Kinetics of the simultaneous production of β- and γ-cyclodextrins catalyzed by CGTase from alkalophilic Bacillus sp.

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ABSTRACT. The cyclodextrins (CDs) are cyclic maltoligosaccharides obtained by cyclization of linear chains of starch, catalyzed by the enzyme cyclomaltooligosaccharides catalyzed by CGTase. The interest in CD production results from the formation of inclusion complexes, which allow many important applications, especially in food, pharmaceutical and cosmetic industries. The substances complexed generally have their properties modified by complexation. It is appreciated if increased solubility and higher thermal and chemical stabilities are obtained. In this work, a kinetic model was developed for the production of cyclodextrins in the presence of CGTase from alkalophilic Bacillus sp., taking into account the reversibility of the cyclization reaction, the simultaneous production of β and γ-CD and also the inhibitory influence of the substrate and products (CDs), on the enzymatic activity of the CGTase. The substrate formed from a solution of maltodextrins was treated as a single substrate. The model was compared with experimental results of 24h of reaction and this comparison demonstrated that there was a very good representation of the data throughout the test period. The model also allowed explaining the observation of different experimental values for each Michaelis-Menten constant and substrate inhibition constant for each CD, although the CDs are produced from the same substrate.

Keywords: cyclic oligosaccharides, cyclization, enzyme kinetics, inclusion complexes, intramolecular transglycosylation.

Cinética da produção simultânea de β- e γ-ciclodextrinas catalisada por CGTase de Bacillus sp. alcalofílico

RESUMO. As ciclodextrinas (CDs) são maltoligosaccharídicos cíclicos obtidos pela ciclização de cadeias lineares de amido, catalisada pela enzima ciclomaltooligosaccharides catalyzed by CGTase. O interesse na produção de CDs resulta da formação de complexos de inclusão, que permitem inúmeras aplicações importantes, principalmente nas indústrias alimentícia, farmacêutica e de cosméticos. As substâncias complexadas têm, geralmente, suas propriedades alteradas, sendo apreciado o aumento de solubilidade e maior estabilidade térmica e química. Neste trabalho, um modelo cinético foi desenvolvido para a produção de ciclodextrinas, na presença da CGTase de Bacillus sp. alcalofílico, levando em conta a reversibilidade da reação de ciclização, a produção simultânea de β- e γ-CD e também a influência inibitória do substrato e dos produtos (CDs), na atividade da enzima. O substrato, formado de uma solução de maltodextrinas, foi tratado como substrato único. O modelo, confrontado com resultados experimentais de 24h de reação, demonstrou que houve uma representação muito boa dos dados, em todo o período de teste. O modelo também permitiu explicar a observação de valores experimentais diferentes para cada constante de Michaelis-Menten e de inibição pelo substrato, para cada CD, embora as CDs sejam produzidas a partir do mesmo substrato.

Palavras-chave: oligosaccharídicos cíclicos, ciclização, cinética enzimática, complexos de inclusão, transglicosilação intramolecular.

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of glucose monomers linked together by glycosidic linkages of the type α-1,4. The best known CDs are composed of 6 (α-CD), 7 (β-CD) and 8 (γ-CD) glucose units. They can be obtained by cyclization of linear starch chains by means of intramolecular transglycosylation reactions, which are catalyzed by the enzyme cyclomaltooligosaccharides catalyzed by CGTase (FRENCH, 1957; SZEJTTLI, 1988).

The interest in the production of CDs results from the formation of inclusion complexes, which allows numerous important applications, especially in the food, pharmaceutical and cosmetic industries. The complexed substances generally have their properties changed and some
alterations of interest are an increase of solubility and greater thermal and chemical stabilities. Therefore, the cyclodextrins can be used to stabilize emulsions and foams and materials that are sensitive to light, heat and oxygen. They can also be employed to control volatility, change the color, flavor, smell and texture of cosmetics and condiments (LANTE; ZOCCA, 2010; LOFTSSON; DUCHÈNE, 2007; SZEJTLI, 1988; MARTIN; VALLE, 2004).

