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A glucose-tolerant β-glucosidase from *Prunus domestica* seeds: Purification and characterization

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ABSTRACT

A glucose-tolerant β-glucosidase was purified to homogeneity from prune (*Prunus domestica*) seeds by successive ammonium sulfate precipitation, hydrophobic interaction chromatography and anion-exchange chromatography. The molecular mass of the enzyme was estimated to be 61 kDa by SDS-PAGE and 54 kDa by gel permeation chromatography. The enzyme has a p*I* of 5.0 by isoelectric focusing and an optimum activity at pH 5.5 and 55 °C. It is stable at temperatures up to 45 °C and in a broad pH range. Its activity was completely inhibited by 5 mM of Ag^+ and Hg^{2+}. The enzyme hydrolyzed both *p*-nitrophenyl β-D-glucopyranoside with a K_m of 3.09 mM and a V_{max} of 122.1 μmol/min mg and *p*-nitrophenyl β-D-fucopyranoside with a K_m of 1.65 mM and a V_{max} of 217.6 μmol/min mg, while cellobiose was not a substrate. Glucono-δ-lactone and glucose competitively inhibited the enzyme with K_i values of 0.033 and 468 mM, respectively.

1. Introduction

β-Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of glycosidic linkages in aryl and alkyl β-D-glucopyranosides as well as diglucosides and oligosaccharides. In plants, β-glucosidases play an important role in diverse fundamental biological processes including defense against pathogens and herbivores, scent release, phytohormone conjugate activation and the degradation of cell wall oligosaccharides during germination [1]. In the last two decades, β-glucosidases have attracted attention due to their applications in food detoxification, biomass conversion and flavor enhancement in wines and beverages [2–4]. In addition, β-glucosidases can be used for the synthesis of various glycosides [5–7] that are of commercial interest to the food, cosmetic, pharmaceutical and detergent industries. For example, alkyl glycosides, one of the most promising nonionic surfactants, have a variety of unique features such as a good surfactant performance, low toxicity and good biodegradability [8,9]. Furthermore, many glycosides possess a number of biological activities including antioxidant, lowering of the blood glucose levels, protecting the membrane from freeze–thaw damage and inhibiting tyrosinase [10].

While most microbial β-glucosidases are inhibited by glucose [11,12], β-glucosidases of plant origin, especially those from plant seeds, are very useful for glycosides synthesis [13] or biomass conversion [3], because these enzymes are tolerant to high concentrations of glucose [14]. Large amounts of fruit seeds are annually disposed of as waste in the food processing industry [15], and the use of the glycosidases from this industrial waste as biocatalysts would provide an added value for these byproducts. The development of a green enzymatic process for alkyl glycosides synthesis could greatly reduce the production costs. Recently, we found a novel β-glucosidase with a high activity from a group of fruit seeds [16]. The crude enzyme from prune seeds was capable of catalyzing the synthesis of a variety of alkyl β-D-glucopyranosides via reverse hydrolysis with satisfactory yields. Prunes are a functional food containing phenolic compounds, dietary fiber and potassium and are thus beneficial for cardiovascular and bowel health. The chemicals present in prunes have been previously examined [17,18]; however, there are no reports of functional enzymes in prune seeds. To better understand the characteristics of this novel β-glucosidase and extend the knowledge about its potential roles, we purified and characterized a prune seed β-glucosidase.

2. Materials and methods

2.1. Materials

Prune seeds were obtained from Yikang Food Processing Co. (Guangzhou, China). *p*-Nitrophenyl β-D-glucopyranoside (pNPG), 2-mercaptoethanol, esculin, arbutin

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and glucono-δ-lactone were purchased from Sigma (St. Louis, MO, USA). Molecular weight marker proteins for SDS-PAGE were obtained from TaKaRa (Dalian, China). The gel filtration calibration kit (high molecular weight) for molecular weight determination was purchased from GE Healthcare (USA). *p*-Nitrophenyl β-D-fucopyranoside (pNPF), *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl β-D-xylopyranoside and *p*-nitrophenyl β-D-glucuronide were purchased from TCI (Japan). Salicin and salicyl alcohol were obtained from Aladdin (Shanghai, China). All other reagents were obtained from commercial sources and were of analytical grade.

2.2. Enzyme extraction and purification

Seeds were washed with deionized water, air-dried, shelled and powdered. The meal was defatted three times with cold acetone (−20 °C), and then air-dried and stored at 4 °C. The crude enzyme extract was prepared as follows: 20 g of defatted prune seed meal was added to 50 mL of phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride and 100 mM NaCl. The suspension was combined with quartz sand and milled using a mortar and pestle. The extraction was performed for 1 h at 4 °C with gentle stirring after addition of 950 mL of phosphate buffer (50 mM, pH 7.4). The supernatant was collected as the crude enzyme extract after centrifugation at 10,000 × *g* (4 °C) for 15 min.

