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Subsite structure of the β-glucosidase from *Aspergillus niger*, evaluated by steady-state kinetics with cello-oligosaccharides as substrates [1]

Terutaka Yazaki [a], Masatake Ohnishi [a,*], Souji Rokushika [b], Gentaro Okada [c]

[a] *The Department of Agricultural and Biological Chemistry, College of Agriculture, University of Kyoto Prefecture, Shimogamo, Kyoto 606, Japan*

[b] *Department of Chemistry, College of Science, Kyoto University, Kitashirakawa, Kyoto 606, Japan*

[c] *Department of Biology, Faculty of Education, Shizuoka University, 836 Oya, Shizuoka 422, Japan*

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Abstract

The β-glucosidase from a commercially available preparation from *Aspergillus niger* was highly purified. The Michaelis constant K_m and the molar activity k_0 for cello-oligosaccharide substrates G_n ($n = 2$–6) were obtained by steady-state kinetic analysis on the β-glucosidase-catalyzed hydrolysis at 25 °C and pH 5.0. Stoichiometric production of G_{n-1} by the β-glucosidase reaction for G_n was confirmed by HPLC techniques. Based on K_m and k_0 for G_n, subsite affinities (A_i, $i = 1$–6) were estimated as follows (kcal/mol): $A_1 = 1.3$, $A_2 = 5.2$, $A_3 = 0.65$, $A_4 = -0.10$, $A_5 = -0.65$, and $A_6 = -0.26$, of which A_1–A_3 are much higher than those of the β-glucosidase of *Candida wickerhamii*. The subsite structure is quite similar to that of the α-glucosidase of *A. niger*, whereas the dependence of k_0 on n is highly characteristic for β-glucosidase, and decreases with n, suggesting some interaction between the particular subsites.

Keywords: Subsite; Steady-state kinetics; Binding affinity; β-Glucosidase; Cello-oligosaccharides

1. Introduction

β-Glucosidase (EC 3.2.1.21; β-D-glucoside glucohydrolase), which occurs ubiquitously in fungi, bacteria, plants, insects, and animals, catalyzes the hydrolysis of mainly (1 → 4)- and (1 → 6)-β-D-glucosidic bonds in aryl and alkyl β-D-glucosides as well as in cello-oligosaccharides [1–15]. These β-glucan-related saccharides occur in many glycosides and sugar chains found in living organisms, and serve an essential physiological role, sometimes intimately related to hydrolysis–transfer reactions of glycosidic bonds. This enzyme has received attention in many fields of science and technology, not only from a basic standpoint, but also for practical applications (biomass

* Corresponding author.

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conversion, cyanogenesis, Gaucher's disease, etc.) [9,16–21].

Kinetic analysis is an effective technique for characterizing enzyme-catalyzed mechanisms and substrate specificity. Numerous β-glucosidases have been purified from different sources, and studied by techniques. In some of these studies, the kinetic parameters (the Michaelis constant K_m and the molar activity k_0) have been determined for cello-oligosaccharides and also for aryl or alkyl glucosides to describe the substrate specificity and mechanism. These kinetic parameters can be used to evaluate the subsite affinity at the active site of enzymes. However, the subsite structure, affinity, and number of subsites have not been analyzed for β-glucosidases. Thoma et al. and Hiromi et al. have proposed that the active site of such enzymes as glucohydrolases is composed of several subsites, which accommodate individual glucose residues of the substrate [22–24]. The subsite structure, namely, the number and affinity of subsites, can be evaluated by quantitative analysis of the products in enzyme-catalyzed reactions [23] or by steady-state kinetic studies [22,24] on a series of *n*-mer saccharide substrates. Determination of the subsite structure is important in characterizing the structure and function of the enzyme active site.

Preparations of β-glucosidase purified from *Aspergillus niger* have been described by several research groups, and the kinetic and molecular properties have been investigated by steady-state kinetic and biochemical techniques [25–29]; however, the experimental results reported are not consistent among the research groups. Thus, the structural properties (molecular weight and subunit composition) and the kinetic properties (K_m, V_{max}, and k_0) for sugar ligands are important in investigating the β-glucosidase preparation from *A. niger*.

The present study describes the subsite map, namely, affinities and number of subsites, and the specificity of the β-glucosidase from *A. niger*.