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 (GASTÓN et al., 2009; MAZZER et al., 2008; SANTOS et al., 2013; SZERMAN et al., 2007). The kinetics of reactions involved in the production of CDs are represented schematically by the following reactions.

\[
\begin{align*}
G_n & \quad \text{cyclization} \quad \rightarrow \quad G_{(n-x)} + cG_x \\
G_n + G_m & \quad \text{coupling} \quad \rightarrow \quad G_{(n-x)} + G_{(m+x)} \\
G_n + G_m & \quad \text{disproportionation} \quad \rightarrow \quad G_{(n-x)} + G_{(m+x)}
\end{align*}
\]

where:
- \(G_n\), \(G_m\), and \(G_x\) are oligosaccharides with \(n\), \(m\) and \(x\) glucose units, respectively;
- \(cG_x\) is the \(\alpha\)-CD for \(x = 6\);
- \(\beta\)-CD for \(x = 7\);
- \(\gamma\)-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 VEEEN et al., 1980). 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 (BRUNEI et al., 1998; MÄKELÄ; KORPELA, 1988).

Matioli et al. (2002) investigated the influence of substrate and products on the initial rate of production of CDs catalyzed by the enzyme CGTase from \(B. \text{firmus}\) and concluded that increased concentrations of the substrate and products of cyclization significantly reduces the enzyme activity by inhibition. This conclusion agrees with the observations of Bender (1985) and Mäkelä and Korpela (1988).

Hamon and Moraes (1990) studied and characterized the enzyme CGTase from alkalophilic \(Bacillus\) sp. cloned in \(E. \text{Coli}\) (manufactured by WACKER Company) and proposed a kinetic model for the cyclization catalyzed by this enzyme. The main products of this cyclization are the \(\beta\)- and \(\gamma\)-CD (since \(\alpha\)-CD is produced in insignificant amounts). In their proposed model, the authors assume that substrate and product are single molecules and the reaction is irreversible, taking into account that this enzyme is inhibited by the presence of substrate and product, in addition, the authors considered the isolated formation of each CDs, however it is known that the cyclization reaction catalyzed by CGTase from alkalophilic \(Bacillus\) sp. produces simultaneously \(\beta\) and \(\gamma\)-CD. With this model the theoretical curve that describes the concentration of product (\(\beta\)-CD and \(\gamma\)-CD) as a function of time was obtained. Comparing the curve with the experimental data, Hamon and Moraes found that the proposed kinetic model was only able to adequately describe the data for a range of reaction time up to 60 minutes.

The objective of this work is to propose a new kinetic model that takes into account the simultaneous production of both CDs, for longer time intervals, greater than 60 minutes.

The kinetic models as developed in this work are used as tools to aid in the analysis of the influence of reversibility, concentration of substrate and products, on the production yield of \(\beta\)-CD and \(\gamma\)-CD, contributing to elucidation of the mechanism of action of CGTase.

**Material and methods**

The enzyme used in this work was the CGTase from alkalophilic \(Bacillus\) sp. cloned in Escherichia coli provided by the company WACKER. Knowing that
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this enzyme produces mainly β- e γ-CD, the methodology described below to obtain the kinetic model considers the simultaneous formation of β- e γ-CD, plus the reversibility of the cyclization reaction. However, this model does not include the disproportionation reactions because these reactions are very fast in comparison with cyclization and can form a quasi-equilibrium condition for the concentration of the linear dextrins, which will be transformed into CDs by the reactions considered in the model.

The substrate was maltodextrin 10 from FLUKA CHEMIE AG, which had a dextrose equivalent (DE) of 10 and average molecular weight of 1672 g gmo1^-1.

Kinetic model

The model proposed for the simultaneous production of β- and γ-CD 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 (ESβ or ESγ) 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; MORAES, 1996; ZHANG; CHUANG, 1999);
- Inhibition by substrate: High concentrations of substrate leads to the formation of ternary complexes SESβ or SESγ. This prevents the conversion of linear dextrins into cyclodextrins;
- Competitive inhibition by 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.

The proposed model and its considerations are shown in Figure 1. In this figure, k1, k-1, k3, k-3, k5, k-5, k7 and k-7 are kinetic constants related to the production of β-CD and ESβ, Eβ, Eβ, SESβ and SESγ are intermediates complexes of the reaction.

The reaction rate was determined by applying the pseudo steady-state hypothesis. This eliminates the concentrations of the intermediate complex from the model (SEGEL, 1975), resulting in the equations:

\[
V_\beta = \frac{d\beta}{dt} = V_{max\beta} \left( \frac{S}{K_{ms_\beta}} + \frac{\beta}{K_{mp_\beta}} \right) - V_{max\beta} \beta \\
\]

\[
V_\gamma = \frac{d\gamma}{dt} = V_{max\gamma} \left( \frac{S}{K_{ms_\gamma}} + \frac{\gamma}{K_{mp_\gamma}} \right) - V_{max\gamma} \gamma 
\]

