Effect of Glycosylation of β-Glucuronidase on its Catalytic Properties in Ionic Liquids

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The purification of glycosylated recombinant β-glucuronidase from *Pichia pastoris* GS115 (PGUS-P) was investigated by a novel two-step process: ammonium sulfate precipitation and molecular sieve chromatography. The highest purification fold obtained was 66.79. The catalytic properties of glycosylated PGUS-P in hydrophobic ionic liquids (ILs)/buffer biphasic system were investigated. A 2.2-fold enhancement in the catalytic efficiency was observed using 50% (v/v) 1-butyl-3-methylimidazolium hexafluorophosphate, in comparison with the acetate buffer medium. When compared with the glycosylated PGUS-P, the deglycosylated enzyme at T_{55} and T_{65} exhibited low activity and low thermal stability in both ILs and acetate buffer. It was also observed that ILs had effect on the pH profile of deglycosylated PGUS-P — the optimum pH was extended from 5.0 (acetate buffer) to 5.0-7.0 (ILs). Therefore, this study indicates that the glycosylation of PGUS-P plays an important role in both catalytic activity and stability in hydrophobic ionic liquids (ILs)/buffer biphasic system.

Key words:
β-glucuronidase, glycosylation, ionic liquids, thermostability

Introduction

Licorice, the root of *Glycrrhiza* spp. *(Fabaceae)*, has been used for thousands of years in China. In traditional Chinese medicine, licorice is one of the most frequently used drugs[1]. Glycyrrhizin (GL), an important triterpenoid saponin[2], is the main active pharmacological ingredient of licorice. GL has anti-inflammatory, anti-ulcerous, anti-viral, and anti-allergic efficacy, and is also commonly used as sweetener and toner in the food industry. GL has two derivatives: glycyrrhetic acid 3-O-mono-β-D-glucuronide (GAMG) and glycyrrhetic acid (GA), which can be produced by hydrolyzing one or two glycosidic bonds from GL. GAMG is attractive due to its stronger physiological functions in comparison with GL, and its sweetness is 5-fold higher than that of GL with lower calories[3]. In addition, GA can be used as an additive in cosmetics due to its scavenging ability of oxygen free radicals. Therefore, GAMG and GA are expected to be better food additives as well as therapeutic agent, which has more commercial potential than GL[4].

In our previous work, we screened a fungal strain *Penicillium purpurogenum* Li-3, which used GL as a carbon source and converted it to GAMG by the secreted β-glucuronidase[5]. The gene was cloned (GenBank Accession No. EU095019) and overexpressed in *Pichia pastoris* GS115 for the high production of β-glucuronidase. Although a large amount of N-glycosylated PGUS-P wasproduced, the glycan content of PGUS-P was estimated to be 14.42 %[6]. Glycosylation is one of the major naturally occurring modifications of protein's structure in eukaryotic cells, and the attached carbohydrate chains plays an integral role in the functional properties of glycoproteins[7–10]. Basically, there are two different types of protein glycosylation: N-glycosylation, at asparagine residues within the consensus sequence Asn-X-Ser/Thr and O-glycosylation, at hydroxyl groups of serine and threonine residues[11,12]. It has been enumerated that over half of the proteins present in nature are glycosylated, with more than three-quarters of these glycoproteins containing N-linked carbohydrates[13]. Scientists have assessed that glycosylation plays an important role in maintaining the thermostability and activity of proteins[14–16].

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Ionic liquids (ILs) are functional solvents which are used as the reaction media in many biocatalytic processes[17,18]. Many reactions such as organic, inorganic, and organometallic have been reported to be performed in ILs[19,20]. In contrast to conventional organic solvents, ILs have many favorable properties, such as low vapor pressure, high ionic conductivity, wide liquid range and high dissolving ability[21]. ILs like 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) and 1-butyl-3-methylimidazolium tetrafluoroborate ([EMIM]BF_4) are known to be good alternatives to organic solvents in bioconversion[22]. The use of ILs in enzymatic reactions leads to remarkable improvements in thermostability, stereoselectivity, regioselectivity, and inhibition of side reactions[23–27]. ILs are also known as rather toxic solvents, since they can influence the stability and activity of enzyme through the anions' nucleophilic properties and hydrogen bonding[28–30].The interaction formed by hydrogen bonds between anion and enzyme is very strong, which may cause a conformational change in the enzyme, thus affecting the enzyme activity. Baker *et al.*[31] found that the thermostability of green fluorescent protein (GFP) decreased in ILs containing [Bmim][Cl] compared to aqueous solution at relative low temperature. For most ILs, solvents may interact with the enzyme's secondary structure via many different but simultaneous solute-solvent interactions (hydrogen bonding or other strong interactions). The effect of ILs may change with the change of these interactions: some solvents strip off the tightly bound water molecules that may result in deactivation of the enzyme, while others elevate reaction kinetics by pulling open the enzyme substrate channels. Many studies have expressed the need for quantitative parameters to describe the ILs in cases where the individual interactions have a direct effect on the reaction products, product ratio, kinetics, or enzyme activity[32,33]. We previously found that enzyme activity changed in ILs but the substrate-specificity remained the same. This demonstrates that ILs can affect enzyme conformation but this effect remains limited[34].