2. Experimental

Materials.—A commercial preparation (Lot No. 7655-A) of β-glucosidase from *Aspergillus niger* (Novozyme 188) was purchased from Novo Nordisk Bioindustrial Co. Ltd. and was purified to homogeneity by procedures described in previous papers [28,29]. The concentration of the enzyme was determined spectrophotometrically, using $A^{1\%}_{280\,nm} = 15.4\ cm^{-1}$ and a molecular weight of 137,000. Cello-oligosaccharides G_n, having a degree of polymerization $n = 2–6$, were prepared as follows: Avicel hydrolyzed with fuming hydrochloric acid was purified by carbon–Celite column chromatography with EtOH–water gradient elution, and paper chromatograms were developed with water as solvent. The products, G_n, were confirmed to be more than 95% pure by TLC on silica gel plates.

The enzymatic glucose-determination kit, Glucose C-II Test Wako, was purchased from Wako Pure Chemicals Co. and used after dilution 1:3 with 0.06 M phosphate buffer, pH 7.1. Other chemicals and organic solvents, of guaranteed grade, were purchased from Nacalai Tesque Inc. and used without further purification.

Assay of the β-glucosidase-catalyzed hydrolytic activity.—Time-curves of the β-glucosidase-catalyzed hydrolysis for cello-oligosaccharides G_n were obtained at pH 5.0 and 25.0 °C as follows: The solutions of enzyme β-glucosidase (60 μL), substrate G_n (60 μL), and buffer (120 μL) were mixed, and aliquots (40 μL) were removed at adequate time-intervals and mixed with 0.5 M NaOH to stop hydrolysis. The quantity of glucose liberated from the substrate G_n was determined with the enzymatic glucose-determination reagent Glucose C-II Test Wako at 505 nm, using glucose for calibration.

Based on the initial velocity v obtained as the slope of the reaction time-curve, the kinetic parameters (the Michaelis constant K_m and the molar activity k_0) were determined for the cello-oligosaccharides (G_n; $n = 2–6$) substrates. The kinetic (rate) parameters and the standard deviations were obtained by using an NEC PC-9801 Personal Computer and Bio-graph software (Kyoto Soft Co.).

HPLC analysis on the products of the β-glucosidase-catalyzed hydrolysis of cello-oligosaccharides G_n.—Identification and quantitative determination of the products G_n were carried out using a Shimadzu LC-3A HPLC apparatus [30]: a sample (10–20 μL) was removed from the reaction mixture and applied to an aminopropyl-silica column (4.6 mm × 25 cm) eluted with 83:17 isopropyl alcohol–water at a flow rate 0.5 mL/min and 50 °C. An Erma ERC-7510 RI detector was used for the detection of the saccharides.

3. Results and discussion

Evaluation of the kinetic parameters for cello-oligosaccharides.—Based on Eq. (1), the kinetic (rate) parameters, the Michaelis constant K_m and the

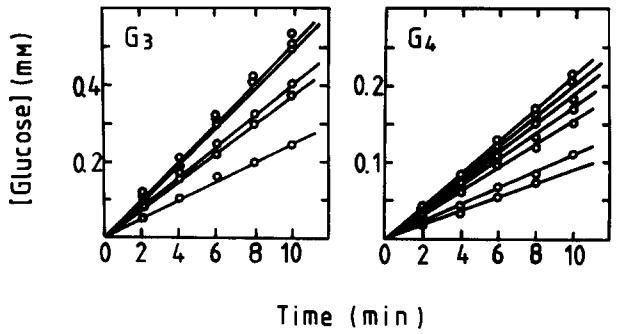


Fig. 1. Progress curves of the β-glucosidase-catalyzed hydrolysis for cello-oligosaccharides. $[E]_0$: 3.22 nM (cellotriose), 1.40 nM (cellotetraose). $[S]_0$: 0.2, 0.4, 0.6, 0.8, and 1.2 mM (cellotriose); 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, and 1.6 mM (cellotetraose); pH 5.0, 25.0 °C.

