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CHEMICAL KINETICS AND CATALYSIS

Study of the Kinetic Parameters for Synthesis and Hydrolysis of Pharmacologically Active Salicin Isomer Catalyzed by Baker's Yeast Maltase[1]

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Abstract—One of the key elements for understanding enzyme reactions is determination of its kinetic parameters. Since transglucosylation is kinetically controlled reaction, besides the reaction of synthesis, very important is the reaction of enzymatic hydrolysis of created product. Therefore, in this study, kinetic parameters for synthesis and secondary hydrolysis of pharmacologically active α isosalicin by baker's yeast maltase were calculated, and it was shown that specifity of maltase for hydrolysis is approximately 150 times higher then for synthesis.

Keywords: synthesis and hydrolysis of salicin isomer, catalysis by maltase, kinetic parameters.

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INTRODUCTION

Many glycoside hydrolases exhibit transglycosylation activity, and transglycosylation reactions are well known and widely used methods for glycoside synthesis [1]. Transglycosylation is a mechanism for glycosidic bond formation in which a glycosyl moiety from a donor compound is transferred to the hydroxyl group of an acceptor molecule other than water [2] (Fig. 1). Transglucosylation is subtype of transglycosylation, where enzyme transfers glucose moiety to acceptor molecule.

Principally, all glycosidases can be divided in two classes, depending whether hydrolysis occurs with retention or with inversion of configuration at the anomeric center. Maltase (EC 3.2.1.20; α-glucosidase) belongs to the family of glycosidases which hydrolyze substrate and form products with the same configuration at the anomer atom like substrate [3]. It is one of the most abounded glycosyl hydrolases present in baker's yeast (*Saccharomyces cerevisiae*), and has been used for synthesis of various pharmacologically active glycosides [4]. Sometimes, the glycosidic residue is crucial for glucosides activities; in other cases glycosylation improves pharmacokinetic parameters [5]. We used maltase for synthesis of α-isosalicin [2-hydroxybenzyl-α-D-glucopyranoside] with *ortho*-hydroxybenzyl alcohol as glucose acceptor and maltose as glucose donor in reaction of transglucosylation (Fig. 1). Salicin, [2-(hydroxymethyl)phenyl-β-D-glucopyranoside] and its structural isomers exhibit pronounced anticoagulant activity [5], tyrosinase inhibitory activity [6], and are also used as anti-inflammatory, analgesic and antipyretic prodrugs [7, 8]. Moreover, α-glucoside of *ortho*-hydroxybenzyl alcohol (α-isosalicin) is more potent anticoagulant than naturally occurring salicin [5].

Alpha-glucoside of *ortho*-hydroxybenzyl alcohol, like all other glycosides, is formed in a kinetically controlled reaction that shows optimum in time, when disaccharide is used as donor [9]. Because the reaction is controlled kinetically, it is possible to overshoot the equilibrium conversion of reactant into product. As the reactant is consumed the concentration of the product will reach the peak when rates for its synthesis and debenzylation become equal. At this point kinetic control is lost and the reaction should be stopped before thermodynamic control takes over and the product undergoes enzymatic hydrolysis (secondary hydrolysis in Fig. 1) [3]. Hence, the yield of glucoside is determined by a delicate balance between the rates of [E–G +A] synthesis (K_1, k_3) and hydrolysis (k_4, k_{-3}, K_7, k_2) on the one hand, and product hydrolysis (k_6, k_5, k_{-5}), on the other.

Determination of kinetic parameters that lies in K_1 is earlier described for (β-galactosidase [2], as well as reaction driven with K_7 for β-glucosidase (substrate transglycosylation) [10], although this reaction does not have influence in transglucosylation reaction catalyzed by maltase from baker's yeast. Reaction of inhi-

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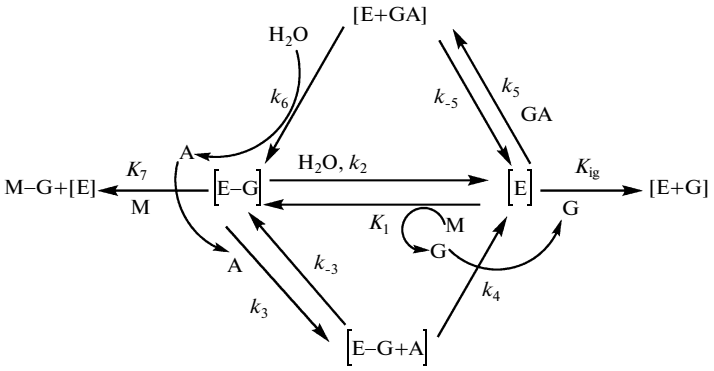