Understanding the impact of glycosylation modifications on enzyme stability in ILs is helpful to rationally engineer enzymes for improved function in ILs environment. Nordwald and Kaar have proved that the modification by ionic liquids can increase the half-life of chymotrypsin, lipase and papain[35]. Here, we tried to improve enzyme properties in ILs and elucidate the effect of glycosylation on the function of enzymes in ILs environments.

In this research, a new purification method of PGUS-P was established to support our further study. We then compared the difference in catalytic efficiency and thermostability between the glycosylated and deglycosylated PGUS-P in ILs.

Materials and methods

Materials

The recombinant strain *P. pastoris* GS115 used in this study was constructed in our previous research work[6]. The 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) and 1-ethyl-3--methyl limidazolium tetrafluoroborate ([EMIM]BF_4) were purchased from Henan Lihua Pharmaceutical Co. Ltd. (Henan, China). Glycyrrhizin and glycyrrhetic acid were purchased from Sigma Chemical Co. Ltd. (USA). The HPLC grade methanol was purchased from Tianjin Fuyu Chemical Co. Ltd. (Tianjin, China). All other chemicals used in this study were of analytical reagent grade.

Growth conditions

A single colony of recombinant *P. pastoris* was isolated from YPD (1 % yeast extract, 2 % peptone, 2 % glucose, and 2 % agar) plate and inoculated into 200 mL BMGY medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 1.61 μM biotin, 0.004 % histidine, 1 % glycerol) in a 500-mL shaker flask, followed by incubation at 30 °C and 220 rpm for 18 hours. The cells were then harvested by centrifugation at 8,000 rpm for 10 minutes, and resuspended in 100 mL BMMY medium (1 % methanol instead of glycerol as the sole carbon source).

Purification of recombinant β-glucuronidase (PGUS-P)

The extracellular β-glucuronidase was isolated by centrifuging the 300 mL fermentation broth at 15,000 rpm at 4 °C for 20 minutes. The supernatant was mixed with $(NH_4)_2SO_4$ (70 % saturation) and stored overnight at 4 °C followed by centrifugation. The precipitate was dissolved in 10 mM, pH 7.4 Tris–HCl buffer (buffer A) and then dialyzed (10,000 molecular weight cut-off) overnight in buffer A. The resultant crude enzyme solution was applied onto a Superdex 75 column (1.6 cm × 20 cm; flow rate 1.0 mL min^{-1}) equilibrated with buffer A. The enzyme was eluted with a linear NaCl gradient (0.1–0.5 M) and the activity of elutes was investigated. The active fractions were preserved at 4 °C.

We calculated the purification fold and recovered enzyme activity as the standard procedure. Firstly, we defined the crude enzyme's purification fold as 1.0 and its recovered enzyme activity as 100 %. After the purification process, we calculated the increase in specific activity as the purification fold, and defined the decrease in the total enzyme activity as recovered enzyme activity.

Deglycosylation by peptide-N-glycosidase F

Peptide-N-glycohydrolase F (PNGase F, Sigma) was used to deglycosylate the recombinant β-glucuronidase (PGUS-P). This was carried out by incubating reaction mixture of 1 mL β-glucuronidase (1 mg mL^{-1}) and 9 mL 50 mM Tris–HCl buffer (pH 7.0), and the reaction was launched by adding 100 IU PNGase F followed by incubation at 37 °C for 12 hours.