molar activity k_0, of β-glucosidase-catalyzed reactions were measured with five cello-oligosaccharides (degrees of polymerization $n = 2$–6) as substrates,

$$v = V_{max}[S]_0/(K_m + [S]_0)$$
$$= k_0[E]_0[S]_0/(K_m + [S]_0) \quad (1)$$

where v and $[S]_0$ are the initial velocity and the initial concentration of substrates, respectively. Two examples of progress curves obtained by the β-glucosidase-catalyzed hydrolysis are illustrated in Fig. 1 for cellotriose and cellotetraose, showing a linear release of product with time. The slope thus indicates the initial velocity, v. The initial velocities v were measured for hydrolysis of cello-oligosaccharides G_n ($n = 2$–6), plotted against the concentration of substrate, $[S]_0$ (shown in Fig. 2). The best-fit values of the kinetic parameters K_m and k_0 were obtained for the oligosaccharides using the nonlinear least-squares method. The parameters K_m, k_0, and k_0/K_m, are

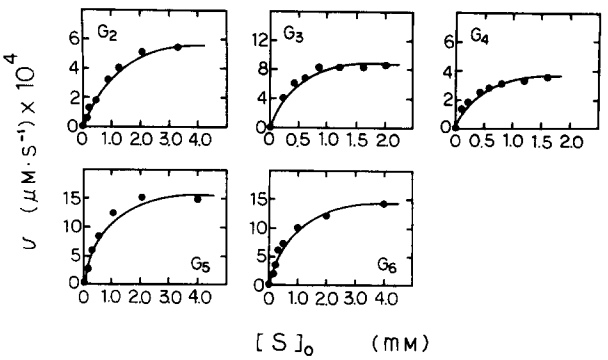


Fig. 2. Dependence of v on $[S]_0$ for the β-glucosidase-catalyzed hydrolysis for cello-oligosaccharides. $[E]_0$: 1.00 nM (cellobiose), 3.22 nM (cellotriose), 1.40 nM (cellotetraose), 10.0 nM (cellopentaose, cellohexaose), pH 5.0, 25.0 °C.

Table 1
Kinetic parameters of the β-glucosidase-catalyzed hydrolysis of cello-oligosaccharides [a]

Substrate (G_n)	K_m (mM)	k_0 (s^{-1})	k_0/K_m ($mM^{-1}s^{-1}$)
G_2	0.85±0.10	360±40	440± 80
G_3	0.26±0.05	330±30	1300±140
G_4	0.25±0.08	270±30	1100±360
G_5	0.46±0.05	170±15	370± 12
G_6	0.69±0.03	160± 6	240± 10

[a] pH 5.0, 25.0 °C. ±, Standard deviation.

listed in Table 1. The theoretical values (Fig. 2, solid lines) were calculated from the obtained K_m and k_0 values (Table 1) using Eq. (1), and confirm that the reaction follows Michaelis–Menten kinetics.

Stoichiometric observation of the product cello-oligosaccharide using HPLC techniques.—Kinetic parameters for the β-glucosidase-catalyzed G_n hydrolysis indicate that those saccharides with smaller degrees of polymerization have larger values of k_0, as can be seen in Table 1. These findings suggest that one of the products of a reaction can also serve as a substrate for the enzyme-catalyzed reaction:

$$E + G_n \rightarrow EG_n \rightarrow E + G_{n-1} + G \quad (2)$$

$$E + G_{n-1} \rightarrow EG_{n-1} \rightarrow E + G_{n-2} + G, \quad (3)$$

for example, when G_4 is a substrate [$n = 4$ in Eq. (2)], the k_0 of its product G_3 is larger than that of the substrate G_4. Therefore, it is necessary to confirm how far the reaction can proceed before the products G_{n-1} accumulate to a level at which they also become substrates for the enzyme-catalyzed reaction. To confirm this level, a small amount of β-glucosidase was incubated with the individual substrate on cello-oligosaccharides, and the products liberated by the β-glucosidase reaction were analyzed using HPLC as a function of time. As an example of the HPLC chromatograms, where G_3 was used as substrate, the product G_2 was found to be equal to the other product G within the reaction time observed for evaluation of the initial velocity v. For the other substrates G_n, the experimental results were almost the same as those for G_3, and thus it was concluded that the product saccharides did not become substrates within the reaction time used for measurement of the initial velocity, suggesting that the kinetic parameters obtained here are reasonable. These experimental values, K_m and k_0, are plotted against the degree of polymerization, n, in Fig. 3, showing that the kinetic parameters K_m and k_0 depend character-