Fig. 1. Schematic model for different kind of reactions catalyzed by α-glucosidase (E), with maltose (M) as glucose (G) donor. The role of acceptor can be played by water (primary hydrolysis k_1) or by other hydroxylic compounds (transglucosylation) such as, *ortho*-hydroxybenzyl alcohol (A), hydrolysis products (G), or a second substrate molecule, i.e. maltose (M), (substrate transglucosylation), [E–G] represents covalent complex between enzyme and glucose, and GA is α glucoside of *ortho*-hydroxybenzyl alcohol; k_6 corresponds secondary hydrolysis.

bition glycosidase (K_{ig}) by monosaccharide is also described for β-galactosidase [11].

Therefore, in this study a detailed kinetic analysis for synthesis and hydrolysis of pharmacologically active alpha glucoside of *ortho*-hydroxybenzyl alcohol by maltase was performed, because they are essential kinetic parameters crucial for maximal yield of reaction.

EXPERIMENTAL

Enzyme α-glucosidase (E.C. 3.2.1.20) was isolated from baker's yeast by a slightly modified previously published procedure [12]. All commercially available reagents and solvents were used as obtained without further purification.

Determination of kinetic parameters for synthesis of α-isosalicin. Glucose donor for this reaction (Fig. 1) was 1.2 M maltose in 0.1 M phosphate-citrate buffer pH 6.8, and concentration of *ortho*-hydroxybenzyl alcohol was varied from 0 to 40 mM. The enzymatic reaction was stopped by adding 0.1 M HCl to give pH 3.0 and acetonitrile (AN) to give 10% (v/v). The reaction mixtures were then centrifuged, and analyzed using an Akta Purifier HPLC applying 10 μl reaction mixture on it. Column used, was Waters Spherisorb 5 μm ODS 2, 4.6 × 250 mm. Solvent A was 10% acetonitrile (AN) in 1 mM HCl, solvent B was 20% AN in 1 mM HCl. Length of gradient from 0 to 100%B was two column volumes, at flow rate 1 ml/min and UV detector was set at 268 nm. Enzyme concentration was 100 mg lyophilized enzyme (specific activity of 25 U/mg) in 1 ml reaction mixture. 1U of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glucose at 25°C per 1 min from 4-nitrophenyl-α-D-glucopyranoside.

Determination of kinetic parameters for hydrolysis of α-isosalicin. Michaelis constant (K_m) for secondary hydrolysis (Fig. 1), was obtained using 4-nitrophenyl-α-D-glucopyranoside (0.45 mM) as one substrate and α-isosalicin (0.35 mM) as another in simultaneous reaction, in 50 mM phosphate buffer pH 6.8. Concentration of enzyme was 100 U/ml. Blank probe was performed in the same reaction condition only without α-isosalicin and with enzyme concentration of 75 U/ml. Concentration of liberated 4-nitrophenolate ion was monitored in UV/VIS spectrophotometer at 445 nm in kinetic mode.

After the K_m was obtained, k_{cat} has been determined by monitoring rate of hydrolysis α-isosalicin by maltase in condition saturated with α-isosalicin (10 K_m). The kinetic was monitored in the same HPLC condition as described in previous section but with 25 U per mL of reaction mixture.

RESULTS AND DISCUSSION

Determination of kinetic parameters for synthesis of α-isosalicin. Applying method of King and Altman [13] under the conditions of the stady-state for all enzyme substrate complexes [10] at Fig. 1, following kinetic parameters are given as:

$$K_{m_s} = \frac{\text{const} + (\text{coefGA})[\text{GA}] + (\text{coefM})[\text{M}]}{(\text{coefA}) + (\text{coefGA} \cdot \text{A})[\text{GA}] + (\text{coefM})[\text{M}]}, \quad (1)$$

where K_{m_s} represents Michaelis constant for *ortho*-hydroxybenzyl alcohol (A) in transglucosylation reaction, i.e. "constant of decomposition" complex [E–G + A];

$$k_{cat_s} = \frac{V_{max_s}}{E_0} = \frac{k_3 k_4 (k_5 k_6 [\text{GA}] + k_1 k_6 [\text{M}] + k_{-5} k_1 [\text{M}])}{(\text{coefA}) + (\text{coefGA} \cdot \text{A})[\text{GA}] + (\text{coefM})[\text{M}]}, \quad (2)$$

where k_{cat_s} represents turnover number for reaction of α-isosalicin synthesis, i.e. catalytic rate constant for conversion of complex [E–G + A] in free enzyme and α-isosalicin (GA). In practical view, k_{cat} is molecular

