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Highly enhancing the characteristics of immobilized thermostable β-glucosidase by Zn^{2+}

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ABSTRACT

The thermostable GH3 β-glycosidase (Tpebgl3) from *Thermotoga petrophila* DSM 13995 was immobilized on macroporous resin NKA-9 modified with polyethylenimine (PEI) and glutaraldehyde (named NKA-9II). The properties of NKA-9II were as follows: the optimal conditions were the same as that of the free enzyme (pH 5.0; 90 °C), and the highest activity with cellobiose as the substrate approached 1.7 U/g; the thermostability, pH stability and glucose tolerance were greatly improved; the residual activity of NKA-9II was 68% of the initial activity at the end of 10 repeated cycles. Moreover, it was found that 2 mM Zn^{2+} increased the relative activity of NKA-9II to 192% and 199% with cellobiose and *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrates, respectively. Meanwhile, Zn^{2+} could greatly improve the reusability, high-temperature stability, and glucose tolerance of NKA-9II. In particular, 84% of the residual activity of NKA-9II with 2 mM Zn^{2+} was retained, which was 21% higher than that with free metal ion after incubation at 85 °C for 7 h; when the glucose concentration was 400 mM, the free Tpebgl3 was completely inactivated, and NKA-9II with 2 mM Zn^{2+} maintained 63% of its initial activity, which was 19.5% higher than the activity of NKA-9II in the absence of Zn^{2+}.

1. Introduction

Tpebgl3 (EC 3.2.1.21), a thermostable recombinant GH3 β-glucosidase from *Thermotoga petrophila* DSM 13995, is widely used in cellulose hydrolysis and the biological conversion and transformation of flavonoids and saponins. For example, it can transform ginsenoside Rb1 or Rd into the minor ginsenoside 20(S)-Rg3 [1], which has better pharmacological activity than the precursors, especially in the treatment of tumors [2]. Tpebgl3 does not resemble many regular β-glucosidases; it has high catalytic efficiency and high specificity. However, the industrial application of bioactive enzymes requires the ability to resist extreme conditions and changeable environments such as extreme pH values, high temperatures, and high glucose concentration [3]. The costs of preparation and microbial contamination of the enzyme are also substantial barriers to industrial application. Immobilization is one of the common technical means of obtaining efficient biocatalysts using a solid support and could solve the problems mentioned above. Moreover, the advantages of immobilization techniques could allow repeated and continuous operation of the enzyme and improved enzyme activity, stability and selectivity [4,5]. Further, solid supports enable higher enzyme activity and are environmentally acceptable [6]. Among the many alternative supports, the properties of resins include high specific surface area (250–700 m^2/g) and pore diameter (2–30 nm) that can host enzymes of various sizes. The solid carrier can act as a protective wall by increasing and improving thermostability, pH stability, and enzyme recoverability. Therefore, mesoporous resins demonstrate high potential as solid supports for enzyme immobilization [7]. Immobilization techniques for enzymes include physical adsorption, entrapment, and chemical interactions (either covalent or non-covalent) [8]. Simple covalent linkages between the carriers and protein usually change the structure of the enzymatic protein and the active site of the natural enzyme because of the inflexible linkage between the enzyme and the solid support [9–11]. The adsorption, retention of native protein state, and reuse of the enzyme are considered so that the binding between the support and the enzyme is very weak, leading to enzyme leakage while the biocatalyst is operated in a reaction system, especially in aqueous media [12]. Thus, it is necessary to achieve enzyme binding to supports by physical means or chemical modification. PEI is