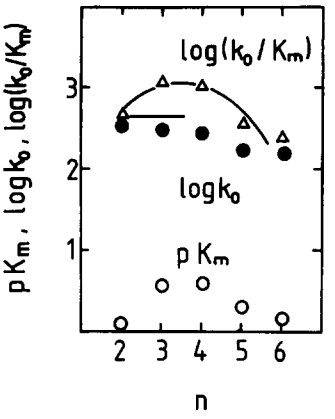


Fig. 3. Dependence of the kinetic parameters on the degree of polymerization (n) of the substrate cello-oligosaccharides. ○, pK_m; ●, $\log k_0$; △, $\log(k_0/K_m)$. The solid line was obtained theoretically by using the k_{int} (469 s^{-1}) and A_i values shown in Table 2.

istically on n. Thus, these kinetic parameters permit calculation of the subsite affinities at the active site of the β-glucosidase.

Determination of the subsite structure of A. niger *β-glucosidase.*—According to the subsite theory developed by Hiromi et al., the rate (kinetic) parameters can be expressed in a unified way in terms of the subsite affinities A_i of m subsites and the intrinsic rate constant k_{int} for glucosyl bond-cleavage [22,24]. The subsite theory has two basic assumptions: (*1*) the subsites have their own proper affinities for a glucose residue of a substrate glucose-polymer and there is no interaction between subsites, and (*2*) the subsite affinities are additive. The binding affinity of a substrate is thus defined by the sum of affinities of the subsites occupied with glucose residues of the substrate. If these assumptions apply, the subsite theory will be valid also for β-glucosidase as outlined next.

When an n-mer substrate S_n is bound to the enzyme, it may be in a productive or a nonproductive complex, $(ES_n)_p$ or $(ES_n)_q$, respectively:

$$E + S_n \underset{K_{n,q}}{\overset{K_{n,p}}{\rightleftharpoons}} \begin{matrix}(ES_n)_p \\ (ES_n)_q\end{matrix} \xrightarrow{k_{int}} E + P \qquad (4)$$

where k_{int} is assumed to be constant irrespective of n and p. $K_{n,p}$ and $K_{n,q}$ are association constants of the n-mer substrate in a binding mode specified by p (productive) and q (nonproductive), respectively. Here $j = p + q$, as illustrated in Scheme 1. Then,

$$K_{n,j} = [(ES_n)_j]/([E][S_n]). \qquad (5)$$

When rapid equilibrium between E and S_n holds, the rate equation obtained is of the same type as the Michaelis equation, and the kinetic parameters K_m and k_0 are expressed with $K_{n,p}$ and $K_{n,q}$ as interpreted previously [22,24]. Then, the ratio k_0 to K_m gives

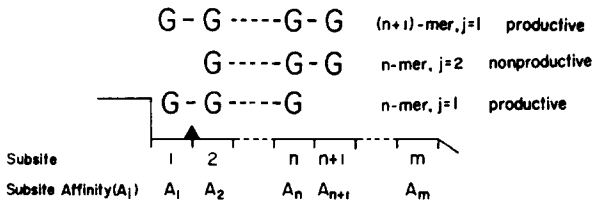
$$k_0/K_m = k_{int} \sum_p K_{n,p}. \qquad (6)$$

Thus, only the productive terms are involved in k_0/K_m, which is important for the determination of the subsite structure. In exo-glucohydrolases, there is only one productive complex for n-mer substrates and thus $K_{n,p} = K_{n,1}$.

The association constant $K_{n,j}$ is related to the molecular-binding affinity $B_{n,j}$, which is the unitary part of the standard affinity $-\Delta G_{n,j}$ as described elsewhere [24,32]. When the molecular-binding affinity $B_{n,j}$ is assumed to be expressed by the sum of the affinities of the subsites occupied by the binding of the substrate molecule, $\sum_i^{cov.}$, $K_{n,j}$ is written as

$$K_{n,j} = (0.018)\exp\left(\sum_i^{cov.} A_i/RT\right)_{n,j} \qquad (7)$$

where the term 0.018 arises from the contribution of the mixing entropy in water at 25 °C, 2.4 kcal/mol. Then, the kinetic parameters K_m and k_0 will be expressed in terms of k_{int} and A_i, as described elsewhere [22,24]. Thus, the subsite parameters k_{int} and A_i can be evaluated using the kinetic parameters K_m and k_0 obtained by steady-state experiments: the difference is the k_0/K_m value between n-mer and $(n + 1)$-mer substrates, $(k_0/K_m)_{n+1} - (k_0/K_m)_n$ gives A_{n+1}, as illustrated in Scheme 1. Then, based on the K_m and k_0 obtained for cello-oligosaccharides



