a reverse reaction to cyclization);
- Single substrate: The substrate is the maltodextrin (a mixture of maltooligosaccharides), which is considered as if it were a single type of molecule. This approach is typical of catalytic processes complexes, with enzymatic or inorganic catalysts (Zanin and Moraes, 1996; Zhang and Chuang, 1999);
- Inhibition by substrate: high concentrations of substrate leads to the formation of ternary complexes SES_β or $SES\gamma$. This prevents the conversion of linear dextrins into cyclodextrins; and
- Competitive inhibition by the products: as the reaction proceeds, a progressive increase in the concentration of β– and γ–CD occurs in the reaction medium. Cyclodextrins produced compete with the substrate for the active site of the enzyme.

A schematic kinetic model representation is shown in Figure 1, where k_1, k_{-1}, k_3, k_{-3}, k_5, k_{-5}, k_7 and k_{-7} are kinetic constants related to the β–CD production and k_2, k_{-2}, k_4, k_{-4}, k_6, k_{-6}, k_8 and k_{-8} are kinetic constants related to the γ–CD production, E represents the free CGTase enzyme, S the substrate (dextrin DE 10), β is β–CD product, γ is γ–CD product and ES_β, ES_γ, $E\beta$, $E\gamma$, SES_β and SES_γ are complex reaction intermediates.

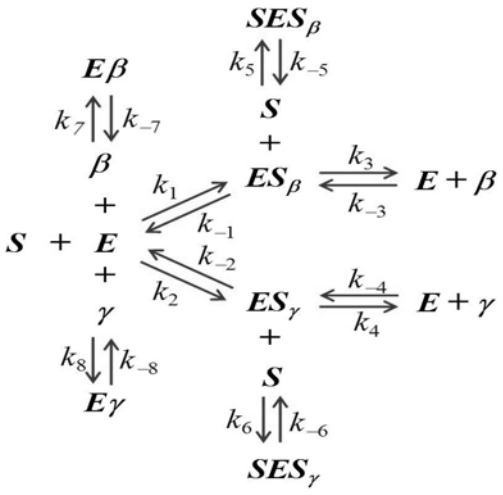

Figure 1: Mechanism considered for the new kinetic model

The reaction rate was determined by applying the hypothesis of steady state for the elimination of the intermediate complexes in the model, resulting the following equations

$$\frac{d\beta}{dt}=\frac{\frac{V_{\max_\beta}}{Kms_\beta}\left(S-\frac{\beta}{K_{eq\beta}}\right)}{1+\frac{\beta}{Kp_\beta}+\frac{\gamma}{Kp_\gamma}+\left(\frac{S}{Kms_\beta}+\frac{\beta}{Kmp_\beta}\right)\left(1+\frac{S}{Ks_\beta}\right)+\left(\frac{S}{Kms_\gamma}+\frac{\gamma}{Kmp_\gamma}\right)\left(1+\frac{S}{Ks_\gamma}\right)} \quad (1)$$

$$\frac{d\gamma}{dt}=\frac{\frac{V_{\max_\gamma}}{Kms_\gamma}\left(S-\frac{\gamma}{K_{eq\gamma}}\right)}{1+\frac{\beta}{Kp_\beta}+\frac{\gamma}{Kp_\gamma}+\left(\frac{S}{Kms_\beta}+\frac{\beta}{Kmp_\beta}\right)\left(1+\frac{S}{Ks_\beta}\right)+\left(\frac{S}{Kms_\gamma}+\frac{\gamma}{Kmp_\gamma}\right)\left(1+\frac{S}{Ks_\gamma}\right)} \quad (2)$$

where:
$Kmp_\beta = (k_{-1}+k_3)/k_{-3}$: Michaelis-Menten constant for coupling reaction
$Kmp_\gamma = (k_{-2}+k_4)/k_{-4}$: Michaelis-Menten constant for coupling reaction
$Kms_\beta = (k_{-1}+k_3)/k_1$: Michaelis-Menten constant for the reaction of cyclization to form β–CD
$Kms_\gamma = (k_{-2}+k_4)/k_2$: Michaelis-Menten constant for the reaction of cyclization to form γ–CD
$Kp_\beta = k_{-7}/k_7$: product inhibition constant (β–CD)
$Kp_\gamma = k_{-8}/k_8$: product inhibition constant (γ–CD)
$Ks_\beta = k_{-5}/k_5$: substrate inhibition constant
$Ks_\gamma = k_{-6}/k_6$: substrate inhibition constant
S = starch concentration (g/mL)
$V_{\max\beta}$ = maximum rate of formation for β–CD per mL of enzyme (mol L^{-1} min^{-1})
$V_{\max\gamma}$ = maximum rate of formation for γ–CD per mL of enzyme (mol L^{-1} min^{-1})
$V_{max-\beta}$ = maximum rate of consumption for β–CD per mL of enzyme by the coupling reation (mol L^{-1} min^{-1})
$V_{max-\gamma}$ = maximum rate of consumption for γ–CD per mL of enzyme by the coupling reation (mol L^{-1} min^{-1})
β = concentration of β–CD (mM)
γ = concentration of γ–CD (mM)

