RS3-2

Lactose Bioconversion by Recombinant β-Galactosidase from *Kluyveromyces lactis*: Kinetic Modeling and Progress Curve Fitting

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Previous models based on the Michaelis-Menten kinetic equation, that glucose was not used as an acceptor, did not explain our experimental data for lactose conversion by a recombinant β-galactosidase from *Kluyeromyces lactis*. In order to create a new kinetic model based on the data, the effects of galactose and glucose on β-galactosidase activity were investigated. Galactose acted as an inhibitor at low concentrations of galactose and lactose, but did not inhibit the activity of β-galactosidase at high concentrations of galactose (above 50 mM) and lactose (above 100 mM). The addition of glucose at concentrations below 50 mM resulted in an increased reaction rate. A new model of *K. lactis* b-galactosidase for both hydrolysis and transgalactosylation reactions with glucose and lactose as acceptors was proposed. The proposed model was fitted well to the experimental data of the time-course reactions for lactose conversion by *K. lactis* β-galactosidase at various concentrations of substrate.

Introduction

Most kinetic studies on *K. lactis* β-galactosidase have been performed with either commercial enzyme preparations extracted from cultured cells as free or immobilized enzymes (1, 2) or with whole washed, permeabilized, or immobilized cells (3). A model based on the Michaelis-Menten kinetic equation has been widely used for lactose hydrolysis by *K. lactis* β-galactosidase (4-7). Yang and Okos (4) presented a model with competitive product inhibition by galactose, assuming that the glucose molecule is the first to leave the active site of the enzyme, leaving a covalent galactosyl-enzyme complex for further hydrolysis. The reaction mechanism for lactose hydrolysis can be described as follows, assuming that the process described by Eq. (2) rapidly reaches its equilibrium state:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}S \xrightarrow{k_2} E\text{-}P + G \quad (1)$$

$$E\text{-}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \quad (2)$$

where *S*, *P*, *G*, *E*, *E:S*, and *E-P* are lactose, galactose, glucose, enzyme, noncovalent enzyme-lactose complex, and covalent galactosyl-enzyme complex, respectively; k_1, k_{-1}, k_2 , k_3, and k_{-3} are primary

reaction rate constants. If $K_i = k_3/k_{-3}$ is the inhibition constant, $K_p = k_2/k_3$ is the rate constant, and the two products are formed evenly ($P \approx G$), the reaction rate equation can be expressed as:

$$v = -\frac{dS}{dt} = \frac{V_{max} S}{K_m(1 + P/K_i) + S(1 + K_p)} \quad (3)$$

where v is the volumetric reaction rate; t is the reaction time; $K_m = (k_{-1} + k_2)/k_1$ is the Michaelis-Menten constant; $V_{max} = k_2 E_0$ is the maximum reaction rate; and E_0 is the initial enzyme concentration. As above, nearly all kinetic models consider only lactose hydrolysis with product inhibition by galactose and/or glucose (4-6).

It is known, however, that the enzymatic hydrolysis of lactose occurs at low lactose concentrations and that oligosaccharide production by the transgalactosylation reaction increases with increasing lactose concentration (8). Recently, models have been developed to describe galacto-oligosaccharide synthesis as well as simultaneous lactose hydrolysis while still including product inhibition (7, 9). The reaction mechanism including transgalactosylation proposed by Zhou et al. (7) is a Michaelis-Menten kinetic model with competitive inhibition by two products, galactose and trisaccharides; but this model does not consider galactosyl-glucose disaccharides formed from galactosyl-enzyme intermediate because the glucose produced during the reaction is not assumed to be an acceptor.

Results and Discussion

Effect of glucose on lactose hydrolysis and transgalactosylation reactions

The effect of glucose on β-galactosidase activity was studied at different concentrations (0, 25, 50, and 100 mM) with *o*NPG, instead of lactose, as a substrate (2.5, 5, 10, and 15 mM) (Fig. 1A). The results suggest that glucose is not an inhibitor for the hydrolysis reaction, but it acts as a better acceptor for transgalactosylation reactions and reacts only with the galactosyl-enzyme intermediate as a galactose acceptor to make galactosyl-glucose disaccharides. The previous models including transgalactosylation did not consider galactosyl-glucose disaccharides (7-9).

Effect of galactose on lactose hydrolysis and transgalactosylation reactions

The Dixon plot (I vs. $1/v_0$) was used to investigate the effect of galactose as an inhibitor. Galactose inhibition was only displayed in ranges below both 50 mM lactose and 50 mM galactose, where the K_i value was 90 mM galactose (Fig. 1B). This means that galactose can bind to the free enzyme to make the galactosyl-enzyme complex for further transgalactosylation reactions with glucose or lactose as the acceptors, but does not bind to the galactosyl-enzyme complex. If exogenous galactose is added to the reaction mixture at a below-critical concentration, galactose can bind to the enzyme to make more galactosyl-enzyme intermediate and thus reduce the rate of formation of the glucose from lactose by competition with lactose for binding to the enzyme.