SDS-PAGE analysis of the recombinant PGUS

The recombinant PGUS in the supernatant was analyzed by polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed with 12 % polyacrylamide gels using the Bis–Tris SDS-PAGE system. The gels were then stained by Coomassie brilliant blue G250 (Amresco), and the recombinant PGUS was identified by densitometric analysis (ProExpress Imaging System, Perkin Elmer).

Determination of enzyme activity and protein concentration

PGUS-P activity was determined by Glycyrrhizin (GL) hydrolysis. The assay mixture consisted of 10 μL enzyme solution and 990 μL 2 g L^{-1} GL (pH 5). After incubation at 37 °C for 30 minutes, the reaction was stopped by incubation in boiled water for 5 minutes, and then centrifuged at 10,000 rpm for 5 minutes. The supernatant (10 μL) was further analyzed by HPLC equipped with a C18 column (4.6 × 250 mm, 5 μm particle size, Kromasil) at 40 °C. The mobile phase was a mixture of methanol and water containing 0.6 % acetic acid (81:19 v/v). Elution was monitored with UV detection at 254 nm. The amount of GAMG wascalculated from the standard curve between the peak area and concentration of GAMG. One enzyme unit (U) of activity was defined as the amount of enzyme that released 1 μmol GAMG in the reaction mixture per minute. PGUS-P concentration was determined by using NanoDrop spectrophotometer 2000C (Thermo Scientific Co., Ltd (USA)). We set the purification buffer (10 mM Tris–HCl, pH 7.4) as blank control, and loaded a 2 μL sample to determine the protein concentration.

GL hydrolysis in ILs system

Three reaction systems were constructed: the first one only contained NaAc-HAc (pH 5.0) in aqueous system as control, the second one was 10 % (v/v) $[BMIM]PF_6$ biphasic system, the third one was 10 % (v/v) $[EMIM]BF_4$ aqueous system. The assay mixture consisted of 10 μL enzyme solution and 990 μL 2 g L^{-1} of GL in these three systems.

Determination of enzyme thermostability

The purified enzyme was incubated at 55 °C and 65 °C. Samples were withdrawn for enzyme assay at specific time intervals. The residual activity was determined by taking the original activity as 100 %.

Results and discussion

Purification of PGUS-P

As shown in Fig. 1, two peaks were obtained after purification with Superdex 75 column. The activity assay by HPLC confirmed that only peak 2 sample showed activity. Then, the molecular weight of sample 2 was investigated by SDS-PAGE. The molecular weight was 78 kDa, consistent with PGUS-P (Fig. 2, Lane 1). The electrophoretic purity

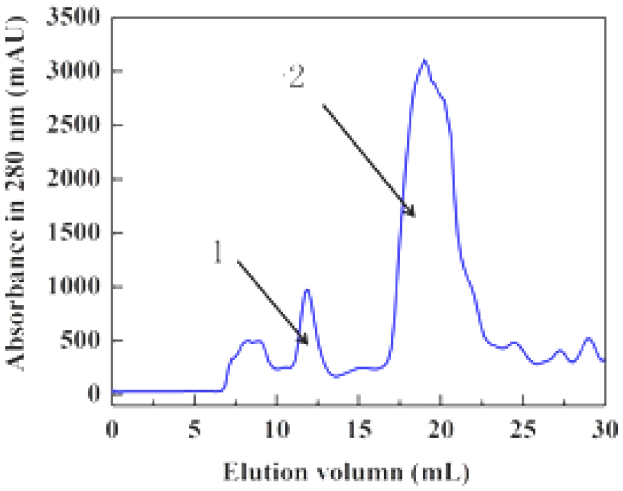

Fig. 1 – *Purification of PGUS-P by gel filtration chromatography on Superdex 75 column. The activity assay by HPLC confirmed that only peak 2 sample showed activity.*