Purification of the enzyme was carried out on an ÄKTA Purifier 100 (GE Healthcare, Piscataway, NJ, USA) at 20 °C.

Finely ground $(NH_4)_2SO_4$ was slowly added to the crude enzyme extract to a 50% saturation level, and after centrifugation at 14,000 × *g* for 15 min, the supernatant was concentrated by ultrafiltration using a 30 kDa cutoff membrane (Pellicon XL Biomax 30 50 cm^2) in a Labscale™ TFF System (Millipore, Bedford, MA, USA).

The enzyme solution was again centrifuged at 14,000 × *g* and then submitted to a Phenyl Sepharose CL-4B hydrophobic chromatography column (1.6 cm × 20 cm, GE Healthcare Amersham Biosciences) pre-equilibrated with 2 M $(NH_4)_2SO_4$ dissolved in 50 mM phosphate buffer (pH 7.0). A stepwise elution was carried out with decreasing concentrations of $(NH_4)_2SO_4$ (2, 0.5 and 0 M) dissolved in 50 mM phosphate buffer (pH 7.0). Active fractions were eluted with 50 mM phosphate buffer (pH 7.0), pooled and concentrated with 10 kDa cutoff microcentrifuge filters (Sartorius, Göttingen, Germany).

The concentrated active fractions were dialyzed overnight at 4 °C in 20 mM Tris–HCl buffer (pH 7.8) to exchange the buffer and remove any remaining $(NH_4)_2SO_4$ in the enzyme solution. The enzyme solution was loaded onto a Mono Q™ 5/50 GL anion exchange chromatography column (GE Healthcare Amersham Biosciences, USA) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.8). A stepwise elution was performed with increasing concentrations of NaCl (0.1, 0.15 and 0.2 M) dissolved in 20 mM Tris–HCl buffer (pH 7.8). The active fractions were eluted with 0.2 M NaCl.

2.3. β-Glucosidase activity assay

The pNPG solution was added to 50 mM citric acid–Na_2HPO_4 buffer (pH 5.5) containing an appropriate amount of enzyme (10 μL, 0.34 mg/mL) at 45 °C to initiate the reaction. If not otherwise specified, the concentration of pNPG was 1.0 mM in the reaction mixture, and the total volume was 3.0 mL. The reaction was quenched after 3 min by the addition of 3 mL of Na_2CO_3 solution (1 M) and the absorbance at 402 nm was recorded by a UV–VIS spectrophotometer (Shimadzu 2550, Japan). A reaction mixture without the enzyme was used as a control. One unit of β-glucosidase activity was defined as the amount of enzyme that releases 1.0 μmol of *p*-nitrophenol per minute under the above-mentioned conditions. For substrates that cannot be assayed spectrophotometrically, the initial hydrolytic rate was determined by measuring the release of the aglycon or glucose with HPLC. For the hydrolysis of arbutin, the initial rate of release of hydroquinol was assayed by HPLC (Waters 1525, USA) using an XBridge™ C18 column (4.6 mm × 250 mm, 5 μm, Waters) and detection at 280 nm. The mobile phase was a mixture of water and methanol (70/30, v/v) with a flow rate of 1 mL/min, and the retention times of arbutin and hydroquinol were 2.92 and 3.56 min, respectively. For the hydrolysis of salicin, the initial rate of release of salicyl alcohol was assayed on the same C18 column as described above with detection at 220 nm. The mobile phase was a mixture of water and methanol (80/20, v/v) with a flow rate of 1 mL/min, and the retention times of salicin and salicyl alcohol were 6.70 and 8.77 min, respectively. For the hydrolysis of cellobiose and esculetin, the initial rate of the release of glucose was assayed on an Aminex® HPX-87H ion exclusion column (BIO-RAD, USA) by HPLC (Waters 600, USA) with a refractive index detector. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.5 mL/min, and the retention times of cellobiose and glucose were 9.71 and 11.99 min, respectively. The detection limits of cellobiose and glucose were 0.02 and 0.05 mM, respectively.

