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Purification and characterization of a glucose-tolerant *β*-glucosidase from black plum seed and its structural changes in ionic liquids

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Abstract

The objective of this study was to characterize a plant origin *β*-glucosidase from black plum seeds and identify its conformational changes in twenty-six imidazolium- and amino acid-based ionic liquids (ILs). The results revealed that the purified 60 kDa enzyme was monomeric in nature, maximally active at 55 °C and pH 5.0, and nearly completely inhibited by Hg^{2+} and Ag^{+}. Attractive peculiarities of the relative low kinetic and higher glucose inhibition constants (K_m=0.58 mM [*p*NPG]; K_i=193.5 mM [glucose]) demonstrated its potential applications in food industry. Circular dichroism studies showed that the secondary structural changes of the enzyme depended not only on the anions, but also on the cations of the assayed ILs. Interestingly, no corresponding relations were observed between the changes in enzyme structure induced by ILs and its catalytic activities, suggesting that the influences of ILs on enzymatic processes don't rely simply on enzyme conformational changes.

Keywords: *β*-Glucosidase; Enzyme structure; Ionic liquids; Kinetics

1. Introduction

β-Glucosidases (E.C. 3.2.1.21) capable of catalyzing the synthesis and hydrolysis of glycosidic linkages are of pivotal importance to plants, animals, and microorganisms in producing functional secondary metabolite glycosides (Cairns et al., 2015; Guadamuro et al., 2017). Over the last two decades, their potential applications have become a hot research topic in food, pharmaceutical, and bioenergy industries (Guo et al., 2015). For example, the competitive advantages of *β*-glucosidase obtained from various sources, in particular, the specificity of the enzyme, have been extendly used in liberating flavor compounds from nonvolatile or odorless glycosides in producing wine and fruit juice, synthesizing functional oligosaccharides, and converting glycosides into their corresponding bioactive aglycones, etc (Guadamuro et al., 2017; Guo et al., 2015; Shin and Oh, 2014). Nowadays, *β*-glucosidase-mediated chemical manufacturing is considered a promising and alternative strategy in organic reactions (Sears and Wong, 2001; Yang et al., 2015).

Generally, *β*-glucosidases from various sources exhibit different reverse hydrolysis and transglucosylation activities during the one-step synthesis of aryl- and alkyl-glycosides, diglucosides, oligosaccharides, and glycoconjugates. However, further studies show that although enzymatic transglucosylations usually exhibit satisfactory product yields or selectivity, the enzyme-poor sources, expensive activated sugar precursors (i.e., UDP-*α*-D-glucose) (Sears and Wong, 2001), and high cost of synthesis inevitably limit their industrial biocatalytic applications. Most recently, *β*-glucosidases from abundant, inexpensive, and available plant seeds have been found superior to other enzymes for synthesizing glycosides and their analogues by reverse hydrolysis (Yang et al., 2015). Moreover, compared to microbial origin *β*-glucosidases, enzymes

of plant origin exhibit unique characteristics, including a high glucose tolerance, broad substrate spectrum, and excellent selectivity (Cao et al., 2015; Yang et al., 2015). Thus far, a series of *β*-D-glucopyranosides have been successfully synthesized using plant origin *β*-glucosidases (Lu et al., 2010; Yang et al., 2012).

With the development of sustainable solvents for green chemistry, ionic liquids (ILs), which possess many potential applications and advantages, are now well recognized and widely considered as useful alternatives to classical solvents or water in biocatalysis (Hallett and Welton, 2011). Enzymatic glycosylation, transglycosylation, transgalactosylation, and saccharification have been demonstrated by several research groups in various neat ILs or IL solution systems (Goswami et al., 2016; Kudou et al., 2014; Xu et al., 2016). For example, Kudou et al. studied cellobiose hydrolysis with *β*-glucosidase from *Thermotoga maritime* in a phosphate buffer containing several ILs and the results showed that 1-butyl-3-methylimidazolium acetate ([C_4MIm][OAc]) presented a positive effect on improving the enzyme activity (Kudou et al., 2014). Our previous studies also revealed that the salidroside yield catalyzed by black plum seed *β*-glucosidase could be significantly enhanced when 1-hexyl-3-methylimidazolium tetrafluoroborate ([C_6MIm][BF_4]) was used as the co-solvent; further, the enzyme behavior depend not only on the alkyl chain lengths of the cation, but also on the type of the anion in imidazolium-based ILs (Bi et al., 2012).

Nevertheless, although ILs have attracted tremendous attention from the academia as well as the industry, their impact on the mechanism of biocatalysis process is still not completely understood, especially the relationship between catalysts and the physicochemical properties of ILs. In the case of quantitative and qualitative analysis of the conformational changes induced by

ILs in enzyme proteins during the course of biotransformations, most of the recent work focuses solely on lipase, *α*-chymotrypsin, tyrosinase, and lysozyme (Esquembre et al., 2013; Lai et al., 2011; Liu et al., 2011; Reddy et al., 2015), while there are very few reports available on the interactions between *β*-glucosidase and ILs. Encouraged by this, based on the enzyme purification, characterization, and secondary structure analysis via circular dichroism (CD) spectroscopy, the present study aims to provide a possible explanation for the observed excellent performance of the black plum seed *β*-glucosidase in IL-containing systems for preparing *β*-D-glucopyranosides (Bi et al., 2012), which might be beneficial to understand the effect of composition, structure, and physicochemical properties of the ILs on the enzymatic glycosylation process.

