0273-2289/05/127/0157/$30.00

Preparation of Trehalase Inhibitor Validoxylamine A by Biocatalyzed Hydrolysis of Validamycin A With Honeybee (*Apis cerana* Fabr.) β-Glucosidase

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Received February 25, 2005; Revised August 4, 2005; Accepted August 24, 2005

Abstract

Validoxylamine A is structurally similar to trehalose and acts a potent competitive inhibitor of trehalase. It has recently been receiving increased attention as a potential material for the development of new insecticides or drugs. In this study, β-glucosidase extracted from honeybees (*Apis cerana* Fabr.) was used as a catalyst to produce validoxylamine A through enzymatic hydrolysis of validamycin A. β-Glucosidase was separated and purified from honeybees, and its characteristics were examined. The results showed that β-glucosidase was stable across a range of temperatures from 30 to 40°C and across a relatively wide range of pH values from 5.0 to 7.5. Investigation of the biocatalyzed hydrolysis process from validamycin A to validoxylamine A with β-glucosidase revealed that both the substrate (validamycin A) and the product (validoxylamine A) inhibited β-glucosidase activity. The inhibition constant of the substrate K_{is} value was 5.01 m*M*, and that of the product K_{ip} value was 1.32 m*M*. This product inhibition was competitive.

Index Entries: Enzyme inhibitor; validamycin A; β-glucosidase; hydrolysis; validoxylamine A; biocatalysis.

Introduction

Validoxylamine A is an aglycon of validamycin A, which is a product of *Streptomyces hygroscopocus* var. *limoneus (1)* or *Streptomyces hygroscopocus*

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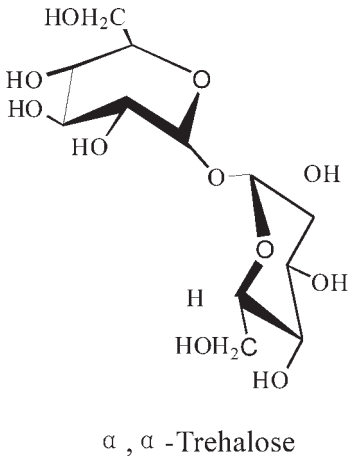


Scheme 1. Structure of α,α-trehalose.

var. *jinggangensis (2)*. Since 1970, it has been used as a control agent against rice sheath blight by *Rhizoctonia solani*. The mode of its fungicidal action depends on the inhibitory activity of validoxylamine A against fungus trehalase *(3)*. Validoxylamine A resembles trehalose in structure (Scheme 1) and is a potent and specific inhibitor of the trehalases (α,α-trehalose glucohydrolase; EC 3.2.1.28) in microorganisms as well as in mammals. Trehalose is a nonreducing disaccharide, which is of importance to some microorganisms for sugar transport and as a readily available energy supply. The sugar has been found in the blood of all insect species examined thus far *(4)*. Metabolic use of trehalose in the organism requires its cleavage by the trehalase that hydrolyzes trehalase to two glucose units *(5,6)*. Trehalase plays a very important role in organisms that store trehalose as a reserve carbohydrate. Validoxylamine A showed strong in vitro inhibitory activity against trehalases of *Spodoptera* larvae and thoracic muscle of *Periplaneta americana (7)*. Injections of validoxylamine A also inhibited trehalase in the ovary and in colleterial gland of the silkworm, in the midgut of the cabbage armyworm, and in thoracic muscle of *P. americana (8,9)*. Elevation of hemolymph trehalose concentration in validoxylamine A–injected flies (housefly, flesh fly, blowfly) suggests that trehalase of the flies was inhibited and the consumption of trehalose was suppressed as in other insects *(10)*. Therefore, the trehalase inhibitor appears to block energy metabolism. It also appears to cause serious abnormalities in insect physiology. Validoxylamine A is a potential raw material for the production of new insecticides or pesticides. The structure of validoxylamine A consists of two pseudoglucose moieties, valienamine and validamine, which show strong glucosidase inhibitory activity because their configurations are similar to that of α-D-glucose *(11–15)*. Through cleavage of the C-N bond of validoxylamine A with *N*-bromosuccinimide in aqueous N, *N*-dimethylformamide, valienamine, and validamine can be obtained *(16,17)*. The two compounds have significant implications for both antiviral and anti-

glucosidase chemotherapy. Thus, validoxylamine A holds some promise in the development of novel drugs.

The structure of validamycin A was found to be a β-glucosidic linkage between D-glucose and validoxylamine A, as shown in Scheme 2. It is suggested that validoxylamine A has just one molecule of glucose less than validamycin A. Therefore, validoxylamine A can be formed by disrupting the C-O bond in validamycin A.

Previously, the resin-catalyzed and microbial degradation of validamycin A for the production of validoxylamine A has been described *(18,19)*. To date, however, the production of validoxylamine A by β-glucosidase hydrolysis has not been reported. Here, we describe the enzymatic hydrolysis of β-glucosidic linkage to produce validoxylamine A with β-glucosidase from honeybees (*Apis cerana* Fabr.).