Scheme 1. Schematic representation of the β-glucosidase active site and the binding modes of n-mer substrates. G, a glucose residue of a substrate; ▲, the catalytic site of the enzyme. Subsites are numbered counting from the terminal one to which nonreducing-end glucose residue of substrate is situated in the productive binding mode.

Table 2
Subsite structure of *Aspergillus niger* β-glucosidase [a]

Subsite (i)	1	2	3	4	5	6
Subsite affinity (A_i) (kcal/mol)	1.34	5.18	0.65	−0.10	−0.65	−0.26
k_{int} (s^{-1})	469					

[a] The catalytic site is situated between subsites 1 and 2.

G_n, we can evaluate the subsite parameters, A_i (i = 1–6) and k_{int}. The results determined for the β-glucosidase preparation are summarized in Table 2, where k_{int} was obtained by procedures described elsewhere [24].

On the other hand, all of the kinetic parameters k_0 and K_m for any n-mer substrate can be obtained theoretically by using the subsite parameters, k_{int} and A_i. Thus, k_0/K_m is given by

$$(k_0/K_m)_n = (0.018)k_{int}\sum_p \exp\left(\sum_i^{cov.} A_i/RT\right). \quad (8)$$

Here the theoretical values obtained for k_0/K_m are represented in Fig. 3 by a solid line, which was calculated with the subsite parameters listed in Table 2. The theoretical k_0/K_m is in excellent agreement with the experimental values, suggesting that the subsite theory is valid for *A. niger* β-glucosidase. Subsite 2 (A_2) was found to have the highest affinity out of all six subsites, whereas A_1 is considerably larger than A_3. This finding reflects the substrate specificity of β-glucosidase.

Based on the subsite theory, the dependence of k_0 on the degree of polymerization n arises merely from the term $\Sigma_p K_{n,p}/\Sigma_j K_{n,j}$, which represents the statistical weight of the productive complex [22,24]. Kita et al. have investigated the α-glucosidase (*A. niger*)-catalyzed reaction for the hydrolysis of malto-oligosaccharides αG_n (n = 2–8) as substrates using steady-state kinetic techniques to obtain the kinetic parameters. The k_0 values for these saccharides are almost identical, that is, the dependence of k_0 on n is nearly constant [31]. Hiromi et al. have studied the glucoamylase (*Rhizopus niveus*)-catalyzed hydrolysis of malto-oligosaccharides αG_n (n = 2–7) and evaluated the kinetic parameters, where the k_0's for G_3–G_7 are almost identical [24,32]. In contrast, the k_0's of the β-glucosidase-catalyzed reaction gradually decrease with increasing n, as seen in Fig. 3 and Table 2. The dependence of k_0 on n is characteristic of β-glucosidase. Two interpretations of this finding are possible, involving (*1*) a transfer reaction and (*2*) properties of the subsites. The first is not likely, since in the β-glucosidase-catalyzed reaction on cello-oligosaccharides (as examined by HPLC), no product was generated via a transfer reaction. Indeed, it was confirmed that a transfer reaction does not cause the decrease in k_0 with n. It is concluded that characteristics of the subsite structure are very important in the investigation of the β-glucosidase-catalyzed mechanism.

