Heat inactivation kinetics of *Hypocrea orientalis* β -glucosidase with enhanced thermal stability by glucose

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Abstract

Thermal inactivation kinetics of *Hypocrea orientalis* β -glucosidase and effect of glucose on thermostability of the enzyme have been determined in this paper. Kinetic studies showed that the thermal inactivation was irreversible and first-order reaction. The microscopic rate constants for inactivation of free enzyme and substrate-enzyme complex were both determined, which suggested that substrates can protect β -glucosidase against thermal deactivation effectively. On the other hand, glucose was found to protect β -glucosidase from heat inactivation to remain almost whole activity below 70 °C at 20 mM concentration, whereas the apparent inactivation rate of BG decreased to be $0.3 \times 10^{-3} \text{ sec}^{-1}$ in the presence of 5 mM glucose, smaller than that of sugar-free enzyme ($1.91 \times 10^{-3} \text{ sec}^{-1}$). The intrinsic fluorescence spectra results showed that glucose also had stabilising effect on the conformation of BG against thermal denaturation. Docking simulation depicted the interaction mode between glucose and active residues of the enzyme to produce stabilising effect.

Keywords: β -glucosidase, thermoinactivation kinetics, glucose, conformation stabilization

1. Introduction

Cellulase is very critical for the second generation technique of bioethanol production which hydrolyzes lignocelluloses for synergic saccharification and fermentation of ethanol [1]. There are various sources of cellulytic fungi, especially *Hypocrea*, *Aspergillus* and *Penicillium*, explored to prepare cellulase enzymes for industrial production of bioethanol. During cellulase hydrolysis, β -glucosidase hydrolyzes cellobiose or oligosaccharide to produce adequate amount of glucose. Usually, most cellulases output the most glucose at around 55 °C. However, β -glucosidase, one of active components of cellulase, tends to be unstable due to heat inactivation during that hydrolysis [3, 4]. Investigations found that most fungal β -glucosidase can retain whole activity below 50 °C for one hour but lost activity dramatically above 60 °C, limiting the further improvement of β -glucosidase activity for enzymatic hydrolysis of oligosaccharides [5].

Directed evolution with multiple mutagenesis and immobilization techniques have been widely used to produce better thermal-resistant β -glucosidase [6]. Molecular structure of β -glucosidase is closely related to thermostability of the enzyme. Investigators modified the enzyme to find that non-covalent attachment of polysaccharides can effectively reduce thermal inactivation rate of β -glucosidase [7, 8]. Kinetic studies found that *Humicola* or *Trichoderma* β -glucosidase can be stimulated by glucose or xylose at low concentration by competitively binding to catalytic site and modulator binding site, which allosterically interact with each other to regulate activity of β -glucosidase [9, 10]. Meanwhile, glucose-6-phosphate has been proved to be able to improve thermostability of *Bacillus* β -glucosidase with through formation of BGLA-G6P complex [11]. Moreover, scientists demonstrated that glycosyl-enzyme intermediate, whose catalytic domain occupied by glycosyl

“slow-substrate”, not only can better resist heat denaturation than the enzymes in free forms but also is more stable against thermolysin-catalyzed proteolysis due to evaluation of unfolding temperature [12]. On the other hand, stabilizing effects of glucose or derivatives generally require structural specificity with low free energy of binding, meaning that hydroxyls on pyranose are the dominants for the affinity of ligands to β -glucosidase [13, 14].

Chen reported that presence of substrate could stabilize the enzyme against thermal inactivation efficiently, according to thermal inactivation kinetics studies of acid phosphatase [15]. Glucose can inhibit β -glucosidase competitively, indicating that it is able to insert the active-site as substrates do to protect the catalytic pocket against thermal denaturation. Inhibition mechanism of glucose on *Hypocrea* β -glucosidase and structure of glucose-enzyme complex have both been clarified [4, 6], but the effect of glucose on the thermal stability of this β -glucosidase has not been reported yet, although that of other sugars such as sucrose and trehalose has been described [16].

2. Materials and Methods

2.1 Reagents

The purified β -glucosidase was prepared as previous procedures [17]. Cellobiose, salicin glucose and *p*NPG were all purchased from Sigma Chemical Company (USA). All other chemicals were local products of analytical grade.

