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Inactivation kinetics and conformation change of *Hypocrea orientalis* β-glucosidase with guanidine hydrochloride

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The relationship between unfolding and inactivation of *Hypocrea orientalis* β-glucosidase has been investigated for the first time. The secretion of β-glucosidase from *H. orientalis* is induced by raw cassava residues. The enzyme was 75 kD without glycosylation. Guanidine hydrochloride (GuHCl) could reversibly inactivate the enzyme with an estimated IC_{50} value of 0.4 M. The inactivation kinetics model by GuHCl has been established and the microscopic inactivation rate constants are determined. The values of forward inactivation rate constants of free enzyme are found to be larger than that of substrate–enzyme complex suggesting the enzyme could be protected by substrate during denaturation. Conformational change of the enzyme during denaturation is observed as the intrinsic fluorescence emission peaks appeared red-shift (334–354 nm) with intensity decreased following increase of GuHCl concentrations. Inactivation extent is found to be greater than conformation change of the whole enzyme, indicating that the active site of *H. orientalis* β-glucosidase might be a more flexible region than the whole enzyme.

[**Key words:** β-Glucosidase; Inactivation kinetics; Conformational change; Guanidine; *Hypocrea orientalis*]

Worldwide escalating energy demands and climate degeneration make renewable and green energy resources gain increasing attention. Cellulosic biomass, mainly consists of cellulose and hemicellulose, is thought to be clean for environment and adequate for production of bioethanol by fermentation (1,2). Cellulose can be enzymatically hydrolyzed into glucose by cellulases through breakdown of O-β-(1, 4) linkage, in which β-glucosidase is responsible to digest cello-oligosaccharides or cellobiose into glucose to accelerate cellulose hydrolysis (3,4).

To date, efficient cellulases are mainly from fungi. Fungal β-glucosidases in CAZy database are mainly divided into two glycoside hydrolysis families, GH1 and GH3. GH3 β-glucosidases are characterized as retaining enzymes with glutamates as conserved key residues for catalysis (5,6). Structure of *Hypocrea jecorina* GH3 β-glucosidase (Cel3A) presented that the flexibility of active site for catalysis can be promoted by the two glycine residues in that site, thus the catalytic specificity and activity are regulated (7,8). It has also been reported that conformation of active site of a β-glucosidase from *H. jecorina* can change for adaption to substrate hydrolysis by pH-induced ionization of amino residues outside of immediate catalytic cleft (9). Moreover, structures of some cellulases present tadpole-like shape, while the extending conformation of the linker in the enzyme molecule can render flexibility for substrate binding and coordinate C- and N- terminal domains work for catalysis (10–12). Enzymes, such as *Aspergillus glaucus* endoglucanase (13) and *Ampullarium crossean* β-glucosidase (14), have been found that unfolding of active sites induced by guanidine hydrochloride (GuHCl) or urea is faster than that of the whole enzyme during denaturation. Accordingly, these investigations point out that conformational transform tightly is critical for activity regulation of β-glucosidase and active sites are more flexible than the whole enzyme (15). On the other hand, GuHCl and urea are found to inhibit the activity of *Trichoderma reesei* cellobiohydrolase competitively (16). Interestingly, these two denaturants, at low concentrations, could enhance the activity of *Aspergillus* endoglucanase (EG) with the increase of flexibility of the enzyme (17). To our best knowledge, however, comparison between the activity change and unfolding of fungal β-glucosidases are still lacking of report.

In previous study, we screened a cellulolytic strain, *Hypocrea orientalis* EU7-22 and found it could secrete high activity cellulase (18). The relationship between conformational change and activity regulation of this β-glucosidase has not been investigated yet. The aim of this paper is to research the inactivation kinetics of the β-glucosidase in GuHCl solution and its conformational change during denaturation.

MATERIALS AND METHODS

Materials and reagents Homogeneous β-glucosidase was isolated from the *H. orientalis* EU7-22 fermentation liquid sequentially through ammonium sulfate precipitation, Sephacryl S200 filtration, DEAE chromatography and Superdex S200 filtration. Guanidine hydrochloride (GuHCl) and pNPG (*p*-nitrophenyl-β-D-glucopyranoside) were from Sigma (St. Louis, MO, USA) and other chemicals were local products of analytical grade. Untreated cassava residues were local products whose component was analyzed before (18). Other carbon resources, pretreated

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wheat straw and sugarcane bagasse were local products in Fujian Province, whose composition have been determined before (19). The carboxymethylcellulose sodium (CMCNa) and glucose was purchased from Sigma.