Materials and Methods

Honeybees and Reagents

Honeybees (*A. cerana* Fabr.) were provided by Hangzhou Changqing Honey Bees (Zhejiang, China). Validamycin A was provided by Qiangjiang Biochem (China). Sephadex G-100 was purchased from Pharmacia. Bovine serum albumin (BSA) and *p*-nitrophenyl-β-glucopyranoside (β-PNPG) were purchased from Sigma. Other reagents were of analytical reagent grade.

Enzyme Assay

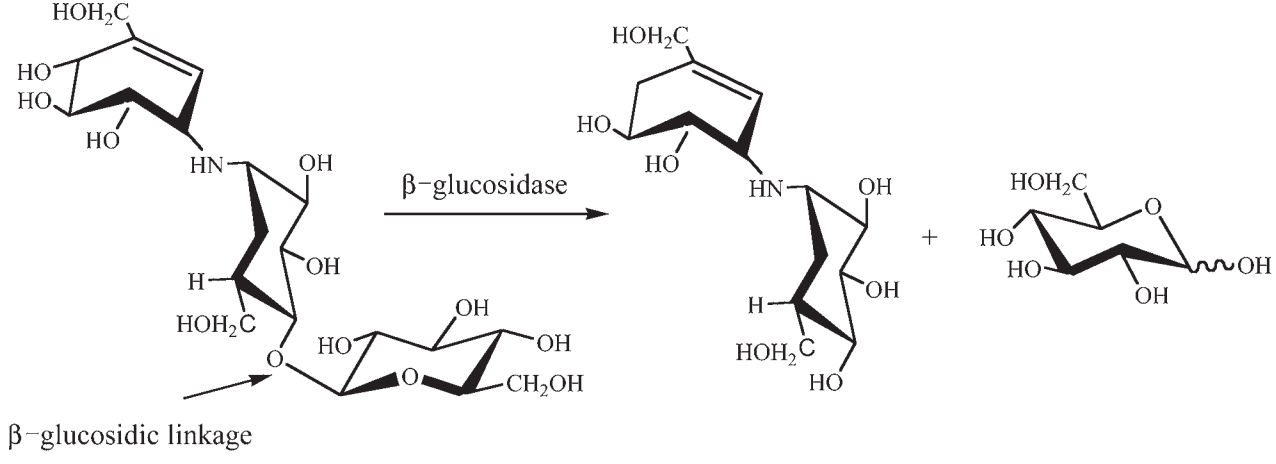
β-Glucosidase activity was determined employing a modification of the Low's method *(20)* of measuring the hydrolysis of β-PNPG. A sample was added to 1.0 mL of 0.02 *M* β-PNPG in 0.02 *M* sodium phosphate buffer at pH 7.5 in a test tube. The mixture was then incubated in a water bath (37°C) for 10 min. To this solution, 0.1 mL of 1.0 *M* Na_2CO_3 was added to stop the reaction. After cooling to room temperature, the solution was transferred into a spectrophotometer cuvet, and the absorbance was measured at 400 nm using a spectrophotometer (722; Shanghai Analysis Instrument, China). With these conditions, one unit of β-glucosidase activity is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol/min.

Protein Assay

Protein concentrations were determined at each stage of enzyme purification by the Bradford method *(21)* with BSA as the standard. Protein concentrations in the column effluents were monitored by measuring A_{280}.

Determination of Substrate and Product

Validamycin A and validoxylamine A were determined by high-performance liquid chromatography (LC-10AS; Shimadzu, Japan) on a 250 × 4.6 mm ODS column (Shimadzu) *(22)*. The eluting solvent was composed of disodium hydrogen phosphate buffer solution (pH 7.0) with 2.5% (in vol-



Scheme 2. Hydrolysis of validamycin A to validoxylamine A by β-glucosidase.

ume) methanol. The flow rate was 1.0 mL/min and the sample volume was 20 µL. Elution was monitored at 210 nm.

Separation and Purification of Enzyme

Approximately 100 adult worker honeybees were killed on site by treatment with dry ice and immediately frozen (–20°C). Because β-glucosidase is present in the honey sac and in organs secreting digestive enzymes in the mouth, each frozen honeybee was cut to collect the head and abdomen from the discarded thorax for isolation of β-glucosidase. The pretreatment is the same as that reported previously *(23)*. All procedures for protein purification were conducted at 4°C or in an ice bath. Ventriculi, honey sacs, and hypopharyngeal glands collected from the honeybees were mashed with a glass rod in the presence of glass beads containing approx 80 mL of 0.02 *M* sodium phosphate buffer, pH 7.5. Samples were then diluted, with the volume brought up to approx 100 mL with the same buffer. After extraction, the mixture was centrifuged (Avanti J-E; Beckman Coulter) for 30 min at 12,000*g*. The suspension was collected and the precipitate was removed. This solution was designated as the raw extract in purification of glucosidase.