*2.2 Thermostability assay of *Hypocrea orientalis* β -glucosidase*

Thermostability of the β -glucosidase was determined as previous method [17]. In determination of the effect of glucose on thermostability of β -glucosidase, various concentrations of glucose were added into the enzyme solution and incubated at different temperatures for different time. Glucose was removed by ultra filtration

before β -glucosidase activity was determined.

2.3 Determination of microscopic thermal inactivation rate constants

The method described by Tsou [18] was used to measure the heat inactivation kinetics of *H. orientalis* β -glucosidase. The time courses of hydrolysis of pNPG by the enzyme at different temperatures (from 37 °C to 73 °C) were recorded by measuring the absorbance of 405 nm with DU800 as previous procedures [15]. The reaction scheme of the irreversible heat inactivation can be written as follow:

Where S, P, E and E' are the substrate, product, native and thermally denatured enzymes, respectively. ES is the enzyme-substrate complex. k_{+0} and k'_{+0} are the microscopic rate constants of the thermal inactivation of the free enzyme and of the enzyme-substrate complex, respectively. The product formation can be written as:

$$[P]_t = \frac{v}{A}(1 - e^{-At}) \quad (1)$$

$$A = (k_{+0}K_m + k'_{+0}[S])/(K_m + [S]) \quad (2)$$

Where $[P]_t$ is the concentration of the product formed at time t, A is the apparent rate constant of thermal inactivation, $[S]$ is the concentration of pNPG, v is the initial rate of native enzyme and K_m is the Michaelis constant. When irreversible reaction time is sufficiently long, the product concentration approaches a constant final value $[P]_{\infty}$,

$$[P]_{\infty} = \frac{v}{A} \quad (3)$$

Combining Eq. (2) and (3) yields:

$$\frac{1}{[P]_{\infty}} = \frac{k_{+0}K_m}{V_m} \cdot \frac{1}{[S]} + \frac{k'_{+0}}{V_m} \quad (4)$$

Since K_m is a known quantity, the value of $\frac{k_{+0}}{k'_{+0}}$ can be calculated from the

ratio of the slope to the intercept. Combining (1) and (3) yields:

$$\ln\left(\frac{[P]_{\infty} - [P]_t}{[P]_{\infty}}\right) = -At \quad (5)$$

Which shows that the plot of $\ln\left(\frac{[P]_{\infty} - [P]_t}{[P]_{\infty}}\right)$ versus time is a straight line with

slope of $-A$ and the apparent inactivation rate A can be obtained. Therefore, since

$\frac{k_{+0}}{k'_{+0}}$ is already known, the microscopic inactivation rate constants (k_{+0} and k'_{+0}) can be

obtained according to equation (2).

2.4 Fluorescence emission spectra measurement

The β -glucosidase solution (10 $\mu\text{g/mL}$) in the presence (or absence) of 20 mM glucose was incubated at different temperatures for 40 minutes and withdrawn to be cooled on ice for fluorescence scan as the method described earlier [19].

2.5 Docking simulation of β -glucosidase and glucose

Molecular docking of this β -glucosidase was carried out on MOE software. The gene of the enzyme has been cloned and identified before (GenBank: JQ904600.1). Homogenous modeling was realized by I-TASSER service (<http://zhanglab.ccmb.med.umich.edu/>). Ligands (glucose and cellobiose) were obtained from PDB database. Before docking, energy minimize and adding hydrogen of the protein and ligands were realized on MOE program. Parameters for docking were as follows: the refinement was set to Forcefield; the Retain of both the first and second scoring were set to 10; the MM/GBVI binding free energy scoring was used to rank the docking poses [20]. Other parameters were the default settings of the software.

Docked conformation with best energy scoring was selected to analyze the mode of the binding.

3. Results and Discussion

3.1 Thermal inactivation kinetics of *H. orientalis* β -glucosidase

Preliminary test showed that the β -glucosidase from *H. orientalis* was stable below 50 °C, but then its enzyme activity decreased sharply with temperature arising from 60 °C, and lost whole activity at 80 °C (**Fig. 1**), while the most optimum temperature for this enzyme to hydrolyze pNPG was 60-70 °C. The time courses of the catalytic hydrolysis below 50 °C retained linear for initial 30 min, meaning that the activity of β -glucosidase was stable at such temperature (**Fig. 2A**). At 63 °C, the highest amount of hydrolysis product was obtained but the reaction rate obviously occurred to reduce with the increase of reaction time because of thermal inactivation of the enzyme. Over 67 °C, the rate of pNPG hydrolysis decreased rapidly and the amount of product did not notably increase after 30 min for the reason that the enzyme was completely inactivated as the concentrations of product ([P]) reached a constant value finally. Semi logarithmic plots of the product concentrations versus time suggested the thermoinactivation process at different temperatures were monophasic first-order reaction (**Fig. 2B**) and the apparent inactivation rate constant A can be obtained from the slope of the plots (shown in **Table 1**).