Strain culture conditions and enzyme assay *H. orientalis* EU7-22 was isolated and stored in Xiamen University (20). The medium contained, per 100 mL, 0.14 g of ammonia sulfate, 0.02 g of urea, 0.2 g of monopotassium phosphate, 0.03 g of lime chloride, 0.03 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of tryptone, 2 mL of Tween 80, 0.14 mg of $ZnSO_4$, 0.16 mg of $MnSO_4 \cdot H_2O$ and 0.5 mg of $FeSO_4 \cdot 7H_2O$ (MS medium). Cellulase production was induced by raw cassava residues run in flask with rotatory shaking at 37°C for several days.

The β-glucosidase was purified through ammonium sulfate precipitation, DEAE chromatography and Sephacryl S200 filtration. The homogeneity of the purified enzyme was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE). Periodic acid-schiff (PAS) staining was used for the detection of glycolytion of the purified protein after SDSPAGE (21). The amino acid sequence of the enzyme was determined by LC-MS/MS method according to Peng et al. (22) with Eksigent nanoLC 425 chromatography system and TripleTOF 5600+ mass spectrometer from ABSciex. To measure the activity of β-glucosidase, 40 μL of enzyme (0.03 μg) was added into 960 μL of substrate solution containing 0.16 mM pNPG and 100 mM NaAc buffer (pH 5.0). The reaction was run in a 60°C water constant bath for 15 min and stopped by adding 2 mL of 0.5 M NaOH, the adsorption at 405 nm of the reaction solution was measured to quantify liberated *p*-nitrophenyl using a standard curve. The CMCNa hydrolase activity (CMCase) was determined by incubating 50 μL of the enzyme with substrate solution containing 0.25% CMC-Na and 50 mM NaAc buffer (pH 5.0) at 50 °C for 15 min and the released reducing sugars was quantified by 3, 5-dinitrosalicylic (DNS) reagent method (19).

Enzyme denaturation in guanidine hydrochloride solutions Enzyme denaturation was carried out at 4°C with different concentrations of guanidine hydrochloride (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) in 0.01 M Tris−HCl buffer (pH 7.5) for 24 h. Denatured enzyme solutions were used for activity assay and measurement of fluorescence spectra. Fluorescence spectrum was made with a Hitachi 850 spectrofluorometer. The excitation wavelength was 280 nm.

Determination of inactivation rate constants For determination of *H. orientalis* β-glucosidase activity under different concentrations of GuHCl, 40 μL of enzyme solution was added to 0.96 mL of substrate solution containing different concentrations of GuHCl (0, 0.1, 0.2, 0.4 and 0.6 M) to initiate the reaction for following the reaction progress.

The inactivation kinetics by GuHCl was analyzed using the substrate-reaction method as detailed below (15). A reversible enzymatic reaction can be described in Fig. 3A. As is usual for the case that substrate concentration is larger sufficiently than enzyme concentration ([S] >> [E_o]), the product formation can be written as follows:

$$[P]_t = \frac{vk_{-0}}{A} \cdot t + \frac{v}{A^2}(A - k_{-0})\left(1 - e^{-At}\right) \quad (1)$$

where $[P]_t$ is the concentration of the product formed at reaction time t, A is the apparent rate constant of inactivation; k_{-0} is the rate constant of reverse inactivation of free enzyme (Fig. 3A) and v is the initial rate of reaction in the absence of GuHCl,

$$A = \frac{k_{+0} \cdot K_m + k_{+0}[S]}{K_m + [S]} + k_{-0} \quad (2)$$

$$v = \frac{V_m \cdot [S]}{K_m + [S]} \quad (3)$$

where k_{+0} is the rate constant of forward inactivation of free enzyme (Fig. 3A); K_m is the Michaelis constant of β-glucosidase and [S] is the concentration of the substrate; When t is sufficiently large, the curves become straight lines and the product concentration is written as $[P]_{calc}$:

$$[P]_{calc} = \frac{vk_{-0}}{A} \cdot t + \frac{v}{A^2}(A - k_{-0}) \quad (4)$$

Combining Eqs. 1 and 4 yields:

$$[P]_{calc} - [P]_t = \frac{v}{A^2}(A - k_{-0}) \cdot e^{-At} \quad (5)$$

$$\ln([P]_{calc} - [P]_t) = -A \cdot t + \text{constant}. \quad (6)$$

where $[P]_{calc}$ is the product concentration to be expected from the straight-line portions of the curves as calculated from Eq. 4, and $[P]_t$ is the product concentration actually observed at time t. Plots of $\ln([P]_{calc} - [P]_t)$ versus t give a series of straight lines at different concentrations of GuHCl with slopes of $-A$. From Eq. 4, a plot of $[P]_{calc}$ against time, t, gives a straight line with a slope of vk_{-0}/A. From the slope of the straight line, k_{-0} can be obtained.