2. Materials and methods

2.1. Materials

Black plum seeds were obtained from local food processing company. *p*-Nitrophenyl *β*-D-glucopyranoside (*p*NPG), *p*-nitrophenol, choline hydroxide, [C_2MIm][BF_4], [C_4MIm][BF_4], [C_6MIm][BF_4], [C_2MIm][PF_6], [C_6MIm][PF_6], [C_8MIm] [PF_6], [C_4MIm][Cl], [C_4MIm][Br], and [C_4MIm][I] were purchased from Sigma-Aldrich. All other solvents were from commercial sources and of the highest purity available.

2.2. Extraction and purification of β-glucosidase

Crude *β*-glucosidase from black plum seeds was extracted according to the method of Chen et al (Chen et al., 2013). $(NH_4)_2SO_4$ was then slowly added to the crude enzyme extract to achieve 40% saturation in an ice bath, and after centrifuging (Avanti J-26XP, Beckman Coulter, USA) at 11 000 g for 15 min and removing the precipitate, more $(NH_4)_2SO_4$ was added to 65% saturation and the mixture was left to stand at 4 °C overnight. Thereafter, the mixture was centrifuged to

collect the protein precipitation, which was dissolved in a small volume of 20 mM Tris-HCl buffer, pH 7.4 (buffer A) and dialyzed against the same buffer for 24 h.

The dissolved sample was submitted to a DEAE-52 anion-exchange chromatography (20 cm×1.6 cm) previously equilibrated with buffer A. Stepwise elution was used with increasing concentrations of NaCl (0.1, 0.15, and 0.2 M) dissolved in buffer A at a flow rate of 1.5 mL/min and the elution was monitored at 280 nm. Active fractions were pooled and applied onto a phenyl Sepharose CL-4B hydrophobic chromatography column (20 cm×1.6 cm) equilibrated with buffer A containing 1.0 M $(NH_4)_2SO_4$ and eluted using the same buffer at a flow rate of 1.5 mL/min, with decreasing concentrations of $(NH_4)_2SO_4$ (0.75, 0.5, and 0 M). Enzyme fractions were again pooled, concentrated using an Amicon Ultra 10-kDa cutoff centrifugal filter device (Millipore, Bedford, MA), applied to a Sephadex G-75 (100 cm×1.6 cm) column, and eluted at a flow rate of 0.5 mL/min with the equilibrated buffer A. Active fractions were pooled and stored at 4 ℃. Protein concentration was estimated by the Bradford method with bovine serum albumin as the standard protein.

2.3. Assay of β-glucosidase activity

The analysis procedure was carried out according to Chen et al. (Chen et al., 2013), with slight modifications. *p*NPG was used as the substrate to determine the enzyme activity. An assay reaction mixture containing 0.1 mL enzyme solution, 2.9 mL citric acid-Na_2HPO_4 buffer (50 mM, pH 7.0), 15 μL *p*NPG (200 mM) was incubated at 45 ℃ for 5 min followed by adding 3.0 mL Na_2CO_3 (1.0 M) to inactivate the enzyme and measuring the absorption at 405 nm. One unit of activity (U) was defined as the amount of enzyme required to produce 1.0 μmol *p*-nitrophenol in 1.0 min under the above conditions.

2.4. Molecular mass determination

The subunit molecular mass of *β*-glucosidase was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN Tetra Cell (Bio-Rad, USA) with 12% polyacrylamide gels, as described by Laemmli (Laemmli, 1970). The pre-stained protein ladder markers (Takara, Japan) with the apparent molecular weights from 10 to 100 kDa were used to evaluate protein molecular size. Bands from SDS gels were stained with coomassie blue for visualization. The native molecular mass was measured with size exclusion chromatography on a SephacrylS-300 HR column (GE Healthcare, Piscataway, NJ, USA) by comparing it with the elution volume of the reference proteins. The enzyme solution was submitted to the column and eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl at a flow rate of 0.5 mL/min. Standard molecular weight proteins used for calibration were ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), and aldolase (158 kDa) obtained from GE Healthcare.

2.5. Effect of temperature

To identify the optimum temperature for enzyme activity, the reactions were conducted at different temperatures (25−70 °C) in citric acid-Na_2HPO_4 (50 mM, pH 7.0). Aliquots of the mixture were withdrawn at specified intervals and assayed according to the method described earlier. Thermostability was ascertained by incubating the enzyme at the given temperature (45 °C, 50 °C, 55 °C, and 60 °C, respectively) over a duration of 480 min without any substrate and assaying the residual activities. The maximum activity of the unincubated enzyme obtained was defined as 100%.

2.6. Effect of pH

The effect of pH on the purified *β*-glucosidase activity was investigated in 50 mM citric acid-Na_2HPO_4 phosphate buffer with various pH values ranging from 3.0 to 8.0 at 55 °C. After incubating the enzyme (0.1 mL, 0.3 mg/mL) in the buffer for 24 h, the residual enzyme activity was measured to assess the pH stability.

2.7. Effect of metal ions and chemical reagents

The purified enzymes were preincubated in eight metal ion (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, and Ag^{+}) solutions at 4°C for 1.0 h, and then the residual activities were examined. In addition, five chemical reagents (EDTA, DTT, SDS, Triton X-100, and Tween 80) were added directly to the reaction mixture containing the *p*NPG used as substrate to estimate their effects on the activity. Enzyme activity in absence of metal ions or reagents was recorded as 100%.