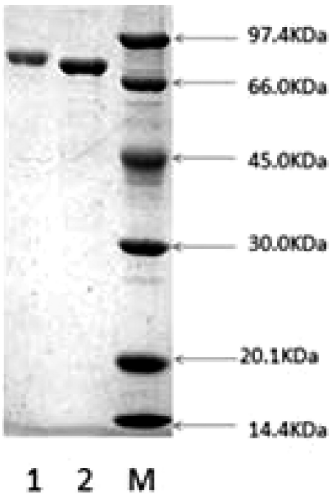

Fig. 2 – *SDS-PAGE analysis of purified PGUS-P treated with PNGase F. Lane 1: PGUS-P (78 kDa); Lane 2: deglycosylated PGUS-P treated with PNGase F (68 kDa); Lane M: marker.*

of PGUS-P was obtained by two-step purification process, including ammonium sulfate precipitation and molecular sieve, which reduced the activity loss and increased enzyme purity. The recovery yield and purification fold at each step is shown in Table 1. After the two-step purification, the specific activity was 506.24 U mg^{-1}, purification fold was 66.79, and the activity recovery yield was 65.0 %. Therefore, the new purification method shortens the purification process from four steps to two steps with a relatively high purification fold, and increases the efficiency of purification compared to previous article[6].

Table 1 – *The purification of PGUS-P*

Steps	Total protein [mg]	Total activity [U]	Specific activity [U mg^{-1}]	Recovery [%]	Purification fold
Fermentation broth	635.6	4821.3	7.58	100.0	1.0
$(NH_4)_2SO_4$ precipitation	59.38	4269.1	71.89	88.5	9.48
Superdex 75	6.19	3133.65	506.24	65.0	66.79

Deglycosylation of PGUS-P

PGUS-P was deglycosylated with PNGase-F under native conditions. As shown in Fig. 2, the molecular weight of PGUS-P decreased from 78 kDa to 68 kDa after treatment with PNGase-F, indicating that the glycan moiety was removed and the corresponding molecular weight was estimated to be 10 Da. The molecular weight of deglycosylated PGUS-P treated with PNGase F (PGUS-P+F) was 68 kDa, which was consistent with our previous work[6].

Effect of N-glycosylation on PGUS-P activity in ILs

The catalytic properties of glycosylated PGUS-P were investigated in the first system containing NaAc-HAc (pH 5.0), and the second system containing [BMIM]PF_6, while other conditions remained constant, as shown in Fig. 3. In the 10 % (v/v) [BMIM]PF_6 biphasic system, the catalytic efficiency increased 2.2 fold when compared with that in the aqueous phase. These results indicated that [BMIM]PF_6 could increase the activity of PGUS-P. We also compared the effect of glycosylation on the catalytic properties of PGUS-P in ILs. In this biphasic system, the catalytic activity of deglycosylated PGUS-P decreased by 12.8 %. With the extension of time, the enzyme stability became the dominant factor. The inactivation rate of deglycosylated PGUS-P was much higher than that of PGUS-P after 90 minutes. In the aqueous system, the inactivation rate of deglycosylated PGUS-P and

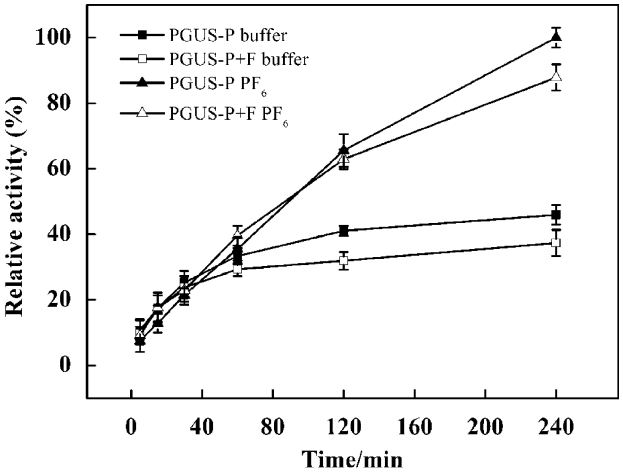

Fig. 3 – *Effect of glycosylation on the catalytic properties of PGUS-P in [BMIM]PF6. Preparations of glycosylated PGUS-P and deglycosylated PGUS-P treated with PNGase F (PGUS-P+F) (0.4 U mL^{-1}) were reacted with GL under the same conditions. The relative activity of PGUS-P and PGUS-P+F was determined in NaAc-HAc buffer or 50 % [BMIM]PF_6. The highest activity was taken as control (100 %). Each value in the panel represents the mean of triplicate ± one standard deviation.*

PGUS-P both greatly increased, and the catalytic activity of deglycosylated PGUS-P was lower than that of PGUS-P in the entire process. Without the protection of ILs and glycosylation, deglycosylated PGUS-P seemed very fragile.