Proposed model for lactose conversion by hydrolysis and transgalactosylation reactions

A new kinetic model for hydrolysis and transgalactosylation reactions of a recombinant b-galactosidase from *K. lactis* was proposed based on experimental kinetic data for the effects of galactose and glucose on β -galactosidase activity. It is assumed that only one rate-limiting step is involved in the reaction mechanism and that the other steps are all reversible. Based on this assumption and the roles of substrate and products investigated in the experiments, the following equations are proposed:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}S \xrightarrow{k_2} E\text{-}P + G \quad (4)$$

$$E\text{-}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P \quad (5)$$

$$E\text{-}P + G \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + D \quad (6)$$

$$E\text{-}P + S \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + T \quad (7)$$

where D is galactosyl-glucose disaccharides and the meanings of other abbreviations are the same as described in Introduction. Assuming that the reactions described by Eqs. (5), (6), and (7) are rapidly equilibrated, the equilibrium constants are $K_H = E\,P/E\text{-}P = k_3/k_{-3}$, $K_D = E\,D/E\text{-}P\,G = k_4/k_{-4}$, and $K_T = E\,T/E\text{-}P\,S = k_5/k_{-5}$. The materials are conserved for the galactose P moiety, $S_0 - S \approx P + D + 2T$ where S_0 is the initial molar concentration of substrate, for the glucose G moiety, $S_0 - S \approx G + D + T$, and for the enzyme, $E_0 = E + E{:}S + E\text{-}P$. The reaction rate expression is:

$$v = -\frac{dS}{dt} = \frac{V_{max}\,S}{K_m(1 + P/K_H + D/(K_D\,G) + T/(K_T\,S) + S} \quad (8)$$

Model evaluation: Lactose conversion by *K. lactis* β-galactosidase

For model evaluation, the initial guess values of reaction rate constants were obtained through trial-and-error processes, beginning with an assumption of 1 mM of enzyme used to get the best fits considering the following criteria: [1] The K_m value of the purified enzyme for lactose at different concentrations up to 200 mM was determined to be 20 mM by initial rate experiments in a previous paper (10); [2] The diffusion rates of substrates and products, the flexibility of the enzyme segments, and the dissociation of enzyme from substrate could be reduced in an enzymatic reaction of a viscous solution with increasing lactose concentrations in the reaction mixture (11-13).

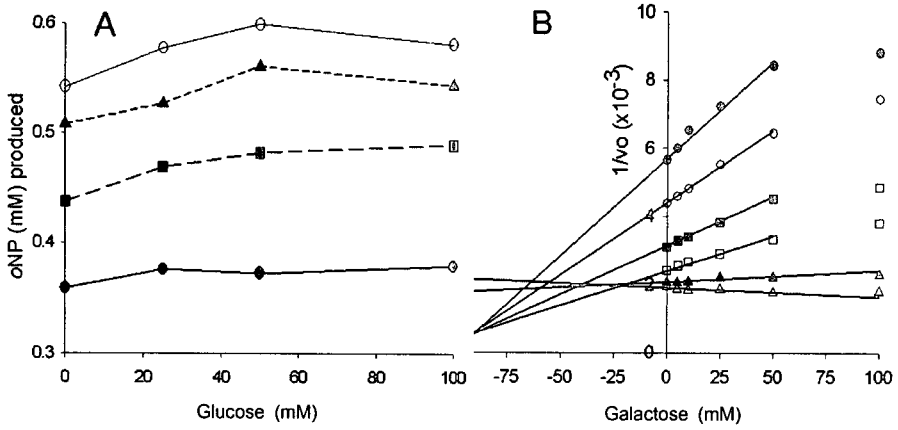

Fig. 1. Effects of glucose and galactose on β-galactosidase activity. (A) At various glucose concentrations of 0, 25, 50, and 100 mM, β-galactosidase activities were studied with respect to *o*NPG of 2.5 (●), 5 (■), 10 (▲), and 15 (○) mM, as a substrate. (B) At various galactose concentrations of 0, 5, 10, 25, 50, and 100 mM, lactose was used as a substrate by varying concentrations of 10 (●), 15 (○), 25 (■), 50 (□), 100 (▲), and 200 (△) mM. The Dixon plot (I vs. $1/V$) used to investigate the effect of galactose as an inhibitor.

The experimental data for the time courses of lactose conversion were obtained with 151, 280, and 880 mM lactose solutions by addition of an equal amount (0.029 mM) of the *K. lactis* β-galactosidase (Fig. 2). The fitted values of reaction rate constants and the time curve fits were obtained by fitting the proposed kinetic model as described by Eq. (8) to the experimental data using the Levenberg-Marquardt method (14), with the initial guess values of reaction rate constants (Table 1). In order to confirm the data fitting, another set of simulations was carried out with Mathematica 4 version (15) by putting the fitted rate constants in the model, using the NDSolve function with derivative mass balance equations and Plot and InputForm functions (data not shown).