Next, the raw extract was salted out by slowly stirring in ammonium sulfate (equivalent to 30% of saturation) over a course of 60 min. The suspension was centrifuged at 18,000*g* for 30 min to remove the precipitate, and additional ammonium sulfate was added into a supernatant fluid to 60% saturation. After gentle stirring for 60 min, the resultant precipitate was collected by centrifugation (18,000*g*, 30 min). The precipitate was dissolved in sodium phosphate buffer (0.02 *M*, pH 7.5) and then stirred gently for 120 min. Insoluble substances were removed by centrifugation (18,000*g*, 30 min). Dissolution of the enzyme precipitate was repeated once to ensure that all the β-glucosidase was collected in the solution. Then the solution was dialyzed against the same sodium phosphate buffer.

The third step of the experiment was to separate β-glucosidase with a column. The enzyme solution was applied to a Sephadex G-100 column (20 × 1.0 cm) that had been equilibrated by washing with 150 mL of sodium phosphate buffer (0.02 *M*, pH 7.5). Proteins were eluted from the column in sodium phosphate buffer at a flow rate of 1.0 mL/min. Fractions containing β-glucosidase activity were collected for further purification.

In the final step, β-glucosidase was purified with DEAE-Sepharose. The sample was injected into a DEAE-Sepharose column (20 × 1.0 cm; Bio-Rad, Hercules, CA). The column was connected to a fast protein liquid chromatography apparatus (Bio-Rad) and equilibrated with sodium phosphate buffer (0.02 *M*, pH 7.5). Proteins were eluted at 1 mL/min with a gradient from 0 to 500 m*M* NaCl. Fractions containing β-glucosidase activity were collected for the hydrolysis of validamycin A.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using a Mini-gel system (Bio-Rad). The gels were cast

Table 1
Summary of Purification of β-Glucosidase From Honeybee (*A. cerana* Fabr.)

Step	Activity (μmol/min)	Protein (mg)	Specific activity (μmol/[min·mg])	Yield (%)	Fold of purification
Extract	6.5	47.3	0.14	100	1.0
30% Salt out	5.8	23.0	0.25	89.2	1.8
60% Salt out	4.6	10.0	0.46	70.8	3.3
Sephadex G-100	2.7	0.5	5.45	41.5	39.1
DEAE-Sepharose	1.3	0.1	12.26	19.7	80.4

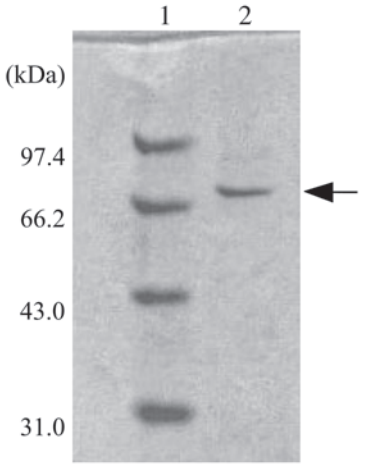


Fig. 1. SDS-PAGE analysis of β-glucosidase. Lane 1, protein markers; lane 2, sample of the enzyme from the final purification step. The arrow indicates the band of the β-glucosidase at 71 kDa.

with 0.75-mm spacers (Bio-Rad). Analysis was performed with a 5% acylamide stacking gel (pH 6.8) and 10% separating gel (pH 8.8) according to the method of Laemmli *(24)*, using the following M_r markers: rabbit phosphorylase B, 97.4 kDa; BSA, 66.2 kDa; rabbit actin, 43.0 kDa; bovine carbonic anhydrase, 31.0 kDa. The gel was stained with Coomassie Brilliant Blue R-250.

Results

Purification of Honeybee β-Glucosidase

Table 1 presents the effects of each purification step on total protein, activity, specific activity, and fold of purification from the honeybee. Through ammonium sulfate precipitation, Sephadex G-100 filtration, and DEAE-Sepharose chromatography, β-glucosidase from honey was purified approx 80-fold, and the final yield was approx 20%.

A homogeneous protein was obtained according to the criteria of SDS-PAGE (Fig. 1). The molecular weight of a single polypeptide chain was estimated to be about 71,000.