The catalytic properties of glycosylated PGUS-P were also investigated in the first system containing NaAc-HAc (pH 5.0) and the third system containing [EMIM]BF_4, while other conditions remained constant. As shown in Fig. 4, in the hydrophilicionic liquids, 1-ethyl-3-methylimidazolium tetrafluorobo-

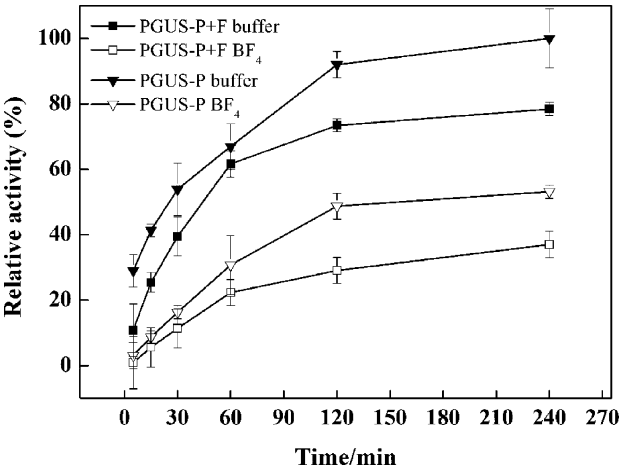

Fig. 4 – *Effect of glycosylation on the catalytic properties of PGUS-P in [EMIM]BF_4. Glycosylated PGUS-P and deglycosylated PGUS-P treated with PNGase F (PGUS-P+F) (0.4 U mL^{-1}) were reacted with GL under the same conditions.The residual activity of PGUS-P and PGUS-P+F was determined in NaAc-HAc buffer or [EMIM]BF_4. The highest activity was taken as control (100 %). Each value in the panel represents the mean of triplicate ± one standard deviation.*

rate ([EMIM]BF_4), a similar trend of enzyme performance between glycosylated and deglycosylated PGUS-P in ILs was observed as that in [BMIM]PF_6. But in this system, the catalytic efficiency decreased about 2-fold when compared with that in the control, which may be due to the fact that the increased polarity of ILs was toxic to the enzymes (Fig. 4). This result is also similar to references. Wang *et al.*[36] investigated the activity and thermostability of horseradish peroxidase in [C_2min][BF_4], [C_4min][BF_4] and [C_6min][BF_4]. It was found that the activity of horseradish peroxidase decreased with increasing ionic liquids concentration.

Based on the above results, it was found that the [BMIM]PF_6 system was favorable for the catalytic activity of PGUS-P, so it was chosen in the following in-depth research.

Effect of [BMIM]PF_6 volumetric ratio on PGUS-P activity

To figure out the best volumetric ratio of 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) in ILs, PGUS-P activity was investigated in ten biphasic systems with different [BMIM]PF_6 volumetric ratio, as shown in Fig. 5. The activity of PGUS-P was relatively high in the 50 % – 70 % (v/v) [BMIM]PF_6 biphasic system. With further increase in the [BMIM]PF_6 volumetric ratio, the enzyme activity gradually decreased and lost almost all the activity in 100 % (v/v) [BMIM]PF_6 ILs. The possible reason may come from two aspects. Firstly, the [BMIM]PF_6 ionic liquids owns a relatively high viscosity (215.4 MPa s)[37] and it may greatly affect

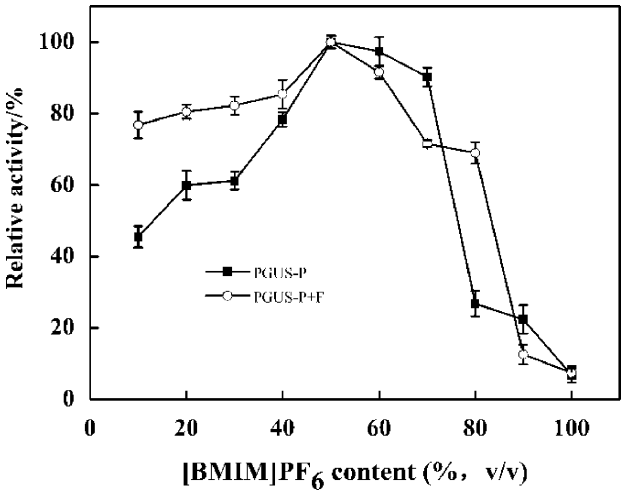

Fig. 5 – *Effect of [BMIM]PF_6 content on the hydrolysis of GL by glycosylated PGUS-P and deglycosylated PGUS-P treated with PNGase F (PGUS-P+F). Preparations of glycosylated PGUS-P and PGUS-P+F were reacted with GL in [BMIM]PF_6 (pH 5.6) at 37 °C for 1 h. The highest activity was taken as control (100 %). Each value in the panel represents the mean of triplicate ± one standard deviation.*