2.4. Molecular mass determination

The molecular mass of the native protein was determined by gel permeation chromatography on a Superdex 200 PG column (1.2 cm × 82 cm) with a flow rate of 1 mL/min and a mobile phase of 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The column was calibrated with ovalbumin (M_w 44,000), conalbumin (M_w 75,000), aldolase (M_w 158,000), ferritin (M_w 440,000) and thyroglobulin (M_w 669,000). The subunit molecular mass was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

2.5. Electrophoresis

2.5.1. SDS-PAGE

For the determination of the subunit molecular weight, SDS-PAGE was performed on a 12.5% polyacrylamide gel in a Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) as described by Laemmli [19]. A mixture of molecular weight marker proteins was used as a reference: lysozyme (M_w 14,300), trypsin inhibitor (M_w 20,100), carbonic anhydrase (M_w 29,000), ovalbumin (M_w 44,287), bovine serum albumin (M_w 66,409) and phosphorylase B (M_w 97,200).

2.5.2. Native PAGE

For the β-glucosidase activity staining, native PAGE was performed on a 8.5% polyacrylamide gel with Tris–glycine buffer (pH 8.8). After electrophoresis, an activity staining was carried out as described by Kwon et al. [20]. The gel was soaked in 0.2 M sodium acetate buffer (pH 5.0) for 10 min to exchange the buffer, and was stained with 0.2 M sodium acetate buffer (pH 5.0) containing 0.1% (w/v) esculin and 0.03% (w/v) ferric chloride. The gel was incubated at 50 °C for several minutes until a black band appeared.

2.5.3. IEF

Isoelectric focusing (IEF) was carried out on 24 cm ImmobilineTM Dry Strips (pH 3–10, GE Healthcare) using an Ettan IPGphor 3 (GE Healthcare, USA), as recommended by the manufacturer.

2.6. Effect of pH

The effect of pH on the activity of β-glucosidase was assessed in a buffer of 50 mM citric acid–Na_2HPO_4 (pH 4.0–8.0). To characterize the pH stability, the activity was measured after the enzyme (10 μL, 0.34 mg/mL) was incubated in the buffer for 24 h at 25 °C and the designated pH.

2.7. Effect of temperature

To determine the optimum temperature, the enzyme (10 μL, 0.34 mg/mL) was preincubated for 3 min at the given temperature, and the activity was determined in a temperature range of 25–65 °C. To characterize the thermal stability, the activity was measured after incubating the purified enzyme (10 μL, 0.34 mg/mL) in 50 mM citric acid–Na_2HPO_4 buffer (pH 5.5) at the designated temperature for specific intervals (15–480 min).

2.8. Effects of metal ions and chemical reagents

The purified β-glucosidase was preincubated for 1 h at 4 °C in the presence of different metal ions, and the activity was measured using the standard assay. The effect of chemical reagents on the activity was examined by adding them directly to the reaction mixture, followed by measuring the activity. The relative activity was defined as the ratio of the enzyme activity in the presence of the tested ions or reagents to that in the absence of metal ions and chemical reagents.

2.9. Kinetic parameters and inhibition constants

The kinetic studies of enzyme hydrolysis were performed in 50 mM citric acid–Na_2HPO_4 buffer (pH 5.5) at 45 °C with pNPG (0.37–8.98 mM) and pNPF (0.35–8.37 mM) as the substrates. The K_m and V_{max} values were calculated from Hanes–Woolf plots. The enzyme inhibition by glucose (49.7–497 mM) or glucono-δ-lactone (0.2–3.0 mM) was studied in 50 mM citric acid–Na_2HPO_4 buffer (pH 5.5) at 45 °C with pNPG as the substrate. The inhibition constants (K_i) were calculated from Dixon plots [12].

2.10. Substrate specificity

To initiate the reaction, the purified enzyme (10 μL, 0.34 mg/mL) was added to 3 mL of citric acid–Na_2HPO_4 buffer (50 mM, pH 5.5) at 45 °C and containing each tested substrate at a 2 mM final concentration.

3. Results and discussion

3.1. Purification

A three-step procedure was designed to purify the prune seed β-glucosidase. The crude extract was first brought to 50% $(NH_4)_2SO_4$ saturation to precipitate a large amount of undesired proteins. After centrifugation, the supernatant was submitted to hydrophobic interaction chromatography on a Phenyl Sepharose CL-4B column that was pre-equilibrated with 2 M $(NH_4)_2SO_4$ (Fig. 1A). A relatively