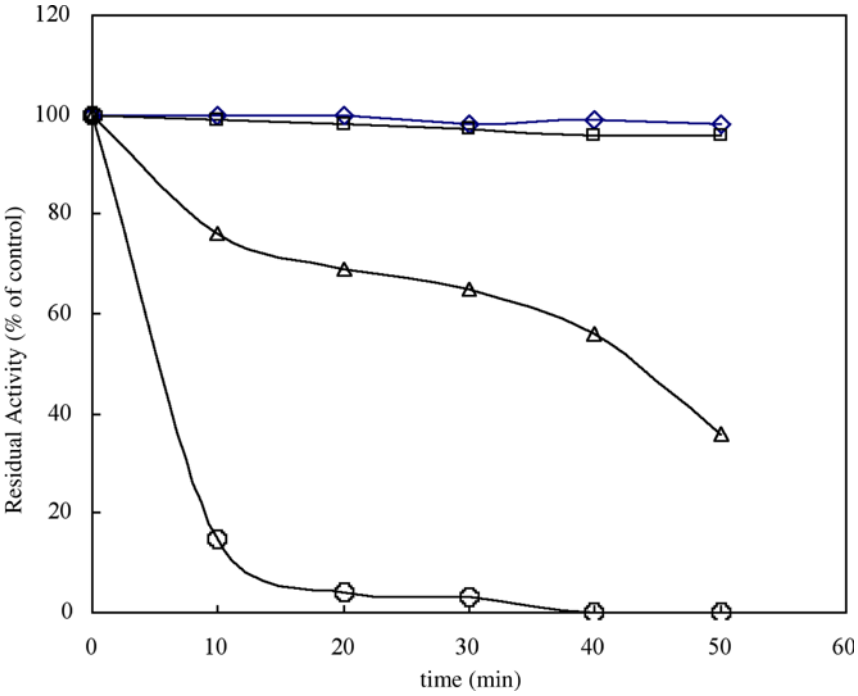


Fig. 2. Effect of temperature on β-glucosidase activity by standard assay using β-PNPG as substrate: (◇) 30°C; (□) 40°C; (△) 50°C; (○) 60°C.

Enzyme Stability

The purified enzyme in sodium phosphate buffer (0.02 *M*, pH 7.5) retained 80 and 30% of its original activity after 20 d of storage at –20 and 4°C, respectively. Less than 5% of the original activity remained after storage at room temperature for 3 wk.

To investigate enzyme stability, the enzyme solution in sodium phosphate buffer (0.02 *M*, pH 7.5) was incubated at various temperatures for 10, 20, 30, 40, and 50 min, and residual enzyme activity was determined by the standard assay using β-PNPG as a substrate (Fig. 2). The enzyme was found to be stable from 30 to 40°C. When incubated for 10 min at 60°C, however, 83% of the original activity was lost. When incubated for 30 min at 60°C, almost no β-glucosidase activity was detectable.

The effect of pH on stability of β-glucosidase was examined by diluting 20 µL of purified β-glucosidase with buffer, ranging in pH from 4.0 to 8.0 (at 1.0-unit increments), to bring the final volume up to 100 µL. The following buffer solutions were used: 0.02 *M* glycine-HCl for pH 3.0, 0.02 *M* sodium acetate buffer for pH 4.0–6.0, 0.02 *M* sodium phosphate buffer for pH 6.0–8.0, and 0.05 *M* glycine-NaOH for pH 8.0–10.0. The resulting solutions were incubated at 37°C for 20 min. Residual enzyme activity was determined with β-PNPG following standard assay conditions; the results are shown in Fig. 3. The pH stability range of β-glucosidase was

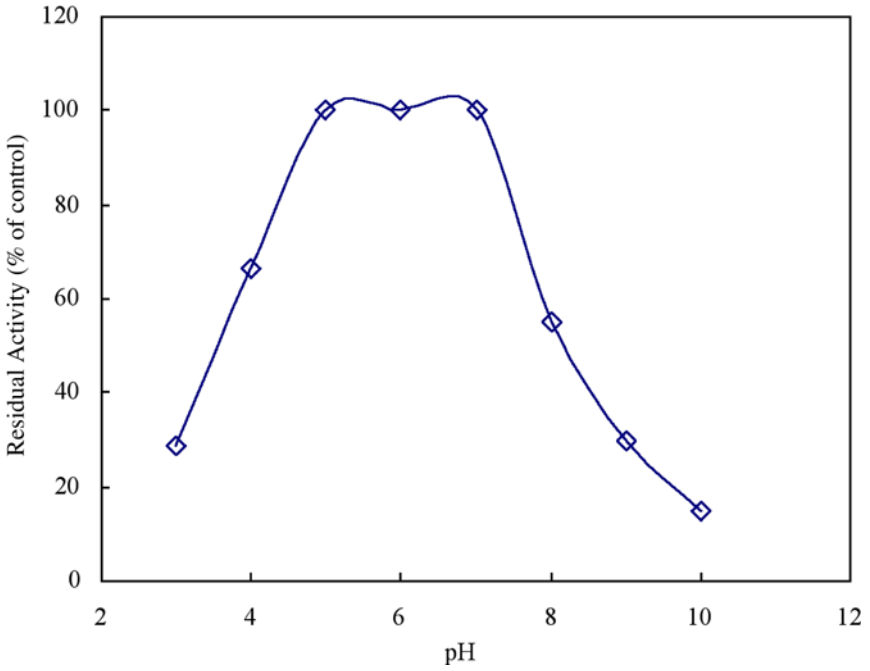


Fig. 3. Effect of pH on β-glucosidase activity by standard assay using β-PNPG as substrate.

4.0–8.0; the optimum pH for β-glucosidase was 5.0–7.5. β-Glucosidase activity decreased dramatically above pH 8.0 or below pH 4.0.