the PGUS-P activity by changing the mass transfer behavior in the reaction system. In addition, ILs are among the most complex solvents. Given their structure and diversity of functionality, they are capable of providing various types of interactions (e.g., dispersive, ð-ð, n-ð, hydrogen bonding, dipolar, ionic/charge-charge). With increasing [BMIM]PF_6 volumetric ratio, the interactions between anion and enzyme becomes stronger, and this may cause a great conformational change to the enzyme, thus affecting enzyme activity[38–42]. Besides, due to the hydrophilic nature of PGUS-P, it gains a low solubility in the hydrophobic ionic liquids, which may also affect the enzyme catalysis.

The activity of deglycosylated PGUS-P was relatively high in the 50 % – 70 % (v/v) [BMIM]PF_6 biphasic system and peak in 50 % (v/v) [BMIM]PF_6. Normally, the activity of deglycosylated PGUS-P was higher than glycosylated PGUS-P, indicating that the affinity between PGUS-P and substrate was improved after removing covalently bound oligosaccharides.

Effect of N-glycosylation on PGUS-P thermostability

In order to investigate the effect of glycosylation on the thermostability of β-glucuronidase, the reactions catalyzed by glycosylated and deglycosylated PGUS-P were performed in aqueous system and biphasic system at 55 °C and 65 °C (Fig. 6). In [BMIM]PF_6 biphasic system, the catalytic activity of both glycosylated and deglycosylated PGUS-P were significantly higher than that in aqueous system. Either in aqueous system or biphasic system, deglycosylated PGUS-P catalytic activity was much lower than that ofglycosylated PGUS-P. In conclusion, glycosylation and [BMIM]PF_6 ionic liquids can promote the thermostability of PGUS-P. After deglycosylation, the thermostability of PGUS-P decreased at both 55 °C and 65 °C.

The possible explanations for these results are that the glycosylation could make the protein structure more rigid and promote the catalytic efficiency. After the deglycosylation, the three-dimensional structure would change and adjust itself thus affecting the cleavage and formation free energy and the stability of enzyme[26,43]. Previous studies have also demonstrated that the N-glycosylation enhanced the structural robustness of proteins and caused great decrease in dynamic fluctuations throughout the entire molecule, which led to an increase in the thermostability[44]. Jafari-Aghdam *et al.*[45] suggested that glycosylation was a key factor to protect the enzyme from heat denaturation.