2.8. Kinetic parameters and inhibition constants determination

Enzyme kinetic parameters were tested with *p*NPG as the substrate in 50 mM citric acid-Na_2HPO_4 buffer pH 5.0 at 55 °C. The K_m and V_{max} values were determined from the Lineweaver-Burk plots using different *p*NPG concentrations (0.33−8.61 mM). Besides, dixon plot analysis was also performed to measure the glucose (48.7−496.7 mM) inhibition constant (K_i) of the purified *β*-glucosidase.

2.9. Preparation of amino acid-based ILs

A group of biocompatible [choline][amino acid] ([Ch][AA]) ILs, such as [Ch][Trp], [Ch][Asp], [Ch][Lys], [Ch][His], [Ch][Ile], [Ch][Gln], [Ch][Leu], [Ch][Phe], [Ch][Met], [Ch][Pro], [Ch][Arg], [Ch][Glu], [Ch][Val], [Ch][Ala], [Ch][Thr], [Ch][Gly], and [Ch][Ser] were synthesized using the method developed by Liu et al (Liu et al., 2012).

2.10. Circular dichroism spectroscopy study

CD spectra studies were explored based on a MOS-450/AF-CD (Bio-Logic, France) in the far-UV region (200−250 nm), at 297 K. Each sample was placed in a 0.1 cm pathlength quartz cuvette filled with the phosphate buffer (50 mM, pH 5.0) containing enzyme protein (0.3 mg/mL) and different ILs (1.0%, v/v), and equilibrated at the corresponding temperature for 30 min before analysis. The scanning rate and spectral resolution were 50 nm/min and 0.1 nm, respectively. Sample spectra were obtained by subtracting the blank buffers (with ILs) from the experimental spectrum and accumulated at three times. The protein second structure (like α-helix and β-sheet) was estimated by using CDPro software package.

3. Results and discussion

3.1. Purification of β-glucosidase

A four-step procedure was explored at 4 °C to purify black plum seed β-glucosidase. After the initial purification of the ammonium sulfate fractionation, the second DEAE-52 anion-exchange chromatography pattern exhibited three peaks corresponding to β-glucosidase activity (see Supplementary material), namely β-glucosidase-1 (BPG for short), β-glucosidase-2, and β-glucosidase-3, respectively. Finally, after subjecting the major fraction of active BPG to sequential phenyl Sepharose CL-4B hydrophobic chromatography and Sephadex G-75 size exclusion chromatography, homogeneous purification was completed. The results are summarized in Table 1. The enzyme was purified by 64.8 fold to a specific activity of 9.1 U/mg with a yield of 1.2%.

3.2. Molecular weight determination

SDS-PAGE as well as size exclusion chromatography were carried out in order to check the purity and molecular weight of the enzyme protein. As shown in Fig. 1A, the purified enzyme

appeared as a single protein band in the SDS-PAGE spectrum, suggesting homogeneous enzyme preparation. Size exclusion chromatography on a SephacrylS-300 HR column with reference proteins revealed that the native molecular mass of BPG was 61 kDa (Fig. 1B), which was almost the same as the single 60 kDa-polypeptide identified by SDS-PAGE. Therefore, the native BPG was identified as a monomeric protein; this observation is similar to previous findings on the subunit structure of several plant *β*-glucosidases such as *prunus domestica* seed, soybean okara, and chayote (Chen et al., 2013; Chiou et al., 2010; Mateos et al., 2015).

3.3. *Effect of temperature and pH on purified BPG*

The chromogenic substrate *p*NPG was selected as a model reactant to assess the effect of temperature and pH on the hydrolytic activity of the purified BPG. It can be clearly seen that the optimal temperature for enzyme activity was 55 °C (Fig. 2A), which is lower than those (60 °C and 70 °C, respectively) of *β*-glucosidases from almond and apple seeds, but higher than those (45−50 °C) from soybean okara and chayote (Chiou et al., 2010; Mateos et al., 2015; Yu et al., 2007). Regarding the thermal stability, Fig. 2B illustrates that the catalytic activity of BPG decreased drastically during a 480 min-incubation period at the optimal temperature, with only 50.0% of its original activity being retained after 120 min. Fortunately, a good residual activity of about 77.2% could be achieved at 45 °C even after a the prolonged 480 min-incubation period.

Of the tested pH values ranging from 3.0 to 8.0 depicted in Fig. 2C, the acidic environment (pH 4.0−6.0) seems to be advantageous for BPG in the production of *p*-nitrophenol; the optimum pH was 5.0, which was relatively low compared to the pH required by most microbial origin *β*-glucosidases (Chan et al., 2016). Moreover, the pH stability assay shows a slight reduction in the enzymatic activity in the pH range of 5.0−8.0. In particular, after 24 h of incubation, BPG from

black plum seeds still maintained 90.4% residual activity, thereby indicating its excellent pH stability and potential industrial applications.