Figure 1. Schematic representation of the kinetic model proposed for the simultaneous production of β- e γ-CD.
where:
\[ K_{\text{mp}} = \frac{\beta}{k_1 + k_2}, \] Michaelis-Menten constant for coupling reaction;
\[ K_{\text{mp}} = \frac{k_2}{k_1 + k_2}, \] Michaelis-Menten constant for coupling reaction;
\[ K_{\text{ss}} = \frac{k_2}{k_1 + k_2}, \] Michaelis-Menten constant for the reaction of cyclization to form β-CD;
\[ K_{\text{ss}} = \frac{k_2}{k_1 + k_2}, \] Michaelis-Menten constant for the reaction of cyclization to form γ-CD;
\[ K_{p} = k_2/k_1, \] product inhibition constant (β-CD);
\[ K_{p} = k_2/k_1, \] product inhibition constant (γ-CD);
\[ K_{s} = k_2/k_1, \] substrate inhibition constant;
\[ S = \text{starch concentration (g mL}^{-1}), \]
\[ V_{\text{max}} = \text{maximum rate of formation for β-CD per mL of enzyme (mol L}^{-1} \text{ min}^{-1}); \]
\[ V_{\text{max}} = \text{maximum rate of formation for γ-CD per mL of enzyme (mol L}^{-1} \text{ min}^{-1}); \]
\[ V_{\text{max}} = \text{maximum rate of consumption for β-CD per mL of enzyme by the coupling reaction (mol L}^{-1} \text{ min}^{-1}); \]
\[ V_{\text{max}} = \text{maximum rate of consumption for γ-CD per mL of enzyme by the coupling reaction (mol L}^{-1} \text{ min}^{-1}); \]
\[ [\beta] = \text{concentration of β-CD (mM);} \]
\[ [\gamma] = \text{concentration of γ-CD (mM).} \]

**Determination of Kinetic parameters**

The kinetic parameters of the direct reactions (\(K_{\text{ss}}, K_{\text{mp}}, V_{\text{max}}\) and \(V_{\text{max}}\)) and substrate inhibition constants (\(K_{p} \) and \(K_{s}\)) were determined from initial rates of production of β-CD and γ-CD for several initial concentrations of substrate. These initial velocities obtained experimentally by Hamon and Moraes (1990), are shown in Tables 1 and 2. The experiment was conducted at a temperature of 50°C and pH 8, using maltodextrin (DE 10) as substrate.

The concentration of enzyme \([E]\) given in mass of protein per volume was \(1.156 \times 10^{-2} \mu\text{g mL}^{-1}\).

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, this provide Equations (3) and (4).

**Table 1.** Data of Enzymatic activity \((S \times V_{\text{ini}})\) of CGTase for the Production of β-CD: \(T = 50^\circ\text{C}, \text{pH} = 8, [E] = 1.156 \times 10^{-2} \mu\text{g mL}^{-1}\) (HAMON, MORAES, 1990).

<table>
<thead>
<tr>
<th>Substrate concentration ((S \text{ (g L}^{-1}))</th>
<th>Enzymatic activity per mL of enzyme (V_{\text{ini}} \text{ (mol kg}^{-1}\text{ min}^{-1}))</th>
<th>(S/V_{\text{ini}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5.40</td>
<td>0.1124</td>
</tr>
<tr>
<td>1.0</td>
<td>7.43</td>
<td>0.1345</td>
</tr>
<tr>
<td>2.5</td>
<td>10.06</td>
<td>0.2486</td>
</tr>
<tr>
<td>5.0</td>
<td>14.92</td>
<td>0.3352</td>
</tr>
<tr>
<td>25.0</td>
<td>19.81</td>
<td>1.2623</td>
</tr>
<tr>
<td>50.0</td>
<td>36.93</td>
<td>2.0309</td>
</tr>
<tr>
<td>75.0</td>
<td>71.04</td>
<td>4.3044</td>
</tr>
<tr>
<td>100.0</td>
<td>88.69</td>
<td>9.2082</td>
</tr>
</tbody>
</table>

**Table 2.** Data of Enzymatic activity \((S \times V_{\text{ini}})\) of CGTase of CD for the Production of γ-CD: \(T = 50^\circ\text{C}, \text{pH} = 8, [E] = 1.156 \times 10^{-2} \mu\text{g mL}^{-1}\) (HAMON; MORAES, 1990).