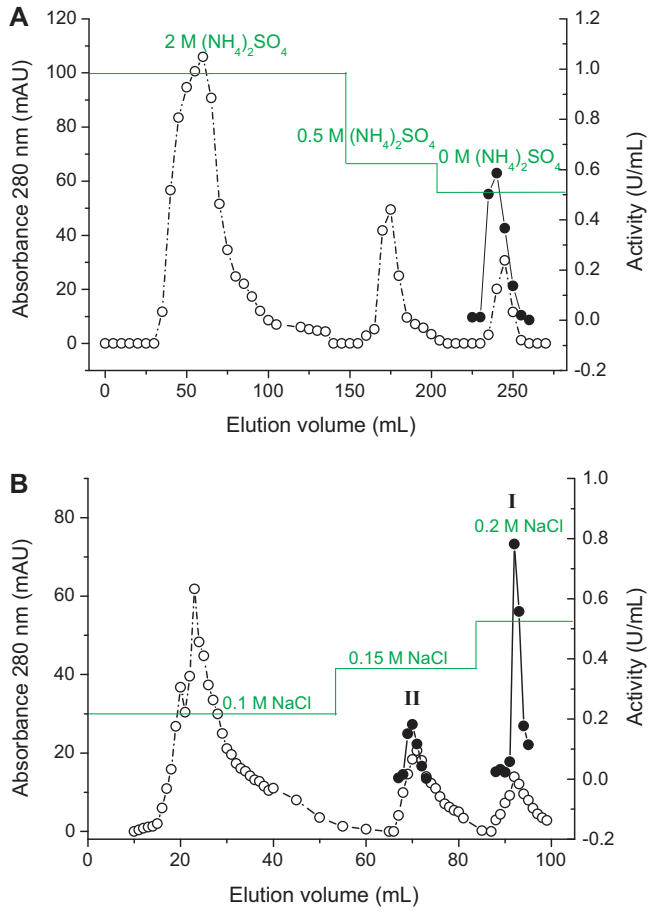

Fig. 1. Purification of β-glucosidase. (A) Hydrophobic interaction chromatography on a Phenyl Sepharose CL-4B column. (B) Anion exchange chromatography on a Mono Q™ 5/50 GL column. The absorbance at 280 nm is shown as open symbols, while the activity is shown as closed symbols.

high salt concentration was used because the main active protein was precipitated at approximately 60% $(NH_4)_2SO_4$ saturation. A stepwise elution was used with decreasing $(NH_4)_2SO_4$ concentrations. The active fractions were collected upon elution with 50 mM phosphate buffer (pH 7.0).

Next, anion exchange chromatography on a Mono Q™ 5/50 GL column was conducted, resulting in the separation of major (I) and minor (II) peaks of activity (Fig. 1B). A stepwise elution, rather than a continuous gradient elution, was chosen for the purification, as this process minimizes the separation time and the consumption of buffer and may be more practical for industrial scale-up. The entire purification process is summarized in Table 1. The major active fraction of β-glucosidase I was purified 59-fold to a specific activity of 23.1 U/mg protein with a yield of 5.4%.

3.2. General properties

The purified β-glucosidase I gave a single major band on SDS-PAGE (Fig. 2A), thus indicating that the obtained enzyme is electrophoretically homogeneous. The subunit molecular mass of this enzyme estimated by SDS-PAGE was approximately 61 kDa, which is similar to several β-glucosidases from other plant sources such as *Prunus serotina* Ehrh. (black cherry) seeds [21], apple seeds [15], rye seedlings [22] and *Citrus sinensis* [23]. The molecular mass of the native protein was estimated to be 54 kDa by gel permeation chromatography, suggesting that β-glucosidase I is a monomer in solution. The β-glucosidase isoenzymes, PH I and PH IIb, from black cherry seeds were also reported to be monomeric glycoproteins [24].

Native PAGE of the purified β-glucosidase I remained homogeneous, because a single band was observed upon staining with Coomassie Brilliant Blue. In addition, the purified enzyme had a single band at the corresponding position after staining for β-glucosidase activity with esculin (Fig. 2B). The p*I* value of β-glucosidase I was determined to be 5.0 by IEF (data not shown), which is higher than those (4.55 and 4.6, respectively) of β-glucosidases from sweet almond [25] and *Prunus avium* L. [26], but lower than those (5.8–6.4) of β-glucosidases from black cherry seeds [24].

As shown in Fig. 3, the optimum pH of β-glucosidase I is 5.5. Similar to β-glucosidases from other plant sources such as apple seeds [15] and soybean okra [27], β-glucosidase I was stable over a broad pH range. For example, it retained approximately 90% of the original activity after incubation at pH 8.0 for 24 h.

As shown in Fig. 4A, the optimum temperature of β-glucosidase I is 55 °C, which is slightly lower than that (60 °C) of almond β-glucosidase [15]. However, β-glucosidase I was not stable at the optimum temperature; the half-life was approximately 23 min (Fig. 4B). The enzyme was fairly stable at 45 °C, as it still maintained 76% initial activity after 480 min of incubation at this temperature. Furthermore, it had excellent storage stability, keeping almost 100% initial activity after storage for 15 days at 4 °C (data not shown).