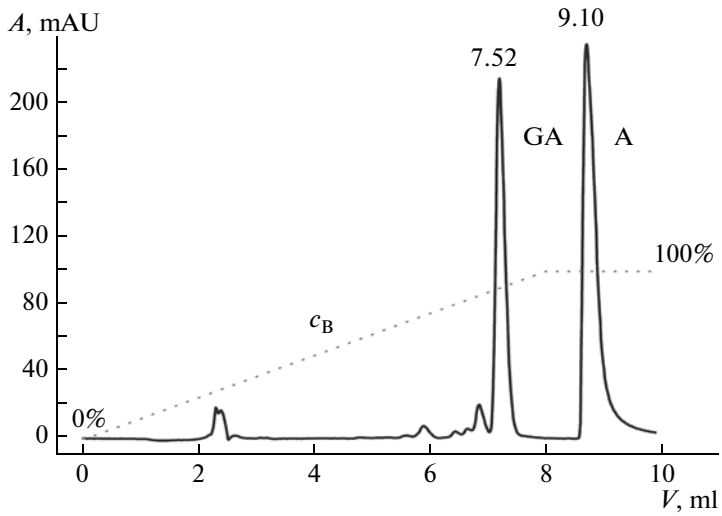

Fig. 2. One of the HPLC chromatograms for transglucosylation reaction with *ortho*-hydroxybenzyl alcohol (A, retention volume 9.10 ml) as glucose acceptor and α-isosalicin (GA, retention volume 7.52 ml) as product of reaction. Yield of reaction was calculated from peak area ratio at 268 nm between GA and A. This chromatogram is obtained when initial concentration of *ortho*-hydroxybenzyl alcohol was 30 mM and reaction was stopped after 30 min. Conditions for HPLC chromatography are described in EXPERIMENTAL section.

activity of enzyme and represent number of molecules of substrate that one molecule of enzyme transforms in one second. Knowing total concentration of enzyme (E_0) and V_{max} is easy to calculate k_{cat} (Eq. 2). Values for specific "coef" and "const" are represented below:

$$\text{const} = k_2(k_6 + k_{-5})(k_4 + k_{-3}),$$

$$(\text{coefA}) = k_3k_4(k_{-5} + k_6),$$

$$(\text{coefGA}) = k_5(k_4 + k_{-3})(k_6 + k_2),$$

$$(\text{coefM}) = K_1(k_6 + k_{-5})(k_4 + k_{-3}),$$

$$(\text{coefGA} \cdot \text{A}) = k_3k_5(k_4 + k_6),$$

$$(\text{coefA} \cdot \text{M}) = k_3K_1(k_{-5} + k_6);$$

[A] is concentration of *ortho*-hydroxybenzyl alcohol; [GA] is concentration of α-isosalicin; [M] is concentration of maltose.

The product formation rate (d[GA]/dt) for each substrate concentration was calculated from HPLC chromatograms (Fig. 2) Michaelis constant and k_{cat} are determined from Lineweaver–Burk (LB) plot, derived from Michaelis–Menten (MM) plot (Fig. 3).

Determination of kinetic parameters for hydrolysis of α-isosalicin. Like in case of synthesis, using King and Altman modified Michaelis–Menten equation based on Fig. 1, following kinetic parameters are given for secondary hydrolysis of created product as:

$$K_{m_h} = \frac{\text{const} + (\text{coefA})[\text{A}] + (\text{coefM})[\text{M}] + (\text{coefA} \cdot \text{M})[\text{A}][\text{M}]}{(\text{coefGA}) + (\text{coefGA} \cdot \text{A})[\text{A}]}, \quad (3)$$

where K_{m_h} represent Michaelis constant for [E + GA] analog that for the synthesis reaction,

$$k_{cat_h} = \frac{V_{max_h}}{E_0} = \frac{k_5k_6(k_3k_4[\text{A}] + k_2k_4 + k_{-3}k_2)}{(\text{coefGA}) + (\text{coefGA})[\text{A}]}, \quad (4)$$

where k_{cat_h} is turnover number for [E + GA] and also has analog meaning as in case of synthesis.