3.4. Effect of metal cations and reagents on purified BPG

Studies on the influence of metal ions and reagents on enzyme activity were also carried out under the reaction conditions described above. The results in Table 2 show that among the eight metal ions examined, the catalytic activity of BPG was clearly enhanced by divalent cations like Mn^{2+}, Ca^{2+}, and Cu^{2+} at a concentration of 5 mM, while no significant inhibition occurred in the presence of Zn^{2+}, Mg^{2+}, and Co^{2+}. However, like most *β*-glucosidases reported (Chen et al., 2013; Krisch et al., 2012; Maitan-Alfenas et al., 2014; Yu et al., 2007), the sulfhydryl oxidants of Hg^{2+} and Ag^{+} nearly completely inactivated the enzyme, which suggestes that both types of ions are competitive to the substrate *p*NPG. In contrast, it was interesting to find that the thiol reducing agent DTT exerted a very slight effect on the enzyme. This means that the thiol groups in BPG protein may not participate in the catalytic reaction, which agrees well with observations made in previous studies (Chen et al., 2013; Lucas et al., 2000). Furthermore, the chelating agent EDTA had no effect on the purified enzyme, implying that this purified BPG is a non-metallic enzyme.

3.5. Studies on kinetic and glucose inhibition constants

To comprehensively analyze the enzyme derived from black plum seeds, the kinetic constants (K_m and V_{max}) corresponding to the activity of BPG against *p*NPG were measured by the Lineweaver-Burk method. As shown in Fig. 3A, the K_m and V_{max} values of the enzyme were estimated to be 0.58 mM and 90.2 μmol/(min·mg protein), respectively. Obviously, the K_m value is significantly lower than that of other well-known *β*-glucosidases from plant sources, such as the apple (1.2 mM) (Yu et al., 2007), almond (2.24 mM) (Romeu et al., 1994), and *prunus domestica*

seeds (3.09 mM) (Chen et al., 2013), which indicates that BPG exhibits an extremely high substrate specificity.

It has been well demonstrated that competitive inhibition by glucose is a common peculiarity of *β*-glucosidases obtained from various sources (Cao et al., 2015; Li et al., 2012). Most microbial *β*-glucosidases, usually present relatively low K_i values, which is considered a major obstacle limiting their industrial applications. Therefore, it is particularly important to discover novel *β*-glucosidases, which have a high resistance towards glucose inhibition. Dixon plots for the *p*NPG hydrolysis by BPG are shown in Fig. 3B. Surprisingly, BPG afforded a much higher K_i value of 193.5 mM compared to that of most microbial *β*-glucosidases (0.6−8.0 mM) (Sinha and Datta, 2016). Furthermore, to the best of our knowledge, normally only plant origin *β*-glucosidases show such a high tolerance to glucose. This suggests that the *β*-glucosidase developed in this study is potentially useful in industrial applications.

3.6. Effect of ILs on the enzyme structure

Although biocatalysis in ILs has been the focus of extensive research and lots of work have been reported on the interactions between enzyme proteins and ILs using different strategies (Kumar et al., 2017), no empirical rules could be established to guide the rational choice of ILs in biocatalytic processes. It is usually time-consuming to find an appropriate IL for a given function by using the trial and error approach. For example, Yang et al. reported that the addition of a certain amount of [C_4MIm][I] into the reaction mixture could increase the yields of arylalkyl *β*-D-glucopyranosides catalyzed by *β*-glucosidase by 0.2−0.5 fold (Yang et al., 2012), while Reddy and co-workers observed the unfolding and denaturation of *α*-chymotrypsin protein in the same IL (Reddy et al., 2015). Furthermore, such denaturation could be successfully offset by adding

[C_4MIm][Br], which usually acts as a powerful devitalizer for biocatalysts. The reason for this phenomenon remains unknown.

Therefore, nine imidazolium-based ILs were firstly selected to elucidate the molecular basis of the impact of IL physicochemical properties on enzyme behavior. As shown in Fig. 4A and 4B, secondary structural changes in the protein not only depend on the anions of the ILs, but also on the cations. The α-helix contents of the protein treated with the chosen ILs were lower than that of the protein in buffer (20.4%) (Fig. 4A), indicating that the protein unfolding and flexibility enhancement occurred. The increased exposure of the hydrophobic cavity and perturbation of the microstructure caused by various ILs in protein might account for the decreases of the α-helix contents (Shu et al., 2011). Most interestingly, upon elongating the alkyl chain length of the cations of PF_6^--based ILs from C2 to C8, the α-helix content gradually increased from 9.2% to 11.4%, while the β-sheet content decreased (Fig. 4B). However, when the anion in the ILs changed from PF_6^- to BF_4^-, the α-helix content decreased while that of β-sheet increased, unlike the case with PF_6^--based ILs. In our early study, [C_6MIm][BF_4] has been proven to be the best co-solvent to achieve a high enzyme activity compared to the other ILs (Bi et al., 2012). Obviously, the experimental data clearly shows that there was no corresponding relation between the secondary structural changes and catalytic activity of β-glucosidase in the examined IL systems. Similar phenomenon was also obtained with regard to the influence of twenty-four eutectic ILs on the performance of horseradish peroxidase (Wu et al., 2014). Moreover, the reduced of hydrogen-bond basicity of the halogen atoms (Cl > Br > I) in ILs may lead to the observed reduction in α-helix contents.