Combining Eqs. 2 and 3 gives:

$$\frac{A}{v} = \frac{K_m}{V_m}(k_{+0} + k_{-0}) \cdot \frac{1}{[S]} + \frac{k'_{+0} + k_{-0}}{V_m} \quad (7)$$

where V_m is the maximum reaction rate of the enzyme; k'_{+0} is the inactivation rate constants of enzyme−substrate complex. A plot of A/v versus $1/[S]$ gives a straight line, whose slope and intercept are $K_m \cdot (k_{+0} + k_{-0})/V_{max}$ and $(k'_{+0} + k_{-0})/V_m$, respectively. Then the rate constants k_{+0} and k'_{+0} can be obtained due to K_m and V_m values of the enzyme are determined before.

Computational docking of *H. orientalis* β-glucosidase and pNPG Due to the amino acid sequence of *H. orientalis* β-glucosidase is 99% identical with *H. jecorina* Cel3A according to the LC-MS/MS sequencing result, the tertiary structure of *H. orientalis* β-glucosidase built by I-TASSER is also 99% identical with *jecorina* Cel3A as our previous modeling. With the Cel3A PDB structure and pNPG molecule, we used AutoDock Vina (23) for in silico docking and the affinity energy was predicted by the program.

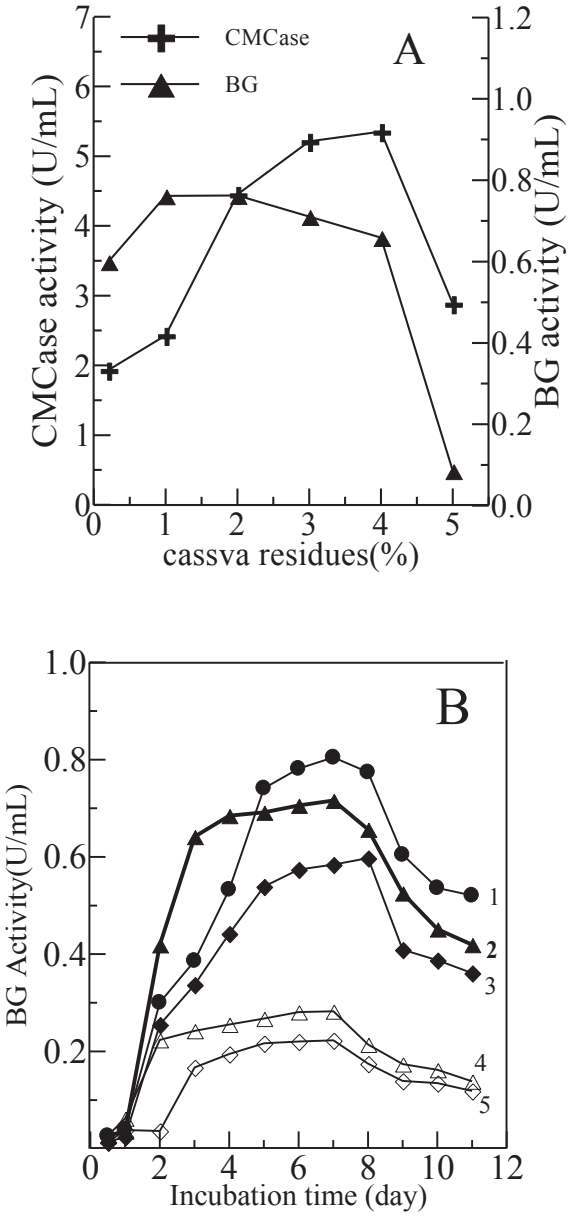

FIG. 1. Cellulase production of *H. orientalis* using different carbon source. (A) Time course of β-glucosidase production by *H. orientalis* EU7-22 using different carbon source. The carbon source (mass ratio were all 3%) for curve 1–5 were respectively wheat straw, raw cassava residues, sugarcane bagasse, CMCNa and glucose. (B) Effect of cassava residue concentrations on the production of *H. orientalis* cellulase. The data were the average of three independent batches.

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