The specific "Coef" and "const" are the same as in explanation of synthetic reaction.

Michaelis constant was determined using one of integrated forms of Michaelis–Menten equation [14]. The traditional technique for determination of enzyme kinetic parameters is to perform numerous experiments measuring the reaction rate, V, as a function of the substrate starting concentration. In a well-known manner, which we used to described synthesis. K_m and V_{max} can be obtained by plotting $1/V$ in a function of 1/[S] (the LB plot). To determine the reaction rate by graphical differentiation of the substrate concentration-time curve, at least several experiments must be performed. This procedure is not only tedious but also inaccurate, especially if a too high substrate conversion during the measurement causes a flat curve instead of a straight line in the substrate concentration-time course [14–17]. The integrated form of Michaelis–Menten method for enzyme kinetic parameter determination is direct and more convenient. This method enables establishment of K_m and

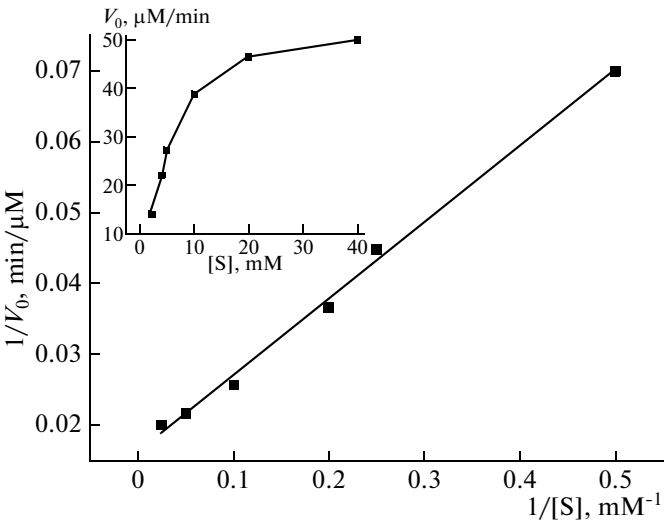

Fig. 3. LB and MM plot for synthesis of α-isosalicin; [M] = 1.2 M; K_{m_s} = 6.62 mM, V_{max} = 0.616 μM/(min mg).

V_{max}, by only one experiment, in which the change of substrate concentration in time is monitored:

$$\frac{\tau}{U} = \frac{K_m}{V_{max}}\left(\frac{1}{U}\ln\frac{1}{1-U} - 1\right) + \frac{c_0 + K_m}{V_{max}}, \quad (5)$$

where τ is the time point of the reaction, U is substrate conversion, and c_0 is initial substrate concentration [14].

Results obtained using this form of integrated equation do not depend on the concentration of substrate used, and therefore allows the use of concentration that are far below K_m. In this way it is possible to use only one, an arbitrary, concentration of substrate, thus saving the total consumption of high cost substrate, like α-isosalicin is, in determination of the constants.

As the maltase is "the most active" toward 4-nitrophenyl-α-D-glucopyranoside [18], so any other substrate can be considered as competitive "inhibitor" for hydrolysis 4-nitrophenyl-α-D-glucopyranoside [19]. Applying Eq. (5) to calculate K_m and V_{max} for 4-nitrophenyl-α-D-glucopyranoside, values obtained matched literature values [18] (Fig. 4).

The results obtained for 4-nitrophenyl-α-D-glucopyranoside in presence of α-isosalicin are shown in Fig. 5.

As it was expected, K_m for hydrolysis 4-nitrophenyl-α-D-glucopyranoside increased (for factor α) and the V_{max} remained the same, suggesting that it is a competitive inhibition [11]. In the case of the competitive inhibition

$$\alpha = \frac{K_m^{app}}{K_m} = \frac{1 + [I]}{K_i}, \quad (6)$$

where [I] is concentration of inhibitor, i.e. in our case α-isosalicin, and K_i is inhibition constant, i.e. "decomposition constant" for enzyme inhibitor complex, or on Fig. 1, "decomposition constant" for [E + GA] complex. Because "decomposition constant" for [E + GA] complex actually represents K_{m_h} for hydrolysis α-isosalicin, K_{m_h} and K_i can be equated. Using Eq. (6) we calculated that K_{m_h} is 0.61 mM.