Green [Ch][AA] ILs, which possess excellent biodegradability and low toxicity, has potential

applications in the fields of biotransformation and biomass pretreatment (Liu et al., 2012). Unexpectedly, black plum seed *β*-glucosidase displayed a poor catalytic performance in these novel solvents (data not shown). To further examine their impact on the catalyst, we assessed the secondary structure of the enzyme protein in buffers containing seventeen [Ch][AA] ILs. As shown in Fig. 4C and 4D, the protein displayed a trend similar to that of imidazolium-based ILs with respect to the variation in *α*-helix and *β*-sheet, except in the case of [Ch][Pro]. Further, it is worth emphasizing that these structural changes did not correlate with the properties of [Ch][AA] ILs and the enzyme activity.

Several research groups suggested that the reduction in *α*-helix content and increase in *β*-sheet content might affect the enzyme active region and improve its activity (Liu et al., 2011; Wu et al., 2014). However, the results obtained in this study do not seem to comply with the above assumptions. It is well recognized that the admirable physicochemical properties of ILs derive from their excellent ability to form intra- and inter-molecular hydrogen bonding, which changes the energy barrier and allows the reaction to progress (Hunt et al., 2015). Therefore, we rather believe that not only the changes in the enzyme structure, but also the multiple interactions occurring between the reactant, product, and solvent with ILs contribute significantly to the biocatalysis process.

4. Conclusions

In conclusion, we have purified and characterized a novel *β*-glucosidase with desired features of good thermal and pH stability, remarkable substrate affinity, and high glucose tolerance from inexpensive and available black plum seeds. Further investigations indicated that the variation trend of the enzyme secondary structure in PF_6^--based ILs was completely opposite to that

observed in BF_4^--based ILs. For amino acid-based ILs, their physicochemical properties also showed ruleless impacts on enzyme structures. These findings will not only provide an efficient plant origin *β*-glucosidase, but also enrich the fundamental theories of the ILs-biomacromolecule relationship.

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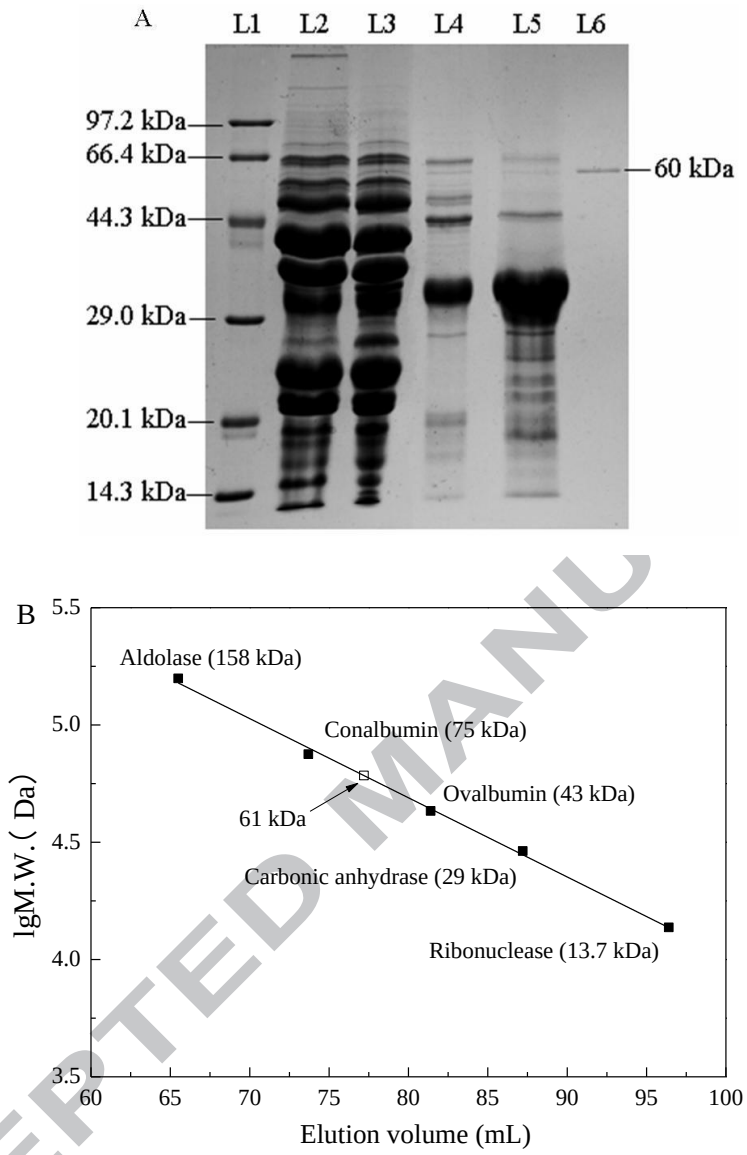
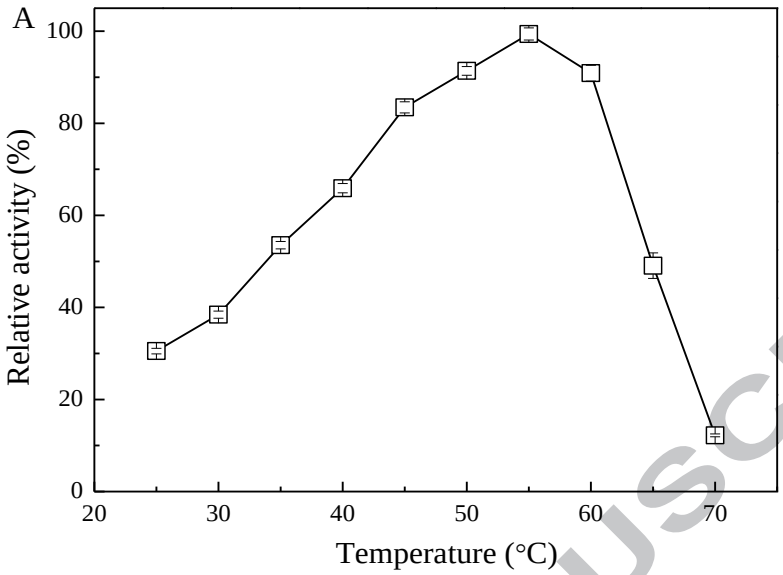
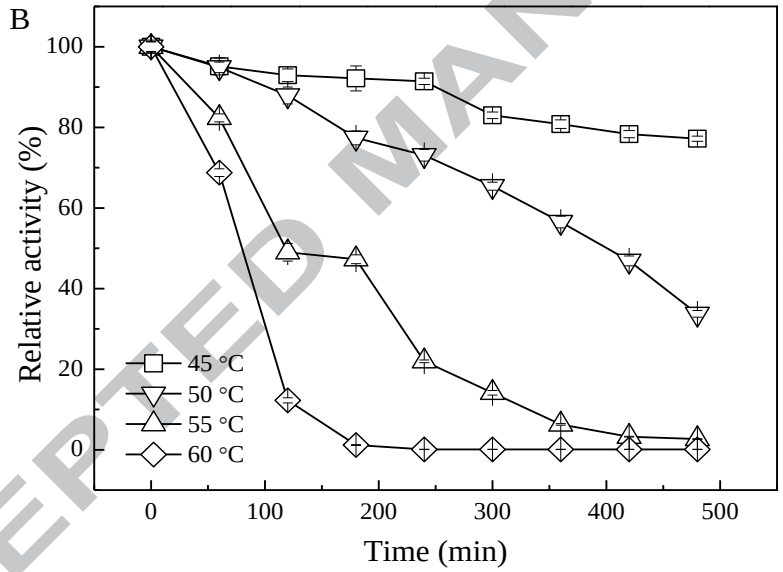