The effects of various cations and chemical reagents on the activity of β-glucosidase I are shown in Table 2. Mn^{2+}, Mg^{2+}, Co^{2+} and Ca^{2+} exerted little effect on the enzyme activity at 5 mM concentration, while the activity was inhibited by 50–60% in the presence of 5 mM Cu^{2+} and Zn^{2+}. And the enzyme activity was not affected by the chelating agent EDTA, implying that divalent cations are not essential for the enzyme activity. Like other β-glucosidases [14,15,28,29], β-glucosidase I was significantly inhibited by the sulfhydryl oxidant Hg^{2+} and Ag^{+} metals ions. However, marginal inhibition of the enzyme activity was caused by thiol-reducing agents 2-mercaptoethanol at 40 mM and DTT at 5 mM (Table 2). Lucas et al. proposed that the inactivation by sulfhydryl oxidant metals might be ascribed to nonspecific salt formation rather than complex formation with thiol groups and/or oxidation of thiol groups [11]. So, thiol groups might be inessential for catalytic activity of β-glucosidase I. The detergents SDS and Tween 80 only had negligible effect on the enzyme activity, but the enzyme lost 35% initial activity in the presence of 1% (v/v) Triton X-100.

3.3. Inhibitory constants

Glucose and glucono-δ-lactone are the two competitive inhibitors of β-glucosidase. Therefore, high resistance to glucose inhibition is one of the most important features of ideal

Table 1
Purification of β-glucosidase I from prune seeds.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Crude extract	1474	3772	0.4	100	1.0
$(NH_4)_2SO_4$ precipitation	1382	318.5	4.3	93.8	11.1
Phenyl Sepharose CL-4B	179.6	14.9	12.1	12.2	31.0
Mono Q™ 5/50 GL	80.3	3.5	23.1	5.4	59.2

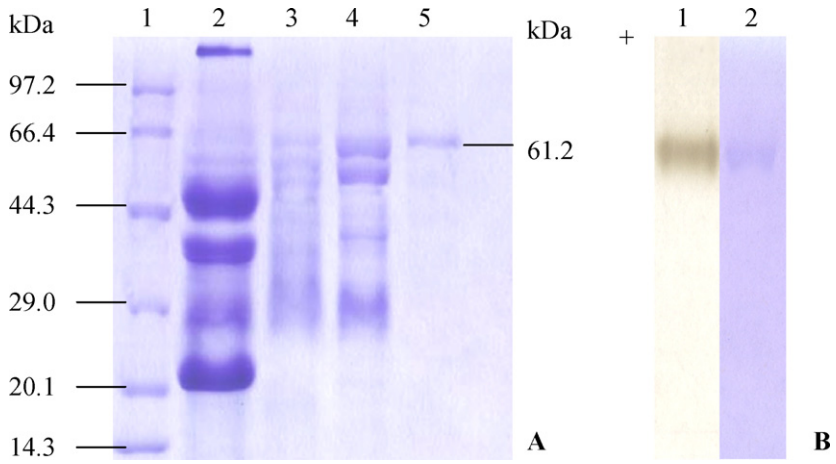

Fig. 2. Electrophoresis. (A) SDS-PAGE analysis (12.5% polyacrylamide) of protein samples from each step of the purification. The protein was stained with Coomassie Brilliant Blue R-250: lane 1, molecular weight markers; lane 2, crude protein extract; lane 3, the supernatant after $(NH_4)_2SO_4$ precipitation and ultrafiltration; lane 4, the active peak from Phenyl Sepharose CL-4B; lane 5, the active peak I from Mono Q™ 5/50 GL. (B) Native PAGE analysis (8.5% polyacrylamide) of the purified β-glucosidase I: lane 1, β-glucosidase activity stained with esculin; lane 2, protein stained with Coomassie Brilliant Blue R-250.

β-glucosidases for industrial applications. Most microbial β-glucosidases that have been tested are strongly inhibited by glucose with K_i values of 1.3–11 mM [11,12,30], although there are several reports on highly glucose-tolerant β-glucosidases from microbial sources, such as *Candida peltata* (K_i = 1.4 M) [31] and *Aspergillus oryzae* (K_i = 1.36 M) [29]. Hence, the effect of the two competitive inhibitors on β-glucosidase I was evaluated with pNPG as a substrate (Fig. 5). Lineweaver–Burk plots revealed that glucose and glucono-δ-lactone followed competitive inhibition (data not shown). Dixon plots were used to determine K_i values of 468 and 0.033 mM for glucose and glucono-δ-lactone, respectively. Glucono-δ-lactone significantly inhibits β-glucosidase I, while glucose does not, β-Glucosidases from black cherry seeds [21] and sweet almond [25] were also inhibited by glucono-δ-lactone with K_i values of 1.3 and 0.16 mM, respectively. However, 200 mM of fructose, galactose, arabinose or xylose did not display the inhibition effect on β-glucosidase I (data not shown), which indicates that this sugar-tolerant enzyme may be promising for industrial applications.