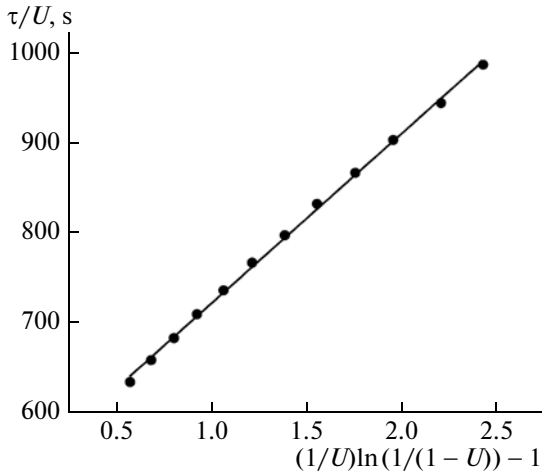

Fig. 4. Plot of integral form of Michaelis–Menten equation for 0.45 mM 4-nitrophenyl-α-D-glucopyranoside as substrate without inhibitor; K_m = 0.27 mM, V_{max} = 25 μM/(min mg). Concentration of enzyme used was 75 U/ml.

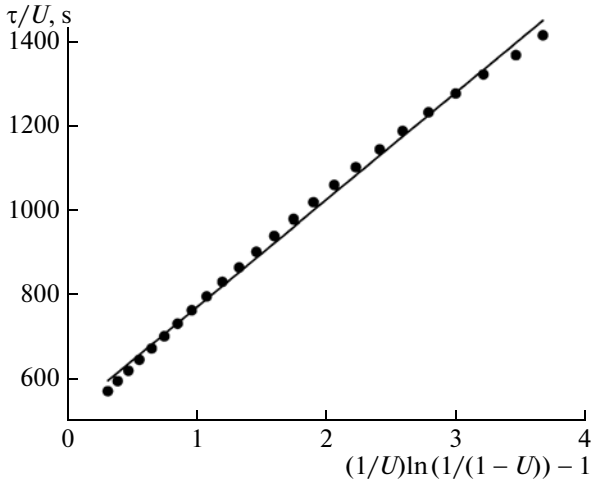

Fig. 5. Plot of integral form for Michaelis–Menten equation for 0.45 mM 4-nitrophenyl-α-D-glucopyranoside as substrate in the presence of 0.35 mM α-isosalicin as the second substrate (i.e. inhibitor), K_m^{app} = 0.45 mM, V_{max}^{app} = 25 μM(min mg). Concentration of enzyme used was 100 U/ml.

Kinetic constants for transglucosylation reaction catalyzed by maltase

A isosalicin		
Constant	synthesis	hydrolysis
K_m, mM	6.62	0.61
V_{max}, μmol/(min mg)	0.616	8.30
k_{cat}, s^{-1}	2.5	33.67
K_{spec}, s^{-1}/mM^{-1}	0.38	55.2

Note: K_{spec} represents real measure of affinity of some enzyme toward its substrate and it is equal to the ratio between k_{cat} and K_m.

Because V_{max} is the rate of the reaction when all enzyme molecules are in complex with its substrate, it is possible to determine V_{max} in the high excess of substrate ([S] ≥ 10 K_m). Therefore V_{max_h} for this reaction was determined from concentration of 6 mM α-isosalicin (10 K_m), and calculated to be V_{max} = 8.3 μM/(min mg).

Results for hydrolysis and synthesis of α glucoside of salicyl alcohol are summarized in table.

CONCLUSION

To the best of authors' knowledge, this is the first time that detailed kinetic study of transglucosylation activity of a glucosidase from *Saccharomyces cerevisiae* was examined. Also this is the first time ever that by using kinetic parameters, secondary hydrolysis of created product by α-glucosidase was described, although it is crucial for maximal yield of transglucosylation. Constants calculated in our study are derivative from our experimental condition, but it is obvious (Eqs. (1)–(4)) that in other concentrations of maltose, or maltose and alcohol in case of hydrolysis, these constants are different.

In order for enzyme to work with maximal rate, minimal alcohol concentration needed for the synthesis of α-isosalicin is about 10 mM, as shown in table. It would be wrong to conclude that when concentration of the product reaches near 1 mM and concentration of alcohol is 10 times smaller than product, secondary hydrolysis starts. It should be emphasized that large amount of maltose is present in the reaction mixture (1.2 M), and as K_m for maltose is 80 mM [18] enzyme practically "does not feel" the α-isosalicin. Secondary hydrolysis starts when concentration of maltose drops to value comparable to α-isosalicin. At this point the reaction should be stopped before the system enters thermodynamic equilibrium.

ACKNOWLEGMENTS

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