<table>
<thead>
<tr>
<th>Substrate concentration ((S \text{ (g L}^{-1}))</th>
<th>Enzymatic activity per mL of enzyme (V_{\text{ini}} \text{ (mol kg}^{-1}\text{ min}^{-1}))</th>
<th>(S/V_{\text{ini}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.06</td>
<td>0.1124</td>
</tr>
<tr>
<td>1.0</td>
<td>1.45</td>
<td>0.1345</td>
</tr>
<tr>
<td>2.5</td>
<td>2.06</td>
<td>0.2486</td>
</tr>
<tr>
<td>5.0</td>
<td>3.45</td>
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<tr>
<td>25.0</td>
<td>5.98</td>
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<tr>
<td>50.0</td>
<td>10.12</td>
<td>2.0309</td>
</tr>
<tr>
<td>75.0</td>
<td>15.04</td>
<td>4.3044</td>
</tr>
<tr>
<td>100.0</td>
<td>16.29</td>
<td>9.2082</td>
</tr>
</tbody>
</table>

Equations (3) and (4) can be rearranged as a second degree polynomial \((y = a + bx + cx^2)\) with \(y = S/V_{\text{ini}}\) and \(x = S\), providing Equations (5) and (6). A nonlinear fit of polynomials represented by Equations (5) and (6) to the data shown in Tables 1 and 2 provides the parameters \(V_{\text{max}}\), \(K_{\text{ss}}\), \(K_{\text{mp}}, K_{\beta}, K_{p}, K_{s}\) and \(K_{s}\).
The kinetic parameters related to the coupling reaction \((V_{max-\beta}, V_{max-\gamma}, Kmp_{\beta} \text{ and } Kmp_{\gamma})\) and inhibition constants for the presence of the products \((Kp_{\beta} \text{ and } Kp_{\gamma})\) were determined by fitting the curves given by the kinetic model to the data of \(\beta\)-CD and \(\gamma\)-CD production as a function of time. The data was obtained experimentally by Hamon and Moraes (1990) for a reaction time of 24 hours. The reaction was carried out at a temperature of 50°C, pH 8, enzyme concentration of 11.56 \(\mu\)g \(\text{mL}^{-1}\), using dextrin (DE 10) as substrate with initial concentration of 100 \(\text{g L}^{-1}\).

### Results and discussion

The kinetic parameters obtained as described above are shown in Table 3. It is observed that the inclusion of reversibility allowed to reduce the apparent value of the inhibition caused by the product \(\beta\)-CD by approximately 50 times in comparison with the model of Hamon and Moraes (1990). Even with a much lower value of the \(\beta\)-CD inhibition parameter the experimental data was adequately represented.

The model proposed here gave for the production of \(\beta\)-CD \((Kms_{\beta})\) a Michaelis-Menten constant that was twice the value obtained by Hamon and Moraes (1990) and for the production of \(\gamma\)-CD there was a reduction in the value of this constant \((Kms_{\gamma})\). 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 \(\beta\)-CD, since this is the main cyclodextrin produced by the CGTase studied.

Table 3 shows also that the inhibition of the production of cyclodextrins by \(\beta\)-CD is approximately twice the inhibition caused by \(\gamma\)-CD.

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:

\[
K_{eq\beta} = \frac{V_{max\beta}/Kms_{\beta}}{V_{max-\beta}-V_{max\beta}/Kmp_{\beta}}
\]  

Similarly, for \(\gamma\)-CD:

\[
K_{eq\gamma} = \frac{V_{max\gamma}/Kms_{\gamma}}{V_{max-\gamma}-V_{max\gamma}/Kmp_{\gamma}}
\]

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 \(\beta\)-CD and also with the results obtained by Tardioli et al. (2000), who report that the CGTase from alkalophilic Bacillus sp. is a \(\beta\)-CGTase, producing CDs with molar ratio \(\beta\)-CD: \(\gamma\)-CD approximately equal to 3:1.