Comparison of the subsite structure with other exo-type glucohydrolases.—When K_m and k_0 values for a series of substrate glucose-polymer are obtained by kinetic analysis, evaluation of the subsite structure is possible in principle, as already described. However, few experimental results on the kinetic parameters of β-glucosidase have been reported. Steady-state kinetic studies have been carried out with the β-glucosidase from *A. niger* by Tavobilov et al., McCleary and Harrington, and Watanabe et al. The kinetics of *A. niger* β-glucosidases are excellently described, as is the substrate specificity. The K_m and V_{max} values were determined, but k_0 values were not presented in these studies [8,25–27,33] and therefore the subsite structure cannot be evaluated. For the β-glucosidase from yeast (*Candida wickerhamii*), steady-state kinetic studies were performed by Freer that gave K_m and k_0 [12], which allows the subsite structure to be evaluated. Using these reported values of K_m and k_0, we attempted a calculation of the subsite structure of the yeast enzyme. Fig. 4 shows the subsite structures evaluated here, with the subsite affinities, A_i, as a histogram, for three kinds of exo-type glucohydrolases: β-glucosidase from *A.*

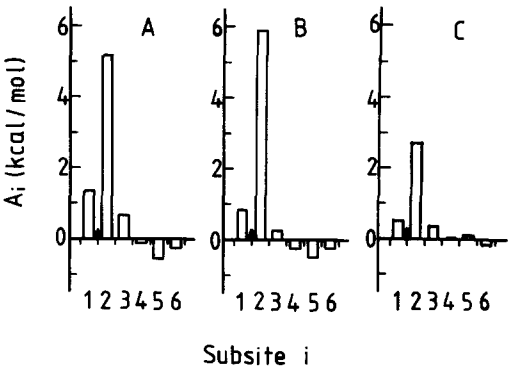


Fig. 4. Subsite structures of exo-glucohydrolases. ▲, the catalytic site. A, β-glucosidase from *A. niger*; B, α-glucosidase from *A. niger* [31]; C, β-glucosidase from yeast (*C. wickerhamii*) [12]. The subsite affinity is represented by the histogram.

niger, α-glucosidase from *A. niger* (α-glucosidase) [31], and β-glucosidase from yeast [12]. The subsite structure of β-glucosidase is very similar to that of α-glucosidase, that is, A_i of subsites 1, 2, and 3 were much larger than those of subsites 4, 5, and 6. A_1 is larger than A_3, indicating that the substrates are bound mainly in the productive mode and the probability of productive binding of cello-oligosaccharides is certainly high. The subsite affinities A_1–A_3 of yeast enzyme (β-glucosidase of yeast) are much smaller than those of the β-glucosidase, suggesting that *A. niger* β-glucosidase is a very effective enzyme (β-glucosidase). No doubt the substrate specificity of β-glucosidase reflects its subsite structure, with distinct high affinities for oligosaccharides having $n = 2$, 3, and 4 (see Table 1).

As has already been described, the molar activity k_0 is given by the statistical weight of the productive binding $k_{int}\Sigma_p K_p/\Sigma_j K_j$. Here, we tried to consider k_0 in relation to the properties of subsites. In α-glucosidase from *A. niger* and β-glucosidase from yeast, k_0's are essentially independent of n. The probability of a productive binding mode is almost identical for all n-mer substrates tested. The subsite structure of β-glucosidase from *A. niger* is very similar to that of α-glucosidase from *A. niger* (see Fig. 4); however, the dependence of k_0 upon n is distinctly different for β-glucosidase, where the k_0's decrease with n. For amylases, as a general rule, the k_0's increase with n of the substrate [34]. Based on the subsite theory, the following cases are proposed: (*1*) the productive binding p decreases, and (*2*) the nonproductive binding q increases, with n. As shown in Fig. 3. A_4, A_5, and A_6 are negative, and therefore the probability of nonproductive binding q has not increased but is decreased by an increase in n of the oligosaccharides G_n. At present, we cannot give a reasonable explanation for this finding, but it cannot be excluded that the subsite(s) 4, 5, or 6 is intimately related with subsite 1, which is critical in productive binding. If this possibility is valid, a decrease in productive binding will occur, resulting in a decrease in k_0 with substrate n. This may be associated with a mechanism for regulation of the enzyme-catalyzed reaction, and thus examination of the reaction mechanism at subsite 1 is extremely important. Recently, Withers et al. investigated the *Agrobacterium* β-glucosidase-catalyzed reaction in detail [35–37] and proposed a mechanism via a transition state in which a glucosyl–enzyme intermediate is produced at the site corresponding to subsite 1. Characterization of the particular subsite(s) in the β-glucosidase from *A. niger* using the steady-state and transient-phase kinetic techniques is currently in progress.

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