Effect of Validamycin A on Hydrolysis

The effect of validamycin A on hydrolysis was studied at different concentrations [*S*] (0.222, 0.334, 0.667, 1.0, 2.0, 3.34, 5.53, 6.67, 8.0, 8.6 m*M*). The reaction mixtures of different tubes consisting of 800 µL of sodium phosphate buffer (0.02 *M*, pH 7.5) and 50 µL of enzyme solution were preincubated at 37°C for 3 min. The reactions were started by adding different volumes of the validamycin A solution and distilled water to a total volume of 1.0 mL, then incubated at 37°C for 1 min. The validoxylamine A concentration was very low after reaction for 1 min, and the effect of validoxylamine A on β-glucosidase activity was negligible. The reaction was stopped by adding 0.1 mL of 1.0 *M* Na_2CO_3 and the amount of validoxylamine A released was determined. Figure 4A shows the effect of validamycin A on β-glucosidase-catalyzed hydrolysis. When the validamycin A concentration was <2.0 m*M*, with increasing validamycin A concentration, the initial rate (v) of validoxylamine A production increased. When validamycin A concentration was >2.0 m*M*, a trend toward a decrease in the initial rate was observed. It is evident that validamycin A has an inhibitory effect on this enzyme. The plot of $1/v$ vs $1/[S]$ (Fig. 4B) was used to obtain the K_m value (K_m = 0.79 m*M*). The plot of $1/v$ vs [*S*] (Fig. 4C) was used

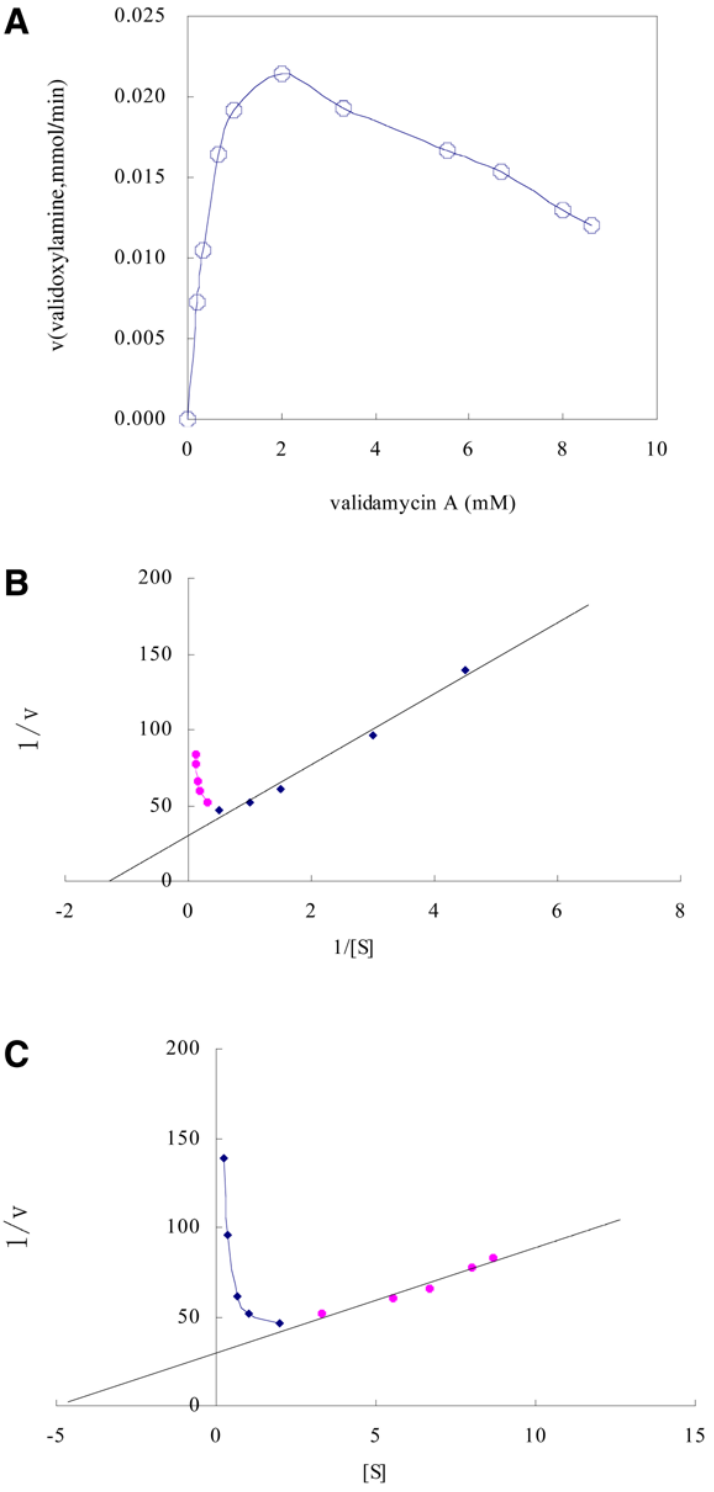


Fig. 4. Effect of validamycin A concentration on β-glucosidase hydrolysis of validamycin A. **(A)** Plot of validamycin A concentration vs initial rate (v) of validoxylamine A release; **(B)** Lineweaver-Burk plot of $1/v$ vs $1/[S]$ used to obtain K_m (K_m = 0.79 m*M*); **(C)** plot of $1/v$ vs $[S]$ used to obtain K_{is} (K_{is} = 5.01 m*M*).

to obtain the K_{is} value (the inhibitor constant that the substrate acts as an inhibitor; K_{is} = 5.01 m*M*).