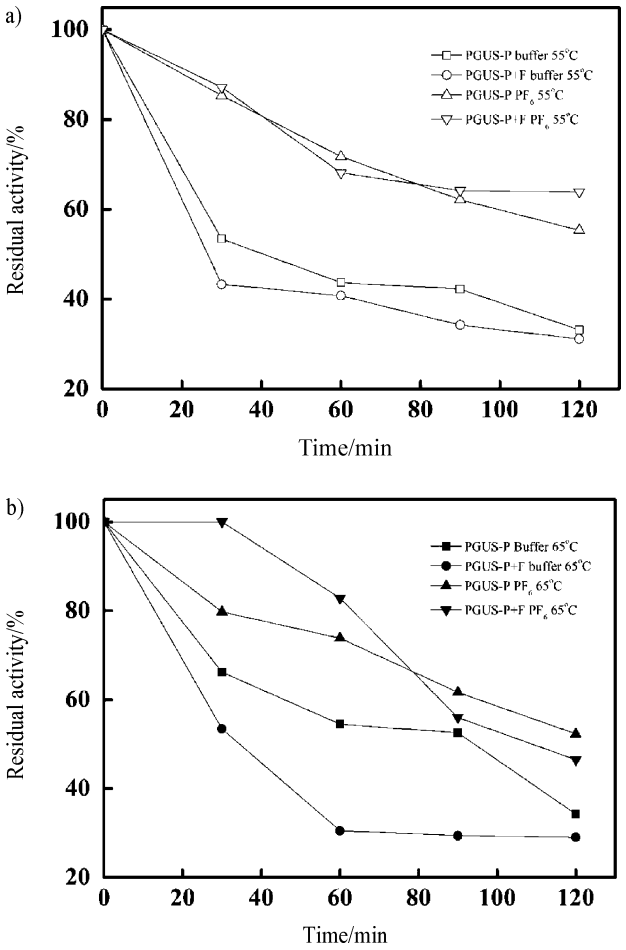

Fig. 6 – *The thermostability of glycosylated PGUS-P and deglycosylated PGUS-P treated with PNGase F (PGUS-P+F) at 55 °C (a) and 65 °C (b). The residual activity of PGUS-P and PGUS-P+F were determined in either NaAc-HAc buffer (pH 5) or 50 % [BMIM]PF_6 buffer (pH 5) at specified temperature. The original activity was taken as control (100 %).*

Effect of pH on PGUS-P activity

The effect of pH (4.0 – 8.0) on the activity of deglycosylated and glycosylated PGUS-P in aqueous and ILs systems was investigated, as shown in Fig. 7. In the aqueous system, the extent of catalysis by deglycosylated PGUS-Pwas very similar to the level observed for glycosylated PGUS-P. However, the catalytic activity of deglycosylated PGUS-P was slightly higher than that of glycosylated PGUS-P, and increased drastically reaching a maximum at pH 5. Both of them dropped sharply above pH 5.0. In the biphasic system, the activity of glycosylated PGUS-P dropped sharply from pH 5.0 compared with deglycosylated PGUS-P, which still exhibited substantial catalysis from pH 5.0-7.0. Apparently, the hydrophobic ionic liquid [BMIM]PF_6 enhanced the catalytic efficiency of deglycosylated PGUS-P under neutral conditions, and N-glycosylation played a vital role in the pH tolerance of PGUS-P.

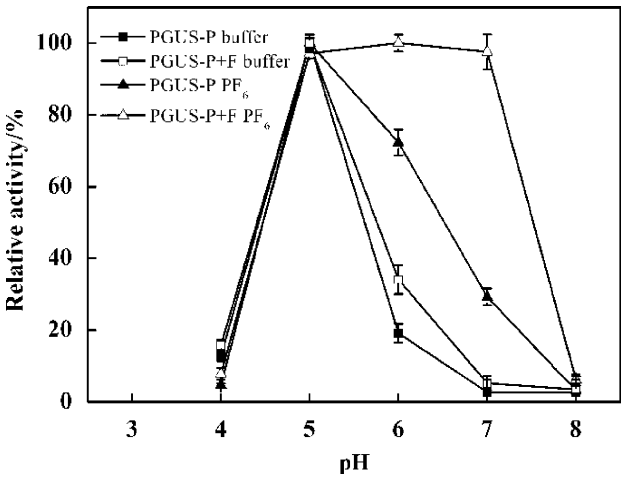

Fig. 7 – *Effect of pH on the activity of glycosylated PGUS-P and deglycosylated PGUS-P treated with PNGase F (PGUS-P+F)*

Conclusion

In this study, we found that the thermostability of PGUS-P was closely related to the glycosylation. Although both forms of PGUS-P shared the same optimal temperature of 40 °C, the deglycosylated PGUS-P was significantly less active than the glycosylated PGUS-P at 55 °C and 65 °C. Efficiency and operational stability of PGUS-P was also investigated in aqueous and ILs media. PGUS-P displayed significantly higher catalytic efficiency and production yield in [BMIM]BF_6 biphasic medium compared to the aqueous medium. Moreover, the hydrophobic ionic liquids was beneficial to the catalytic behavior of deglycosylated PGUS-P under normal conditions.

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Nomenclature

U – activity unit
IU – international unit

Abbreviations

PGUS-P – recombinant β-glucuronidase from *Pichia pastoris*
[BMIM]PF_6 – 1-butyl-3-methylimidazolium hexafluorophosphate
[EMIM]BF_4 – 1-butyl-3-methylimidazolium tetrafluoroborate

GL – glycyrrhizin
GAMG – glycyrrhetic acid 3-O-mono-β-D-glucuronide
GA – glycyrrhetic acid
GFP – green fluorescent protein
PNGase F – peptide-N-glycohydrolase F
PAGE – polyacrylamide gel electrophoresis
HPLC – high-performance liquid chromatography

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