Kinetic parameters of direct reactions (Kms_β, Kms_γ, $V_{\max\beta}$, $V_{\max\gamma}$) and the inhibition constants by the substrate (Ks_β, Ks_γ) were determined from the initial rates of β– and γ–CD productions as a function of the initial substrate concentration, obtained experimentally by Hamon and Moraes (1990). The initial rate of production of CDs can be obtained by writing Equations (1) and (2) in an irreversible way, considering that the reaction starts in the absence of products.

$$V_{ini\beta}=\frac{d\beta}{dt}=\frac{\frac{V_{\max_\beta}}{Kms_\beta}S}{1+S\left(\frac{1}{Kms_\beta}+\frac{1}{Kms_\gamma}\right)+S^2\left(\frac{1}{Ks_\beta Kms_\beta}+\frac{1}{Ks_\gamma Kms_\gamma}\right)} \quad (3)$$

$$V_{ini\gamma}=\frac{d\gamma}{dt}=\frac{\frac{V_{\max\gamma}}{Kms_\gamma}S}{1+S\left(\frac{1}{Kms_\beta}+\frac{1}{Kms_\gamma}\right)+S^2\left(\frac{1}{Ks_\beta Kms_\beta}+\frac{1}{Ks_\gamma Kms_\gamma}\right)} \quad (4)$$

Equations (3) and (4) can be rearranged as a second degree polynomial ($y = a + bx + cx^2$) with $y = S/V_{ini}$ and $x = S$. In this case:

$$\frac{S}{V_{ini\beta}} = \frac{Kms_\beta}{Vmax_\beta} + \left(\frac{Kms_\beta}{Vmax_\beta}\right)\left(\frac{1}{Kms_\beta} + \frac{1}{Kms_\gamma}\right)S + \left(\frac{Kms_\beta}{Vmax_\beta}\right)\left(\frac{1}{Ks_\beta Kms_\beta} + \frac{1}{Ks_\gamma Kms_\gamma}\right)S^2 \quad (5)$$

$$\frac{S}{V_{ini\gamma}} = \frac{Kms_\gamma}{Vmax_\gamma} + \left(\frac{Kms_\gamma}{Vmax_\gamma}\right)\left(\frac{1}{Kms_\beta} + \frac{1}{Kms_\gamma}\right)S + \left(\frac{Kms_\gamma}{Vmax_\gamma}\right)\left(\frac{1}{Ks_\beta Kms_\beta} + \frac{1}{Ks_\gamma Kms_\gamma}\right)S^2 \quad (6)$$

A nonlinear fit of polynomials represented by Equations (5) and (6) to the data of initial rates of β– and γ–CD productions obtained by Hamon and Moraes provides the parameters $V_{max\beta}$, $V_{max\gamma}$, Kms_β, Kms_γ, Ks_β and Ks_γ. The kinetic parameters related to the coupling reaction ($V_{max\text{-}\beta}$, $V_{max\text{-}\gamma}$, Kmp_β, Kmp_γ) and the inhibition constants by the products (Kp_β, Kp_γ) were determined by adjusting the curves described by the kinetic model to the data set of Hamon and Moraes (1990) for the production of β– and γ–cyclodextrin for a period of 24 hours

3. Results and discussion

The kinetic parameters obtained as described above are shown in Table 1. It is observed that the inclusion of reversibility allowed to reduce the apparent value of the inhibition caused by the product β–CD by approximately 50 times in comparison with the model of Hamon and Moraes (1990). Even with a much lower value of the β–CD inhibition parameter the experimental data was adequately represented.

The model proposed here gave for the production of β–CD (Kms_β) a Michaelis-Menten constant that was twice the value obtained by Hamon and Moraes (1990) and for the production of γ–CD there was a reduction in the value of this constant (Kms_γ). As *Kms* measures the affinity between the enzyme and the substrate, the intermediate enzyme-substrate complex which has higher concentration should be the one that leads to the production of β–CD, since this is the main cyclodextrin produced by the CGTase studied.

Table 1 shows also that the inhibition of the production of cyclodextrins by β–CD is approximately twice the inhibition caused by γ–CD.

Table 1 - Kinetic parameters for the model of Hamon and Moraes (1990) and for the model proposed in this work for the simultaneous production of β– and γ–CD catalyzed by CGTase of alkalophilic Bacillus sp.