Fig. 1. SDS-PAGE analysis and molecular mass determination of BPG. (A) SDS-PAGE analysis of purified protein samples from black plum seed (12% polyacrylamide). Lane 1, molecular weight markers; Lane 2, crude enzyme extract; Lane 3, dissolution of $(NH_4)_2SO_4$ precipitation; Lane 4, fractions after anion-exchange chromatography; Lane 5, fractions after hydrophobic chromatography; Lane 6, fractions after size exclusion chromatography. (B) Determination of molecular mass of BPG by size exclusion chromatography. The reference proteins (■) are ribonuclease A, carbonic anhydrase, ovalbumin, conalbumin, and aldolase, respectively. The purified BPG (□) eluted at a volume corresponding to 61 kDa.





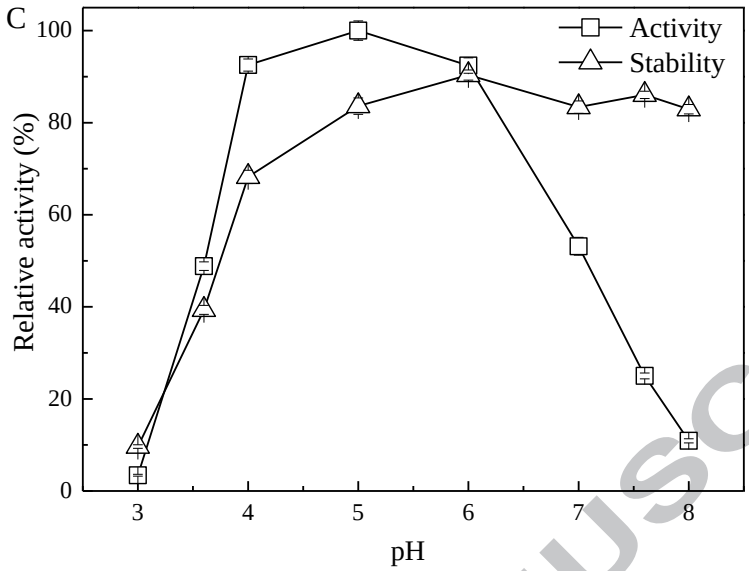


Fig. 2. Effect of temperature and pH on BPG activity. (A) The activity was measured in the temperature ranging from 25 °C to 70 °C in buffer (50 mM, pH 7.0). (B) To characterize the thermal stability, the enzyme was incubated at the indicated temperatures for 480 min. (C) The activity was measured in the pH values ranging from 3.0 to 8.0 at 55 °C. For pH stability, the enzyme was incubated at the indicated pH values for 24 h.