3.4. Substrate specificity

The initial hydrolysis rates of various substrates and relative activities of β-glucosidase I are shown in Table 3. Similar

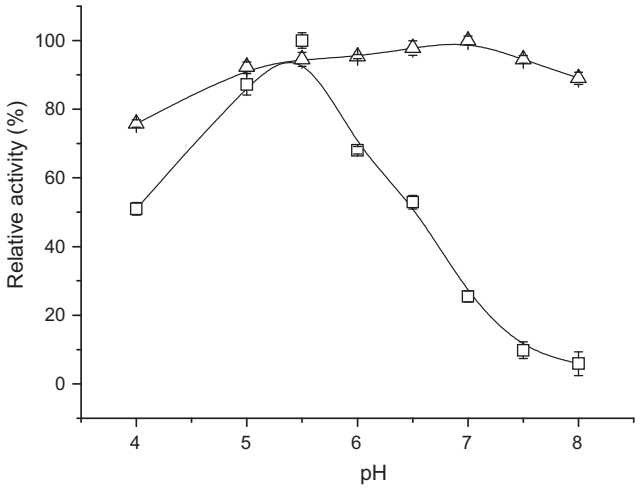

Fig. 3. Effect of pH on the hydrolysis of pNPG catalyzed by β-glucosidase I. Symbols: activity (□); stability (△). The activity was measured in the pH range of 4.0–8.0. To characterize pH stability, the activity was measured after incubating for 24 h at 25 °C and the designated pH.

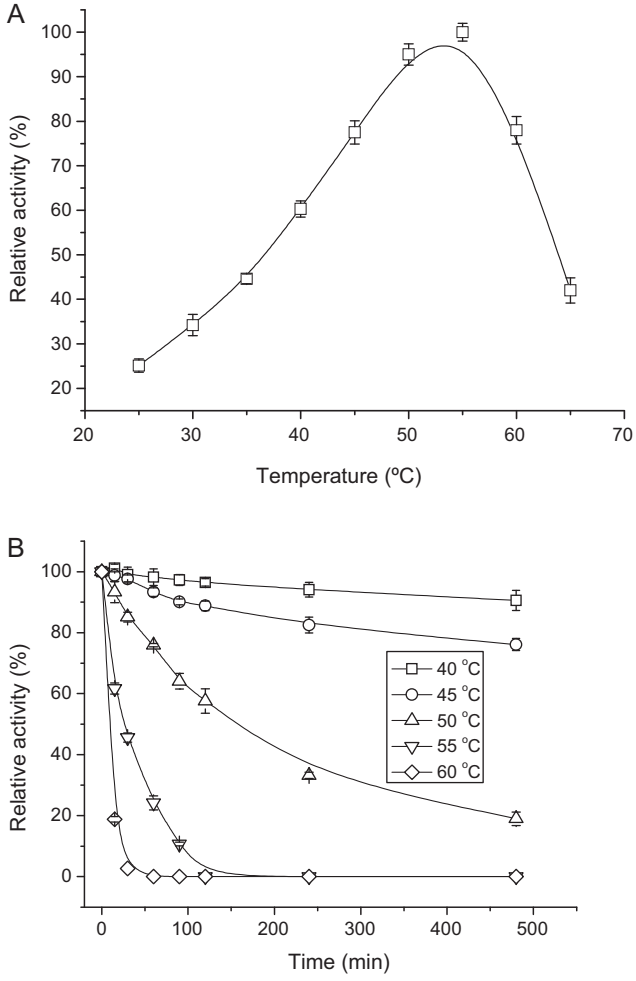

Fig. 4. Effect of temperature on the hydrolysis of pNPG catalyzed by β-glucosidase I. (A) Activity; (B) stability. The activity was measured in the temperature range of 25–65 °C. To characterize the thermal stability, the activity was measured after incubating at the designated temperature and pH 5.5 for the designated time.

Table 2
Effects of metal ions and reagents on the activity of β-glucosidase I.