Parameter		Hamon and Moraes	Proposed model
$Vmax_\beta$	$(\frac{mol/L}{min})$	1.259×10^{-3}	2.660×10^{-3}
$Vmax_{-\beta}$	$(\frac{mol/L}{min})$	----	58.595
Kms_β	(mol/L)	2.596×10^{-4}	5.483×10^{-4}
Ks_β	(mol/L)	2.279×10^{-2}	1.767×10^{-2}
Keq_β	(--)	----	1.962×10^{-1}
Kp_β	(mol/L)	6.000×10^{-7}	2.760×10^{-5}
Kmp_β	(mol/L)	----	7.854×10^{-5}
$Vmax_\gamma$	$(\frac{mol/L}{min})$	2.152×10^{-4}	4.552×10^{-4}
$Vmax_{-\gamma}$	$(\frac{mol/L}{min})$	----	18.312
Kms	(mol/L)	1.320×10^{-3}	4.929×10^{-4}
Ks_γ	(mol/L)	3.604×10^{-2}	2.795×10^{-2}
Keq_γ	(--)	----	5.263×10^{-2}
Kp_γ	(mol/L)	----	5.290×10^{-5}
Kmp_γ	(mol/L)	----	5.976×10^{-4}

At the condition of chemical equilibrium: $V_\beta = 0$, $S = S_{eq}$, $\beta = \beta_{eq}$ and $K_{eq\beta} = \beta_{eq}/S_{eq}$. The substitution of these values in Equation (1) gives:

$$K_{eq\beta} = \frac{Vmax_{\beta} / Kms_{\beta}}{Vmax_{-\beta} - Vmax_{\beta} / Kmp_{\beta}} \quad (7)$$

Similarly, for γ-CD:

$$K_{eq\gamma} = \frac{Vmax_{\gamma} / Kms_{\gamma}}{Vmax_{-\gamma} - Vmax_{\gamma} / Kmp_{\gamma}} \quad (8)$$

The ratio of 3.73 between the equilibrium constant $K_{eq\beta}$ and $K_{eq\gamma}$ is physically consistent with the fact that the major product is β-CD and also with the results obtained by Tardioli *et al.* (2000), who report that the CGTase from alkalophilic *Bacillus* sp is a β-CGTase, producing CDs with molar ratio β-CD:γ-CD approximately equal to 3:1.

The model developed in this work resulted in a system of two ordinary differential equations, which were solved numerically using the program MAPLE™. The kinetic equations obtained were used to draw the theoretical curves that describes the concentration of β–CD and γ–CD as functions of time. These curves are shown in Figures 2 and 3. The proposed model was fitted to the data set of Hamon and Moraes (1990), providing an excellent fit.

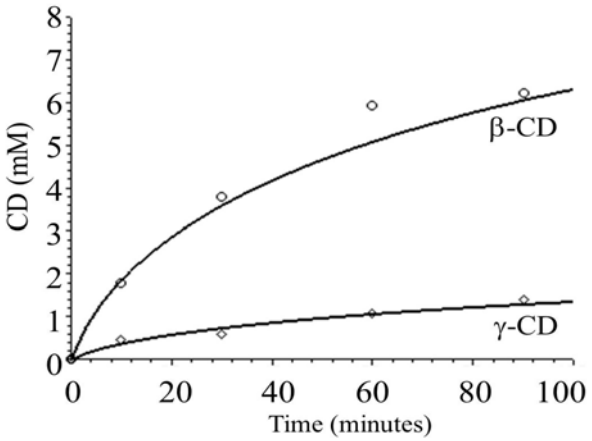

Figure 2: Modeling the production of β–CD and γ–CD for a period of up to 100 minutes

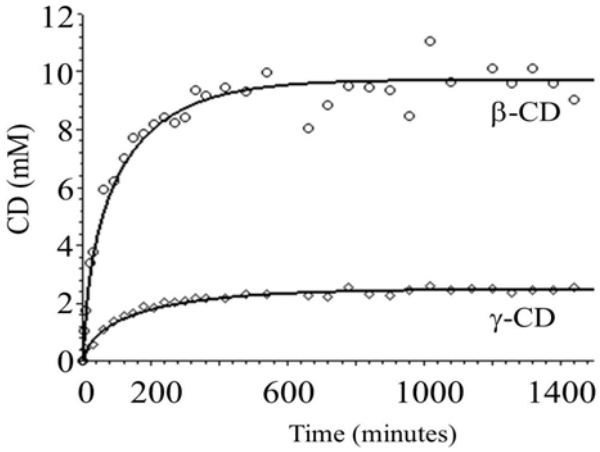

Figure 3: Modeling the production of β–CD and γ–CD for a period of 24 hours

4. Conclusion

The equations presented take into account that the cyclization reaction catalysed by the *Bacillus* sp CGTase produces simultaneously two CDs (β–CD and γ–CD) and they were essential to fully and appropriately represent the physical reality of the production of CDs. The kinetic modeling, as proposed, leads to different *Km* and *Ks* for each product of cyclization. This result agrees with the experimental observations of Hamon and Moraes (1990) and allow to conclude that the manner in which the substrate binds to the enzyme (ES_β or ES_γ) determine which product is formed (β– or γ–CD). The latter explains how the same CGTase enzyme can produce two different products from the same substrate, resulting in different constants of Michaelis-Menten and different inhibition constants by the same substrate.

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