Effect of Validoxylamine A on Validamycin A Hydrolysis

The effect of validoxylamine A on validamycin A hydrolysis was studied at different validoxylamine A concentrations: 0, 1.0, 2.0, 3.0, 4.0 m*M*. Validamycin A was used as a substrate by varying concentrations of 0.222, 0.667, and 2.0 m*M*. The reaction mixtures of different tubes consisting of 800 µL of sodium phosphate buffer (0.02 *M*, pH 7.5) and 50 µL of enzyme solution were preincubated at 37°C for 3 min. The reactions were started by concurrently adding different volumes of validamycin A solution, validoxylamine A solution, and distilled water to a total volume of 1.0 mL, then incubated at 37°C for 1 min. At that time, the substrate inhibitory effect was negligible. The reactions were stopped by adding 0.1 mL of 1.0 *M* Na_2CO_3, and the amount of validoxylamine A released was determined. Plots of validoxylamine A concentration vs initial rate (v) of validoxylamine A (Fig. 5A) show that validoxylamine A acts as an inhibitor. The Dixon plot (I vs $1/v$; Fig. 5B) was used to investigate the effect of validoxylamine A as an inhibitor. The K_{ip} value (the inhibition constant that the product acts as an inhibitor) was 1.32 m*M*.

Effect of Glucose on Validamycin A Hydrolysis

The effect of glucose on validamycin A hydrolysis was studied at different concentrations: of 0, 1.0, 2.0, 3.0, and 4.0 m*M*. Validamycin A was used as a substrate by varying concentrations of 0.222, 0.667, and 2.0 m*M*. The reaction mixtures of different tubes consisting of 800 µL of sodium phosphate buffer (0.02 *M*, pH 7.5) and 50 µL of enzyme solution were preincubated at 37°C for 3 min. The reactions were started by concurrently adding different volumes of validamycin A solution, glucose solution, and distilled water to a total volume of 1.0 mL. The reactions were stopped by adding 0.1 mL of 1.0 *M* Na_2CO_3, and the amount of validoxylamine A released was determined.

Figure 6 shows the effect of glucose on β-glucosidase-catalyzed validamycin A. With an increase in glucose concentration, the amount of validoxylamine A produced was essentially unchanged. These results suggest that glucose does not affect the hydrolysis reaction equilibrium.

Production of Validoxylamine A by β-Glucosidase Hydrolysis

The production of validoxylamine A by hydrolysis with β-glucosidase was examined at various validamycin A concentrations (0.667, 6.67 m*M*). The reactions were carried out at 37°C in sodium phosphate buffer (0.02 *M*, pH 7.5). The amount of validoxylamine A in the reaction systems was determined every 10 min.

Figure 7 shows the time course of validoxylamine A production by hydrolysis of validamycin A. In the first 10 min, the amount of validoxy-