	Relative activity (%)	
Control	100	
Metal ions		
Mn^{2+}	5 mM	105.3 ± 0.3
Mg^{2+}	5 mM	97.0 ± 0.2
Co^{2+}	5 mM	92.2 ± 0.1
Ca^{2+}	5 mM	103.3 ± 0.1
Cu^{2+}	1 mM	63.2 ± 0.2
	5 mM	46.3 ± 0.2
Zn^{2+}	1 mM	64.2 ± 1.3
	5 mM	41.1 ± 0.8
Hg^{2+}	1 mM	27.2 ± 0.3
	5 mM	0
Ag^{+}	1 mM	0
	5 mM	0
Reagents		
2-Mercaptoethanol	40 mM	88.3 ± 0.2
DTT[a]	5 mM	91.7 ± 0.1
SDS	5 mM	101.2 ± 1.5
Tween 80	5% (v/v)	96.1 ± 0.7
Triton X-100	1% (v/v)	65.1 ± 2.2
EDTA	5 mM	105.8 ± 0.1

[a] Dithiothreitol.

Table 3
The activity of β-glucosidase I on various substrates.[a]

Substrate	Initial hydrolysis rate (μmol/min)	Relative activity (%)
pNPG	0.261	100
pNPF	0.633 ± 0.007	242.5 ± 2.1
p-Nitrophenyl β-D-galactopyranoside	0.051 ± 0.001	19.5 ± 0.7
p-Nitrophenyl β-D-xylopyranoside	0.006 ± 0.000	2.3 ± 0.0
p-Nitrophenyl β-D-glucuronide	nd[b]	nd
Arbutin[c]	0.022 ± 0.001	8.4 ± 0.3
Salicin[c]	0.059 ± 0.000	22.6 ± 0.0
Esculetin	0.117 ± 0.003	44.8 ± 1.1
Cellobiose (2 and 10 mM)[c]	nd	nd

[a] Conditions: 50 mM phosphate buffer (pH 7.0), 45 °C, 2 mM substrate.
[b] No activity detected.
[c] Reaction for 1 h.

to β-glucosidases from other sources [11,12], β-glucosidase I was also able to hydrolyze β-galactopyranoside (19.5%) and β-xylopyranoside (2.3%). The enzyme had maximal hydrolytic activity with pNPF as a substrate, which was approximately 2.4-fold higher than that of pNPG at 2 mM. A similar phenomenon was also observed in the cases of vanilla bean and rice β-glucosidases [14,32]. Moderate activity was found in the enzymatic hydrolysis of esculetin (44.8%). Arbutin and salicin were hydrolyzed at very slow rates by β-glucosidase I (8.4% and 22.6%, respectively). No hydrolysis occurred with *p*-nitrophenyl β-D-glucuronide and cellobiose

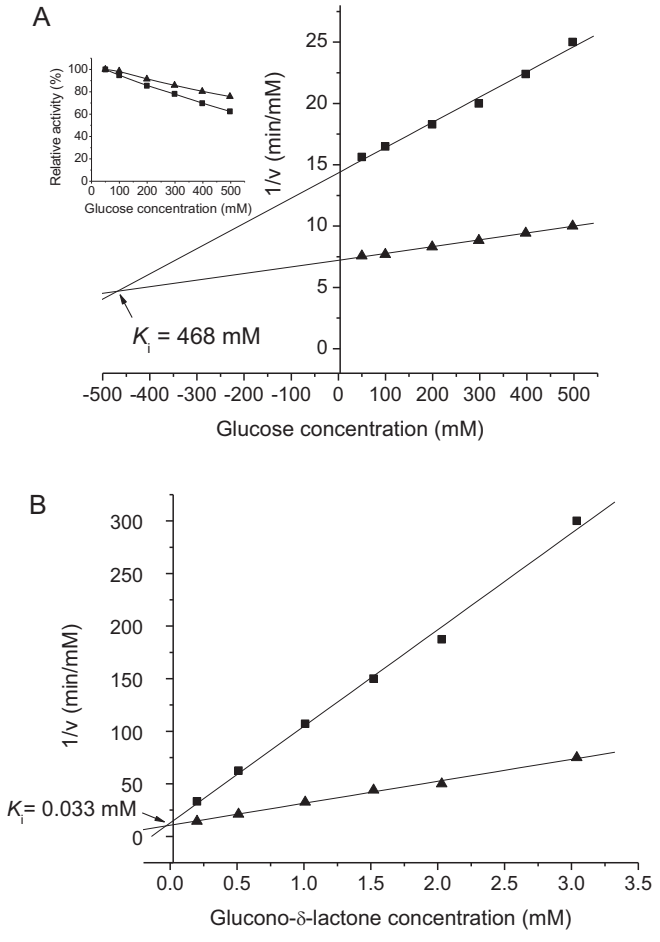

Fig. 5. Inhibitory kinetics. (A) A dixon plot of the inhibitory effect of glucose on pNPG hydrolysis [pNPG: 1.37 mM (■) and 5.46 mM (▲)]. (B) A dixon plot of the inhibitory effect of glucono-δ-lactone on pNPG hydrolysis [pNPG: 1.37 mM (■) and 4.10 mM (▲)].