Fig. 1

Fig. 2

3.2 Determination of microscopic thermoinactivation rate constants of β -glucosidase

Hydrolysis kinetics of pNPG with different concentrations (**Fig. 3A**) showed that inactivation of β -glucosidase completed after 60 min reaction at 67 °C and the product concentrations approached a constant value ($[P]_{\infty}$) when the reaction time were sufficiently long, which decreased with the reduction of substrate concentrations.

From the slope and intercept of the dual reciprocal plot of $[P]_{\infty}$ versus $[S]$ (**Fig. 3B**) the value of $\frac{k_{+0}}{k'_{+0}}$ can be obtained to calculate the microscopic thermoinactivation rate constants of free enzyme and enzyme-substrate complex, k_{+0} and k'_{+0} , respectively (**Table 1**). The microscopic thermal inactivation rate constants of either free enzyme or substrate-enzyme complex (k_{+0} and k'_{+0}) at 70 °C were much larger than that at 67 °C, respectively, and especially, the values of k'_{+0} were distinctly smaller than that of k_{+0} at such temperature, indicating that the presence of substrate has protective effect on β -glucosidase against thermal inactivation.

3.3 Effect of glucose on the thermal stability of *H. orientalis* β -glucosidase

Glucose and cellobiose are products of cellulose hydrolysis of cellulose and thermal inactivation of *H. orientalis* β -glucosidase incubated with glucose, cellobiose, mannose or salicin were investigated in detail. The residual activity of β -glucosidase, co-incubated with different concentrations of glucose, increased due to addition of glucose concentration. At high temperature below 70 °C, about half of native activity retained after incubation with 5 mM glucose, meanwhile 20 mM glucose can save almost all of β -glucosidase activity against heat deactivation (**Fig. 4A**).

Fig. 4

Results in **Fig. 4B** implied that increasing amount of glucose, cellobiose or salicin enhanced the thermal stability of β -glucosidase significantly within 20 mM, while the residual activity nearly remained whole activity after incubation with beyond 10 mM sugar and effects of glucose on thermal stability of β -glucosidase at various concentrations were similar with that of cellobiose and salicin. However, only about 30% activity remained even β -glucosidase was incubated with 30 mM mannose.

Determination of inactivation rate showed that activity of all enzymes decreased fast at initial inactivation phase but relatively slow after 20 min incubation. The

residual activity of β -glucosidase with glucose reduced in same velocity with that of the enzyme co-incubated with cellobiose or salicin. While the enzyme incubated with 5 mM mannose deactivated as fast as sugar-free enzymes (**Fig. 4C**). Semilogarithmic plots (**Fig. 4D**) presented that thermal inactivation of free enzyme followed first-order kinetics, and the inactivation rate k_1 was $1.91 \times 10^{-3} \text{ sec}^{-1}$ obtained from the slope of the line, while thermal inactivation of enzyme incubated with 5 mM glucose contained two phases, inactivation rate was faster at initial step and then much slower at later phase (in accordance with ash line in **Fig. 4D**), with first-order rate constant of $0.3 \times 10^{-3} \text{ sec}^{-1}$ (slope value of the ash line).

3.4 Differences of fluorescence emission spectra of heat treated enzymes

The intrinsic fluorescence emission spectra of *H. orientalis* β -glucosidase after exposure to different temperatures were measured to investigate conformational change of heat treated β -glucosidase and determine the effect of glucose on the enzyme conformation change after thermal deactivation. The results showed that emission peak of intrinsic fluorescence of native β -glucosidase occurred at 334 nm (**Fig. 5A**). Any change of intrinsic fluorescence peak of the enzyme was not observed after exposure to 40 °C for 30 min. Meanwhile, the fluorescence intensity decreased with temperature rising to 60 °C. When the enzyme exposed to 70 and 80 °C, the fluorescence peak occurred red shift with significant elevation of fluorescence intensity, suggesting that conformation of β -glucosidase was unable to recover after such heat treatment upon cooling and hyperthermia induced irreversible unfolding of β -glucosidase molecule to make Trp residues and Tyr residues totally exposed.