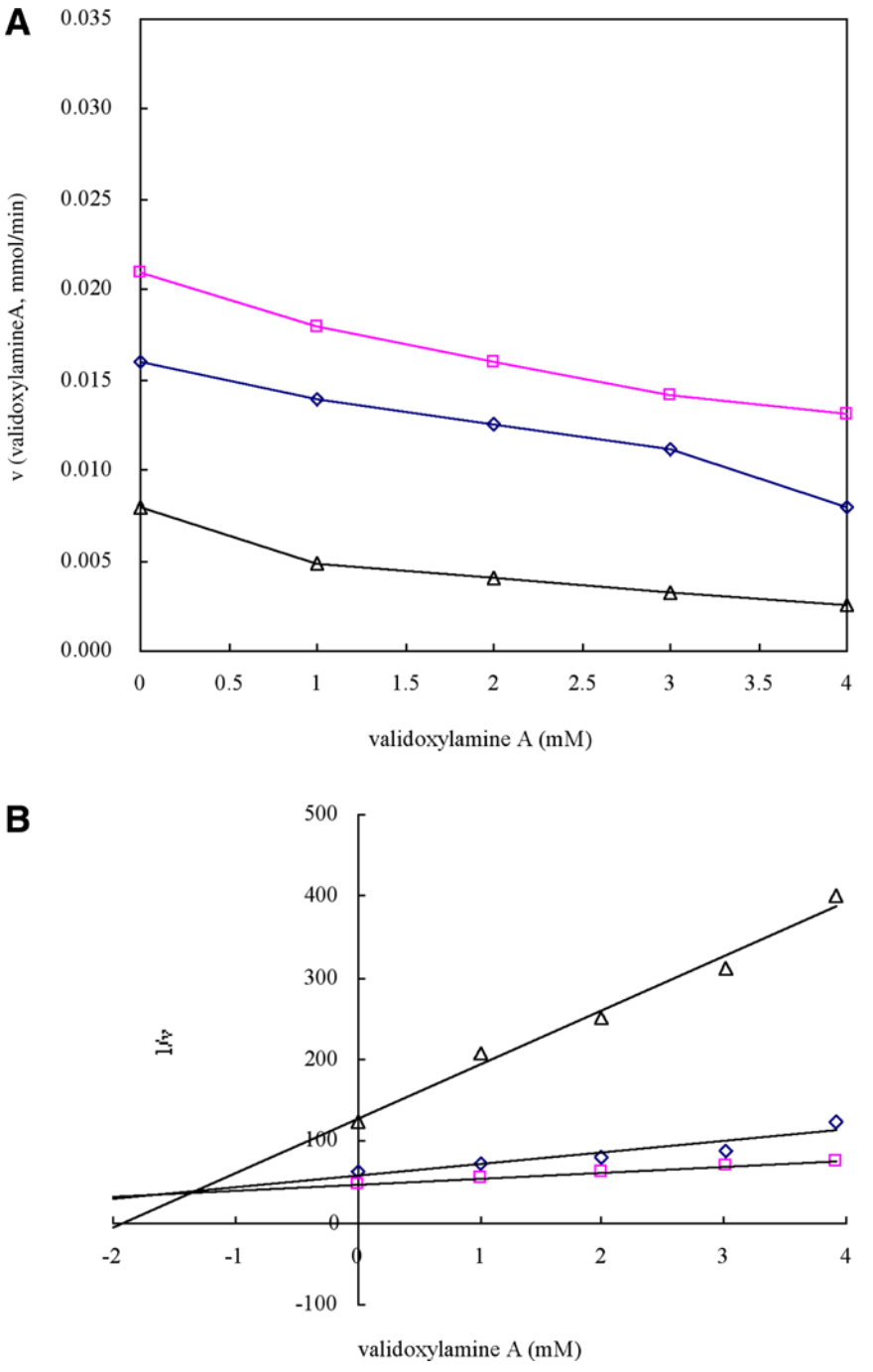


Fig. 5. Effect of validoxylamine A concentration on validamycin A hydrolysis at validamycin A concentrations of (△) 0.222, (◇) 0.667, and (□) 2.0 m*M*. **(A)** The plot of validoxylamine A concentration vs initial rate (v) of validoxylamine A release is shown. **(B)** The Dixon plot of validoxylamine A concentration vs $1/v$ was used to investigate the effect of validoxylamine A as an inhibitor.

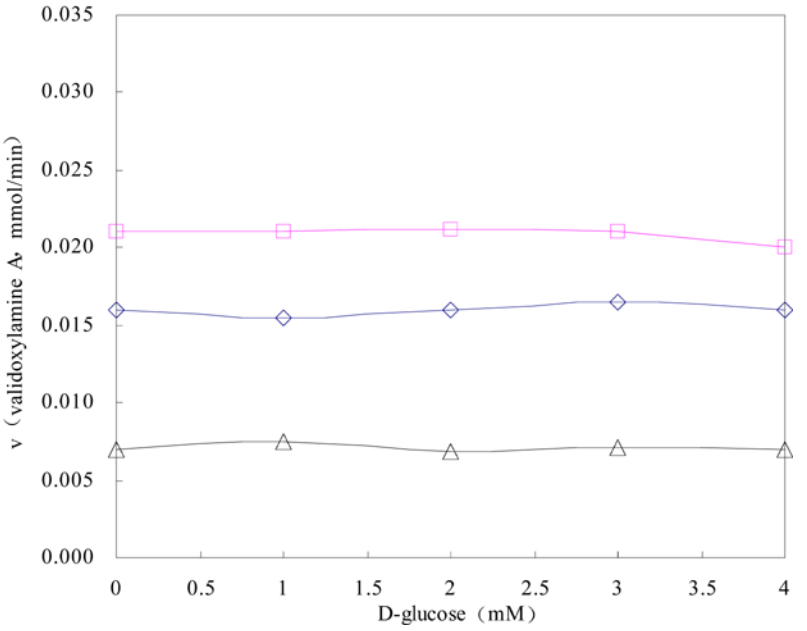


Fig. 6. Effect of glucose concentration on validamycin A hydrolysis at validamycin A concentrations of (△) 0.222, (◇) 0.667, and (□) 2.0 m*M*.