### Table 3. Kinetic parameters for the model of Hamon and Moraes (1990) and for the model proposed in this work for the simultaneous production of \(\beta\)- and \(\gamma\)-CD catalyzed by CGTase of alkalophilic Bacillus sp.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hamon and Moraes</th>
<th>Proposed model</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{max\beta}) (mol L (\text{min}^{-1}))</td>
<td>(1.259 \times 10^{-3})</td>
<td>(2.660 \times 10^{-3})</td>
</tr>
<tr>
<td>(V_{max\gamma}) (mol L (\text{min}^{-1}))</td>
<td>(---)</td>
<td>(58.595)</td>
</tr>
<tr>
<td>(Kms_{\beta}) (mol L (^{-1}))</td>
<td>(2.596 \times 10^{-4})</td>
<td>(5.483 \times 10^{-4})</td>
</tr>
<tr>
<td>(Kms_{\gamma}) (mol L (^{-1}))</td>
<td>(2.279 \times 10^{-2})</td>
<td>(1.767 \times 10^{-2})</td>
</tr>
<tr>
<td>(Kp_{\beta}) (mol L (^{-1}))</td>
<td>(1.962 \times 10^{-3})</td>
<td>(1.962 \times 10^{-3})</td>
</tr>
<tr>
<td>(Kp_{\gamma}) (mol L (^{-1}))</td>
<td>(6.000 \times 10^{-7})</td>
<td>(2.760 \times 10^{-3})</td>
</tr>
<tr>
<td>(V_{max\beta}) (mol L (\text{min}^{-1}))</td>
<td>(2.152 \times 10^{-4})</td>
<td>(4.552 \times 10^{-4})</td>
</tr>
<tr>
<td>(V_{max\gamma}) (mol L (\text{min}^{-1}))</td>
<td>(---)</td>
<td>(18.312)</td>
</tr>
<tr>
<td>(Kms_{\beta}) (mol L (^{-1}))</td>
<td>(1.320 \times 10^{-3})</td>
<td>(4.929 \times 10^{-4})</td>
</tr>
<tr>
<td>(Kms_{\gamma}) (mol L (^{-1}))</td>
<td>(3.684 \times 10^{-2})</td>
<td>(2.795 \times 10^{-2})</td>
</tr>
<tr>
<td>(Kp_{\beta}) (mol L (^{-1}))</td>
<td>(---)</td>
<td>(5.263 \times 10^{-3})</td>
</tr>
<tr>
<td>(Kp_{\gamma}) (mol L (^{-1}))</td>
<td>(---)</td>
<td>(5.290 \times 10^{-3})</td>
</tr>
<tr>
<td>(Kmp_{\beta}) (mol L (^{-1}))</td>
<td>(---)</td>
<td>(5.976 \times 10^{-4})</td>
</tr>
</tbody>
</table>

The mathematical model developed in this work is formed by a system of two ordinary differential equations which were solved numerically using the software MAPLE\textsuperscript{TM}. Equations (1) and (2) were used to obtain the theoretical curve that describes the concentration of \(\beta\)-CD and \(\gamma\)-CD as a function of time. These curves are shown in Figures 2 and 3, where the symbols in the form of circles represent experimental data for \(\beta\)-CD and symbols in the form of diamonds represent the experimental data for \(\gamma\)-CD.

Comparing the model curves to the experimental data it becomes clear that there was a very good representation of the data throughout the entire test period. For the data points of long reaction times fluctuations around the average are observed.
However, it is known that these data are valid as they were obtained with sufficient experimental accuracy. These oscillations occur as a consequence of a very complex kinetics, with simultaneous formation and destruction of the products of the reactions. Only a kinetic model more advanced, yet not available in the literature, could generate the possibility of obtaining the oscillations at the final equilibrium of the reaction. The development of a model with these characteristics would have great scientific interest for the area and is therefore one of the goals for future developments of our research group.

![Figure 2](image)

**Figure 2.** Modeling the reaction of production of β-CD and γ-CD for a period of up to 90 minutes $T = 50^\circ\text{C}$, $\text{pH} = 8$, $[E] = 11.56$ μg mL$^{-1}$, [Dextrin] = 100 g L$^{-1}$.

![Figure 3](image)

**Figure 3.** Modeling the reaction of production of β-CD and γ-CD for a period of 24 hours, $T = 50^\circ\text{C}$, $\text{pH} = 8$, $[E] = 11.56$ μg mL$^{-1}$, [Dextrin] = 100 g L$^{-1}$.

**Conclusion**

Proposed model describes the reaction kinetics for the production of β-CD and γ-CD, considering substrate and product inhibition, reversibility of the reaction and considering that the cyclization reaction catalyzed by CGTase produces simultaneously the two CDs. The model was fitted to the experimental data set providing an excellent fit, proving that it represents appropriately the production of CDs.

Simultaneous production of two CDs leads to different kinetic constants $K_{ms}$ and $K_s$ for each product of cyclization, showing that substrate interacts with the enzyme in a specific manner to produce each of the cyclodextrins (β- or γ-CD). This result agrees with experimental observations of Hamon and Moraes, allows comprehending that the way in which the substrate binds to the enzyme determines which product will be formed (β- or γ-CD), and explains how the same enzyme, with the same substrate, can produce two products with different Michaelis-Menten and substrate inhibition constants.

**References**


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