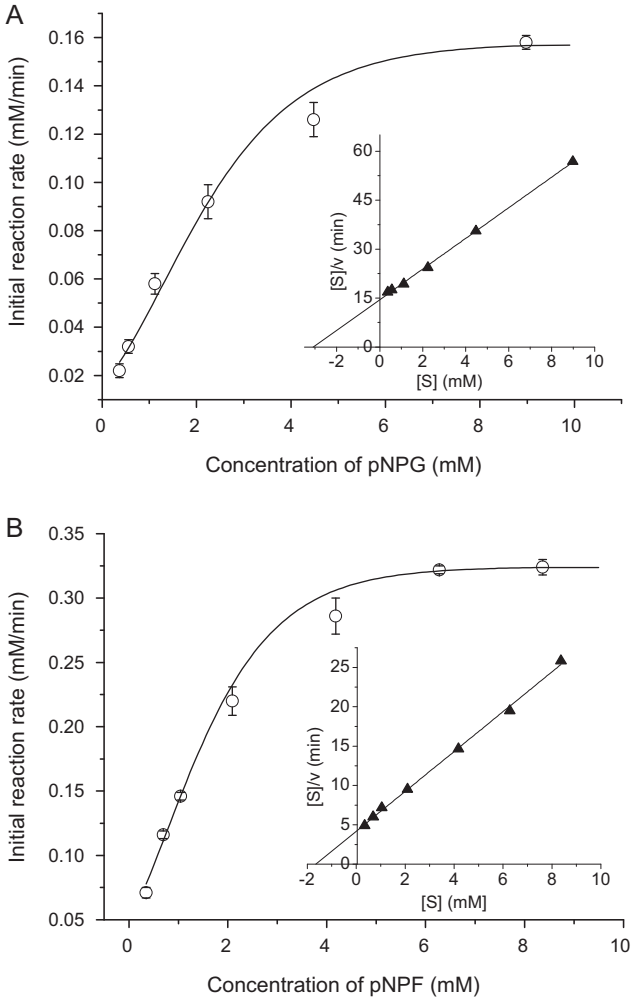

Fig. 6. Dependence of the β-glucosidase I activity on substrate concentrations. (A) pNPG; (B) pNPF. Michaelis–Menten curves are shown with Hanes–Woolf plots inset.

as the substrates. Previously, it was reported that β-glucosidases from black cherry seeds [21,33], vanilla bean [14], maize [34] and *Arabidopsis thaliana* [35] were also unable to catalyze the hydrolysis of cellobiose. Prune seed β-glucosidase I was similar in substrate specificity to vanilla bean β-glucosidase [14].

3.5. Kinetics studies

In the study of substrate specificity, both pNPG and pNPF were found to be good substrates of β-glucosidase I. The effect of the substrate concentration on the enzyme activity was evaluated (Fig. 6). According to the Hanes–Woolf plot, the K_m value was 3.09 mM and the V_{max} was 122.1 μmol/(min mg protein) with pNPG as the substrate. The kinetic parameters of β-glucosidase I are similar to those of β-glucosidases from other plants. For example, the K_m and V_{max} values of almond β-glucosidase were reported to be 2.24 mM and 588 μmol/(min mg protein), respectively [36], and corn stover β-glucosidase had a K_m of 2.3 mM and a V_{max} of 18.6 μmol/(min mg protein) [3]. As shown in Fig. 6B, pNPF did not inhibit the activity of β-glucosidase I with concentrations up to 8.4 mM, and K_m and V_{max} values of 1.65 mM and 217.6 μmol/(min mg protein), respectively, were obtained with this compound. The lower K_m and higher V_{max} suggest that the enzyme has a higher catalytic efficiency with pNPF than with pNPG.

4. Conclusions

β-glucosidase I from prune seeds has potential in industrial applications (the glycosides synthesis, etc.) because of its excellent properties, such as being highly tolerant to glucose and stable in a broad pH range. Indeed, we recently synthesized a group of alkyl glycosides with satisfactory yields using the crude enzyme (in the form of prune seed meal) [16]. In addition, this enzyme can be harvested from waste fruit seeds that are produced by food processing industries, thus possessing the advantages of low cost and availability. According to its substrate specificity, β-glucosidase I was presumed to be unrelated to the degradation of polysaccharides. Like other β-glucosidases in *Prunus* plant seeds [37], it might be involved in cyanogenesis as a chemical defense against herbivores and microorganisms.

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