Fig. 5

Fluorescence emission spectra of β -glucosidase incubated at different temperatures with 20 mM glucose presented similar curves except above 70 °C (**Fig.**

5B). The spectrum of the enzyme incubated with glucose at 70 °C was same as that of 60 °C, implying conformation was stabilized by glucose at that temperature. Moreover, the emission peak of 80 °C treated enzyme with glucose occurred red shift less than that of sugar-free treated enzyme. The results showed that the presence of glucose has stabilising effect on conformation of β -glucosidase.

3.5 Molecular docking

Docking simulation was performed to illuminate the structural mechanism of the interaction between the enzyme and monosugar molecules. **Fig 6** presented overview of 3D structure of *H. orientalis* β -glucosidase. Docking pose of glucose and cellobiose in the catalytic site were shown in **Fig 7**. The docked conformation revealed that hydroxyls of β -D-glucose formed hydrogen bonds with amino residues of the enzyme in the catalytic site, including Trp₆₈, Val₇₄, Asp₉₂, Leu₁₄₁, Lys₁₈₉, His₁₉₀, Met₂₃₂, Tyr₂₃₅, Asp₂₆₇, Trp₂₆₈ and Ser₄₁₅ (**Fig 7b** and **d**). All these residues were involved in the hydrolysis of cellobiose by the enzyme which required 18 amino residues as the simulation result depicted (**Fig 7a** and **c**).

Fig. 6

Temperature is very important for efficiency of enzymatic saccharification of cellulose. Fungal β -glucosidases usually showed the best activity at around 55 °C (**Table 2**) to hydrolyze cellobiose or oligosaccharides during cellulase degradation of cellulose materials. This report indicated that *H. orientalis* β -glucosidase owned good thermal stability below 50 °C, which was adequate for industrial production with endoglucanase and exo-glucanase to degrade cellulosic residues [21]. Determination of microscopic thermal inactivation rate constants at 67 °C and 70 °C suggested that residual activity of *H. orientalis* β -glucosidase decreased mildly at 67 °C but much more rapidly at 70 °C. More importantly, the phenomenon that microscopic thermal

inactivation rate constant (k'_{+0}) of enzyme-substrate complex was much smaller than that of free β -glucosidase suggested that substrates could improve thermal resistance of β -glucosidase against inactivation, which is in accordance with that of *Scylla serrata* alkaline phosphatase and *Pieris rapae* NAGase [15, 19]. Cellulase could produce a large amount of cellobiose during cellulose hydrolysis, and this study proved that the reaction temperature can be furtherly raised to enhance the activity of β -glucosidase.

Table 2

Despite glucose is a competitive inhibitor of β -glucosidase, this study presented that thermal stability of β -glucosidase can be improved by glucose under high temperature. The protective effect of glucose for β -glucosidase on thermal stability was similar with that of cellobiose and salicin. This supports the conclusion that size exclusion effect of sugar not be useful for the stabilization of the active-site against thermal deactivation [22] and structural specificity is essential for combination with sugar to make the active center stable [8, 23], that's why mannose could not stabilize the enzyme against thermodenaturation. On the other hand, comparison between inactivation course and conformational change of heat treated β -glucosidase represented that inactivation occurs before the change of conformation of the enzyme as a whole, which supports that active-site has more flexibility in a limited region than the enzyme as a whole [24]. Importantly, the intrinsic fluorescence emission spectra results that heat treated enzyme in the presence of glucose occurred less red shift than that without glucose showed glucose contributed to conformational stabilization of the whole enzyme molecule against thermal denaturation.