The fitted k_{cat} ($\approx k_2$) values steadily decreased with the increase in initial lactose concentration. This means that the initial guesses were properly deduced from enzymatic reactions in a viscous solution as described above. The fitted reaction rate constants for disaccharides (k_4 and k_{-4}) were found to be higher than those for trisaccharides (k_5 and k_{-5}); glucose is a better glycosyl acceptor to form disaccharides, but the disaccharides produced are more easily broken down; lactose is a poorer acceptor, but has more chances to make trisaccharides at high concentrations of lactose, and the trisaccharides formed are much more stable.

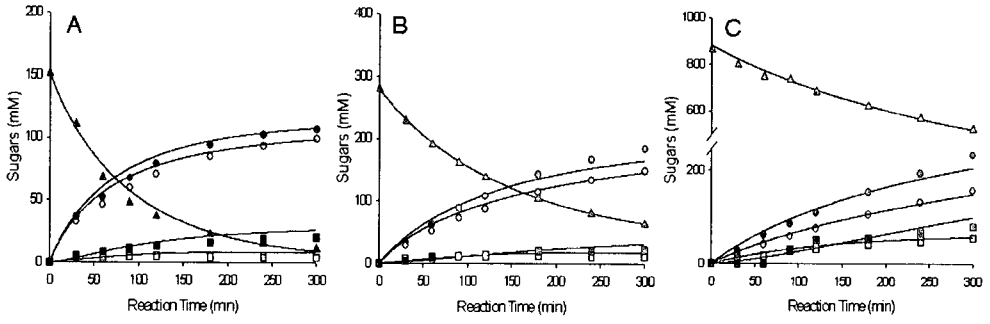

Fig. 2. The time-course reactions for lactose conversion by addition of equal amount (0.029 mM) of *K. lactis* β-galactosidase with three different initial concentrations of lactose; (A) 151 mM, (B) 280 mM and (C) 880 mM. Lactose (▲), glucose (●), galactose (○), disaccharides (■), and trisaccharides (□). Symbols are the points of experimental data and lines are the curve fits of the proposed model. Please note the different concentration scales.

The proposed model explained well not only the lactose hydrolysis but also the galacto-oligosaccharide synthesis by the *K. lactis* β-galactosidase at various concentrations of substrate (Fig. 2). The conversion data are in good agreement with the predictions of the derived reaction rate equation. In all cases, the glucose concentration was higher than the galactose concentration, indicating that the difference between the amounts of glucose and galactose produced was used in the transgalactosylation reaction for the formation of trisaccharides. With the increase of initial substrate concentration from 151 and 280 to 880 mM, the concentration ratio of glucose to galactose increased from 1.08 and 1.24 to 1.49 at the reaction time of 300 min, respectively and the production of galacto-oligosaccharides also increased. While in a diluted lactose solution, water rather than other sugars such as glucose and lactose can be more competitive as an acceptor for galactosyl-enzyme intermediates; therefore, galactose is formed. On the other hand, in a high lactose-content solution, more galactosyl residues of galactosyl-enzyme intermediates are transferred to acceptors such as glucose and lactose rather than to water, and thus disaccharides and trisaccharide are formed due to lower water activity (16-18). These results imply that the lactose hydrolysis reaction by the *K. lactis* β-galactosidase takes place concomitantly with the transgalactosylation reaction at high substrate concentrations.

Acknowledgments

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Table 1. The initial guess values and the fitted values of reaction rate constants

	Initial guess values			Fitted values		
Lactose (mM)	151	280	880	151	280	880
k_1 ($mM^{-1}s^{-1}$)	28.7	31.6	43.1	27.9	31.0	42.5
k_{-1} (s^{-1})	575	517	431	591	527	437
k_2 (s^{-1})	1.61	1.44	1.09	1.58	1.41	1.12
k_3 (s^{-1})	4.60	4.31	2.87	4.49	4.17	2.64
k_{-3} ($mM^{-1}s^{-1}$)	0.632	0.862	1.44	0.648	0.883	1.41
k_4 ($mM^{-1}s^{-1}$)	0.0230	0.0287	0.0431	0.0237	0.0317	0.0529
k_{-4} ($mM^{-1}s^{-1}$)	1.44	5.75	11.5	1.40	5.21	9.51
k_5 ($mM^{-1}s^{-1}$)	0.000718	0.000862	0.00144	0.000826	0.000991	0.00153
k_{-5} ($mM^{-1}s^{-1}$)	0.0287	0.144	1.01	0.0203	0.125	1.19

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