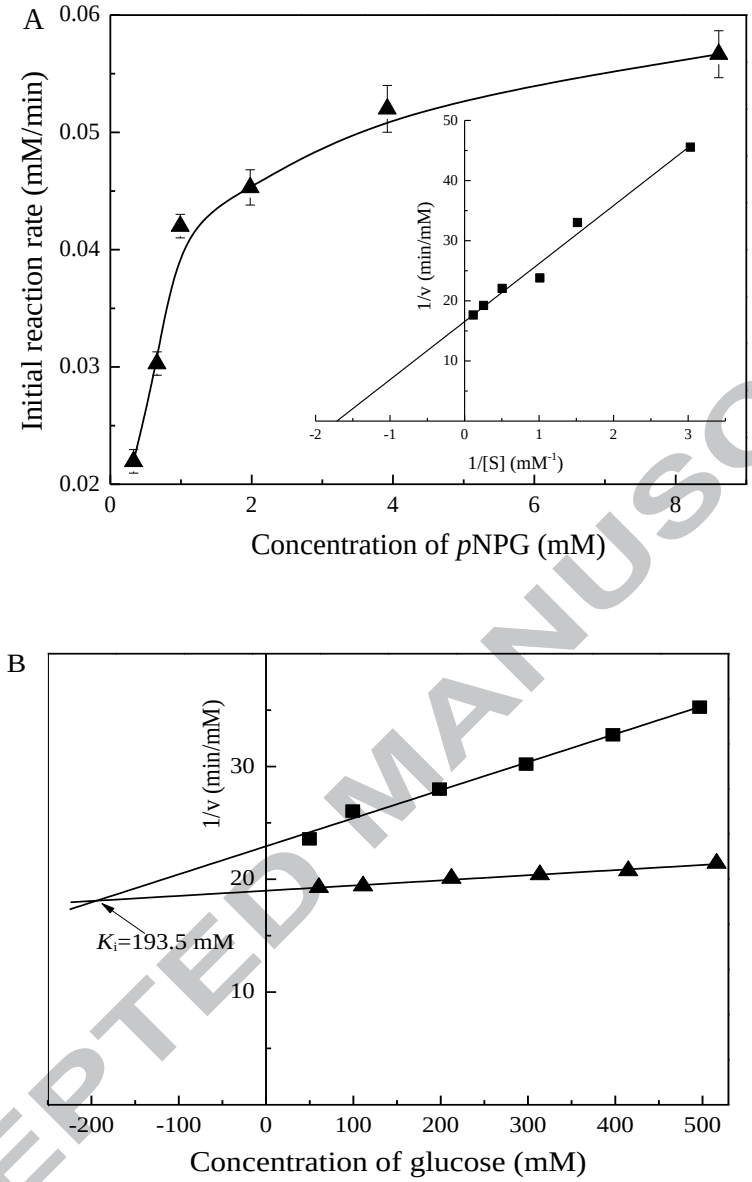
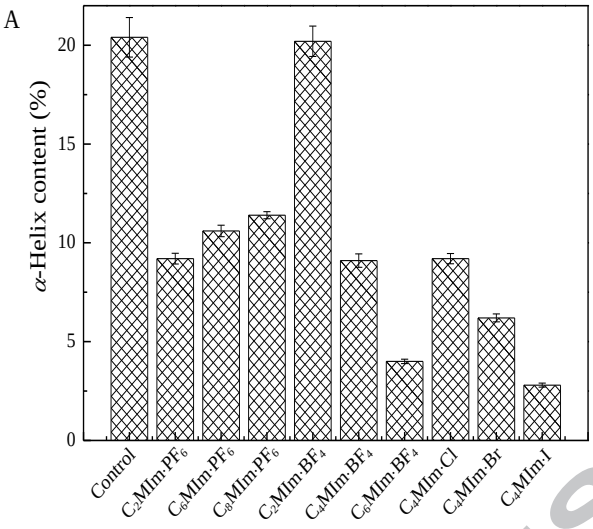
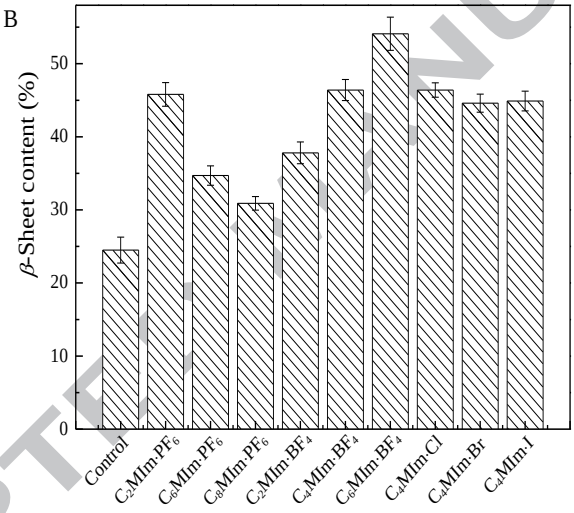
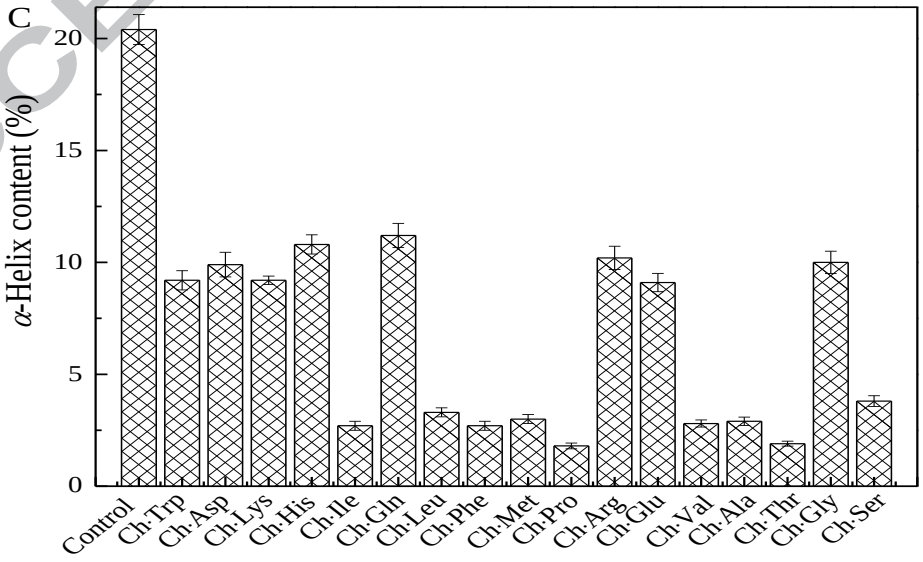