Fig. 7

Structural analysis mentioned that one β -glucosidase molecule combined with

one glucose molecule in active center [25]. Glucose molecules could form hydrogen bonds to combine with cellulase in active center as glycoside substrate does. To clarify interaction mode of glucose and β -glucosidase, MOE 2010 program was used to simulate the molecular docking for better understanding of the stabilising effect of glucose. The selected docked conformation owned the best free energy of binding and was closed to a *Hypocrea* β -glucosidase published in PDB database [25] with similar values of docking potential energy (data not listed), indicating our simulation result was reasonable. The model exhibited the 11 active amino residues in catalytic pocket which were responsible for glucose to protect the enzyme from thermo-denaturation were totally overlapped by the catalytic section for cellobiose hydrolysis (**Fig 7c and 7d**). And it can be seen that Lys₁₈₉ connected synchronously to hydroxyls of second position and third position on glucose molecule, which may be the reason why D-mannose could be effective like glucose. That simulation results provided structural basis for stabilising effect of glucose on the enzyme, which is hydrogen bonds between the glucose and the active residues (from Trp₆₈ to Ser₄₁₅, shown in **Fig 7d**) formed in the mode could make the conformation of the β -glucosidase stable. Tsou described that conformational flexibility of active-site leads enzymatic activity to lose in prior to denaturation of enzyme molecule [18], and it is conceivable that stabilization of the flexible amino residues, shown in the catalytic site, could contribute to keep the whole conformation of the β -glucosidase steady.

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Figure captions

Fig. 1 Thermostability (*closed circles*) and optimum temperature (*open circles*) of *H. orientalis* β -glucosidase.

Fig. 2 Hydrolysis kinetics of pNPG by β -glucosidase at various temperatures. (A) Hydrolysis kinetics of pNPG by β -glucosidase with 0.16 mM pNPG. Temperatures for curves 1-8 are 37, 45, 53, 60, 63, 67, 70 and 73 °C, respectively. (B) Semilogarithmic plots of the product at 67 °C and 70 °C.

Fig. 3 Determination of microscopic thermal inactivation rate constants. (A) Hydrolysis kinetics of pNPG by β -glucosidase with different substrate concentrations at 67 °C. Substrate concentrations for curves 1-4 are 0.48, 0.36, 0.24 and 0.16 mM, respectively. (B) Plot of $1/[P]_{\infty}$ vs. $1/[S]$ at 67 °C.

Fig. 4 Effects of glucose, cellobiose and salicin on the thermal stability of *H. orientalis* β -glucosidase. (A) Thermostability of β -glucosidase with glucose at 0 mM (●), 5 mM (▲) and 20 mM (△). (B) Effects of glucose (●), cellobiose (△), salicin (○) and mannose (+) on the residual activity of β -glucosidase after 70 °C incubation for 30 minutes. Activity of native β -glucosidase is set as control. (C) Time courses of inactivation with cellobiose, salicin, glucose, mannose and the absence of sugar. (D) Semilogarithmic plots of heat treated β -glucosidase with the presence of 5 mM glucose (●) and the absence of sugar (○), data were obtained from (C).

Fig. 5 Fluorescence emission spectra of heat treated β -glucosidase at 70 °C incubated without glucose (A) and with 20 mM glucose (B).

Fig. 6 Predicted 3D structure of *H. orientalis* β -glucosidase.

Fig. 7 Simulation of β -glucosidase docking with sugars. Panel (a) and (c) are docking mode of cellobiose in catalytic pocket, (b) and (d) are that of glucose.

Table 1. Microscopic thermal inactivation rate constants of *H. orientalis*

β -glucosidase

Temperature /°C	k_{+0}/k'_{+0}	Inactivation rate constants		
		$A (\times 10^{-3} \text{ s}^{-1})$	$k_{+0} (\times 10^{-3} \text{ s}^{-1})$	$k'_{+0} (\times 10^{-3} \text{ s}^{-1})$
67	6.04	0.3	0.41	0.068
70	5.76	1.95	2.69	0.46

Table 2. Effects of temperature on activity and stability of various fungal β -glucosidase

species	opt. temp (°C)	optional incubation conditions to lead half activity lost (°C, h)	ref
<i>A. niger</i>	70	70 , 1	15
<i>A. oryzae</i>	50	60 , 0.5	16
<i>T. viride</i>	60	70 , 0.5	17
<i>P. occitanis</i> (BGL2)	60	65, 0.1	18
<i>Penidiella</i> sp.	60-70	70 , 1	19
<i>Haliotis discus</i>	50	60 , 0.5	20
<i>Paecilomyces</i> sp.	60	70-75 , 0.5	21
<i>H. orientalis</i>	60	65, 0.5	This work

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7