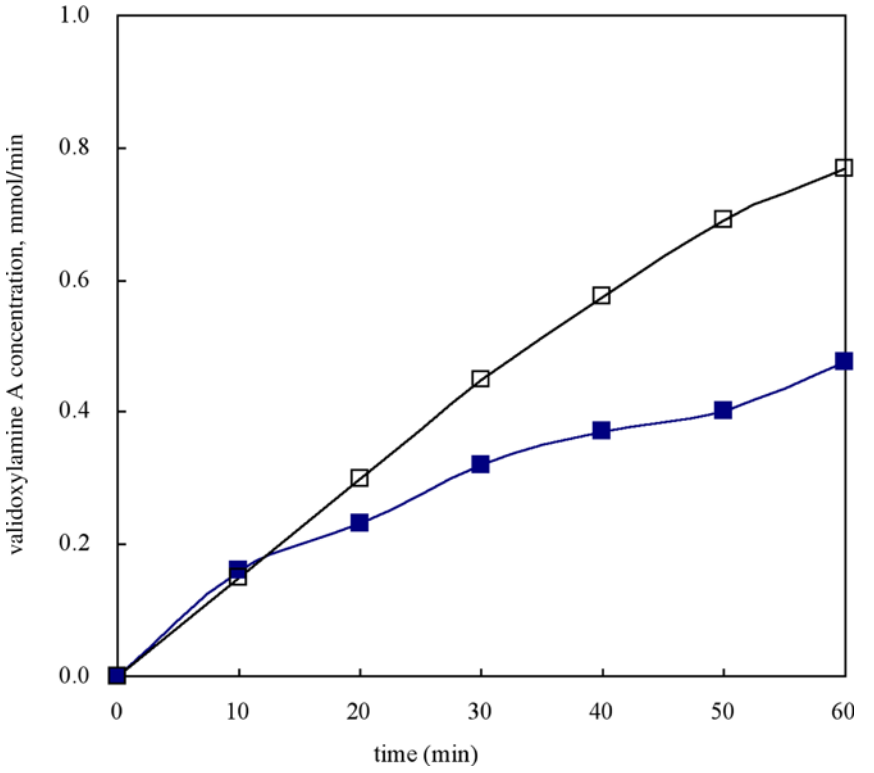


Fig. 7. Time course of validoxylamine A production by hydrolysis of validamycin A with β-glucosidase at validamycin A concentrations of (■) 0.667 and (□) 6.67 m*M*.

lamine A released was almost the same although the substrate concentrations were different. However, after 1 h of reaction, the conversion rate was 71.2% at the lower substrate concentration of 0.667 m*M*, and the conversion rate was only 11.5% as the substrate concentration was increased to 6.67 m*M*.

After enzymatic conversion, the reaction broth contained validoxylamine A, validamycin A, glucose, and the enzyme. Validoxylamine A is a weakly basic compound with a pK_a of 6.0 and exists with a positive ion state at acidic condition, so we adjusted the pH of the reaction broth below 6.0 with 2.0 *M* HCl, then separated validoxylamine A with an ion-exchange resin of Amberlite IR-120 (H^+) and eluted with 1.0 *M* aqueous ammonia. The eluate was concentrated. Afterward, a column of Dowex 1 × 2 (OH^-) was applied to further purify the validoxylamine A, and the active eluate was concentrated. Finally, ethanol was added to the concentrated liquid to give a white powder of validoxylamine A.

Discussion

β-Glucosidase (EC 3.2.1.21; β-glucosidase glucohydrolase) comprises a heterogeneous group of enzymes able to cleave the β-glucosidic linkages of di- and/or oligosaccharides, or other glucose conjugates. β-Glucosidases are widely distributed in the living world and play pivotal roles in many biologic process, such as degradation of cellulosic biomasses, hydrolysis of glycolipids, cyanogenesis, and modification of secondary metabolites *(25)*. They are found widely in fungi *(26)*, yeast *(27)*, bacteria *(28)*, and higher plants *(29)*, and many β-glucosidases have been purified and characterized *(20,30)*. β-Glucosidases have been found in insects of various orders and families, with the majority isolated from the intestinal tract *(31)*. β-Glucosidase activity was detected in the honeybee in 1986 *(32)*, and a number of its physicochemical and kinetic properties have been determined *(23)*. We isolated honeybee β-Glucosidase, which can catalyze the hydrolysis of validamycin A to produce validoxylamine A by cleaving the β-Glucosidic linkage. It was determined that the substrate (validamycin A) and the product (validoxylamine A) could inhibit β-Glucosidase activity during the production of validoxylamine A by hydrolysis of validamycin A with β-Glucosidase. Product (validoxylamine A) inhibition was competitive. Based on the role of the substrate and products investigated in these experiments, the following equations are proposed:

$$\begin{array}{ccccc} & & \overset{K_m}{} & \overset{k_2}{} & \\ E + S & \rightleftharpoons & ES & \longrightarrow & E + P_1 + P_2 \\ & & + & & + \\ & & S & & E \\ & & K_{is} \Big\uparrow\Big\downarrow & & K_{ip} \Big\uparrow\Big\downarrow \\ & & ESS & & EP_1 \end{array}$$

in which P_1 is the product validoxylamine A; P_2 is the product D-glucose; K_m is the Michaelis-Menten constant; k_2 is the rate constant for the formation

of the reaction products; K_{is} is the inhibition constant of the substrate, indicating the dissociation constant of the enzyme-substrate-substrate (*ESS*); and K_{ip} is the inhibition constant of production P_1, indicating the dissociation constant of the enzyme-substrate-P_1 (EP_1). The K_{is} value was 1.32 m*M* and it was approx 1.67 times larger than the K_m value (0.79 m*M*), suggesting that the substrate validamycin A had about 1.67 times greater affinity for the active site of the enzyme than for the *ES* complex. The K_{ip} value was 5.01 m*M* and it was approx 6.34 times larger than the K_m value, suggesting that validamycin A had about 6.34 times greater affinity for the active site of the enzyme than validoxylamine A. Substrate and production inhibition results in a lower conversion rate. To improve the production rate, the substrate concentration should not be high, and the product validoxylamine A should be removed to shift the reaction equilibrium in the desired direction.

Acknowledgments

This work was supported by the Fund of the Major Basic Research Development Program of China (2003CB716005), the National Natural Science Foundation of China (20176055), and the Natural Science Foundation of Zhejiang Province (ZB0106).

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