Fig. 3. Determination of the kinetic and glucose inhibition constants. (A) Effects of different *p*NPG concentrations on BPG activity (*inset* of Hanes-Woolf plot). The reactions were carried out at pH 5.0 and 55°C by adding various amount of *p*NPG into 50 mM buffer. (B) Dixon plot of the inhibition of the glucose. The reactions were carried out in the presence of fixed concentration of *p*NPG [*p*NPG: 1.32 mM (■) and 5.19 mM (▲)] at pH 5.0 and 55°C.







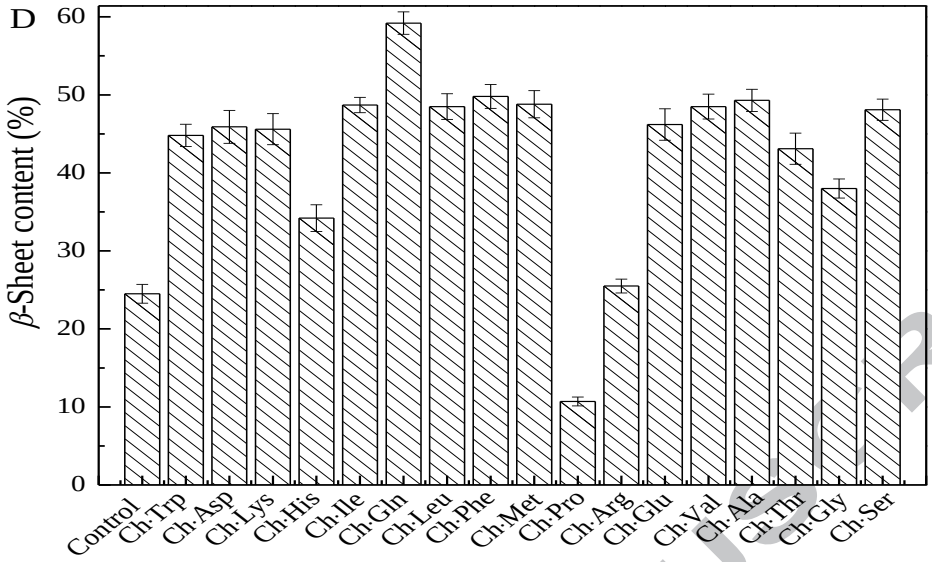


Fig. 4. Secondary structure contents of BPG in the presence of 9 imidazolium- and 17 amino acid-based ILs obtained from CD studies.

Table 1 Summary of purification of *β*-glucosidase from black plum seed

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification (fold)
Crude extract	226.2±6.5	1583.3±10.2	0.14±0.01	100.0±0.0	1.0±0.0
$(NH_4)_2SO_4$ (40-65%)	60.2±1.2	412.4±8.1	0.15±0.01	26.6±0.7	1.1±0.03
DEAE-52	45.8±1.0	26.0±0.5	1.8±0.05	20.2±0.5	12.8±0.3
phenyl Sepharose CL-4B	13.5±0.3	3.3±0.1	4.1±0.2	6.0±0.2	29.5±0.7
Sephade G75	2.7±0.1	0.3±0.01	9.1±0.3	1.2±0.04	64.8±0.8

Table 2 Effects of metal cations and chemical reagents on BPG activity

Reagent	Concentration	Relative activity (%)
No addition	0 mM	100.0±0.0
Zn^{2+}	5.0 mM	98.4±0.2
Mg^{2+}	5.0 mM	95.5±0.1
Mn^{2+}	5.0 mM	133.3±0.3
Ca^{2+}	5.0 mM	116.8±0.3
Cu^{2+}	5.0 mM	117.0±0.6
Co^{2+}	5.0 mM	83.5±1.1
Hg^{2+}	5.0 mM	3.0±0.1
Ag^{+}	5.0 mM	2.4±0.1
EDTA	5.0 mM	107.3±0.8
DTT [a]	5.0 mM	87.7±0.7
SDS	5.0 mM	66.6±0.8
Triton X-100	1.0% (v/v)	49.8±1.3
Tween 80	5.0% (v/v)	90.9±0.4

[a] Dithiothreitol.

Highlights

- A novel *β*-glucosidase from black plum seeds was firstly purified and characterized.
- The enzyme shows lower kinetic K_m value and higher glucose inhibition capability.

- Structural changes in protein depended on the anions and cations of ionic liquids.
- Amino acid-based ionic liquids exhibited ruleless impacts on the enzyme structure.