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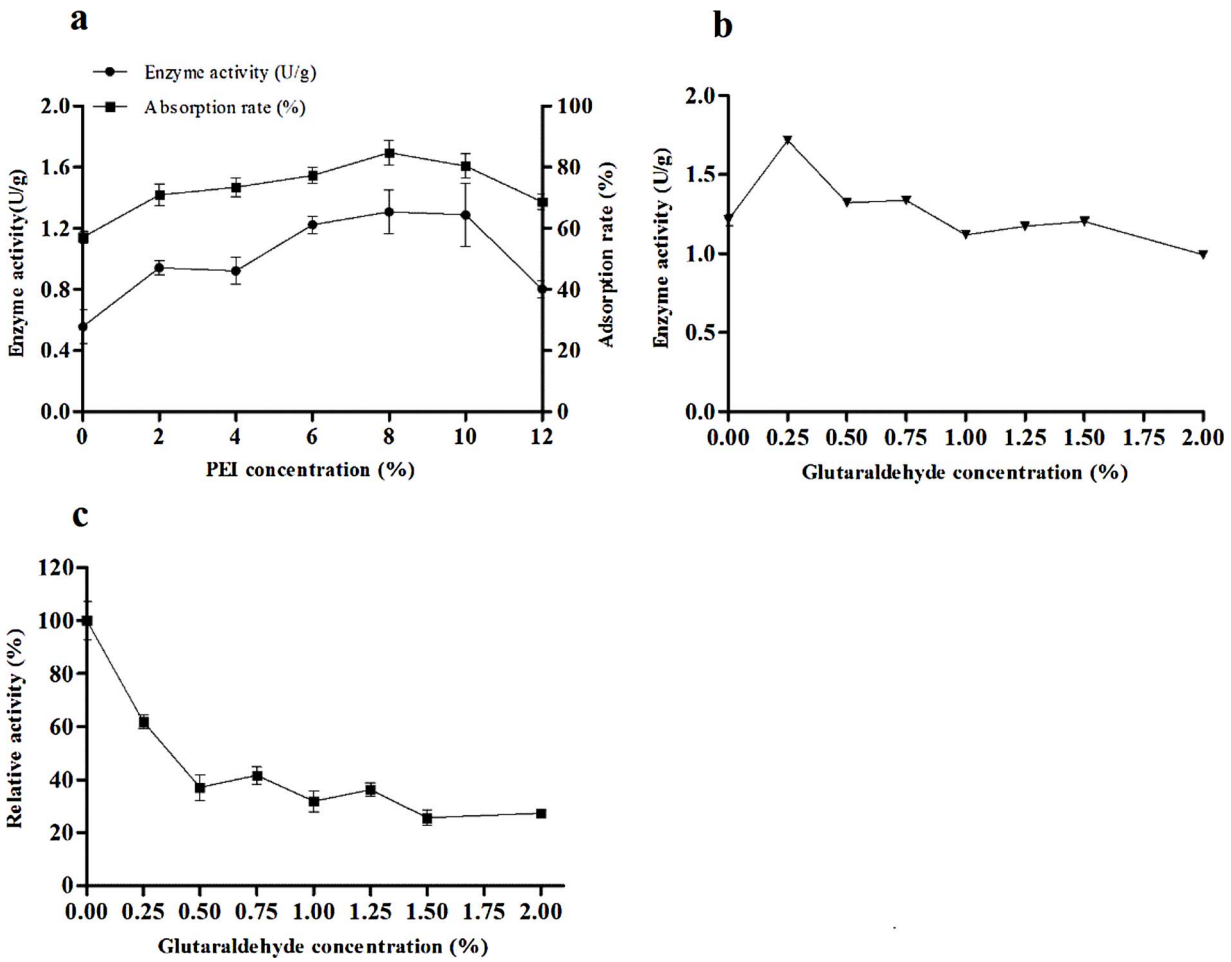

Fig 1. Modification of NKA-9 (a: the activity of immobilized enzyme and the adsorption degree of immobilized protein were determined; b: the activity of immobilized enzyme coated with different concentration of glutaraldehyde, the activity of immobilized enzyme with 0.25% glutaraldehyde was defined as 100%; c: the effect of glutaraldehyde on free enzyme; free Tpebgl3 kept at pH 4.5 and coated with 0, 0.25%, 0.50%, 0.75%, 1.00%, 1.25%, 1.50%, and 2.00% of glutaraldehyde at room temperature for 2 h).

a polymer containing primary, secondary and tertiary amino groups, having strong anion exchange capacity under a broad range of conditions, and the capability to chemically react with different moieties on either an enzyme or a support. Therefore, the support could be modified with polyethylenimine (PEI) to reduce enzyme desorption from the support and confer a strong ion exchange character to the enzyme [13–16]. Moreover, glutaraldehyde, a good cross-linking agent [17], has been widely applied in enzyme immobilization.

In addition, the influence of metal cations on the characteristics of free enzyme is always assayed in many studies [18,19]. However, the effects of cations on the properties of immobilized enzymes, including enhancing catalytic efficiency and increasing reusability, thermal stability [20], and substrate tolerance, were not usually observed. The interaction between metal ions and proteins is based on Lewis acid/base theory [18]; when accepting an electron pair, the metal ion, usually as a Lewis acid, integrates with a protein to affect the activity of the enzyme.

Immobilization of Tpebgl3 can lead to its widespread application in industrial biological catalysis and transformation with higher activity [21,22]. Moreover, because of the high-temperature of the GH3 Tpebgl3, it is important to select a material that is resistant to high temperature as the carrier for immobilization [23]. In this study, NKA-9 was selected as the solid support that immobilized Tpebgl3. The structures of NKA-9 were modified with a certain amount of PEI and glutaraldehyde; the properties of immobilization were greatly improved. Specifically, adding the metal cation Zn^{2+}, the catalytic activity, reusability, thermal stability, and glucose tolerance of the immobilized enzymes were more advantageous than other reports about immobilized enzymes.

2. Materials and methods

2.1. Materials

Tpebgl3 from *Thermotoga petrophila* DSM 13995 was expressed by the recombinant *E. coli* BL21 (DE3) and has been described elsewhere. The macroporous adsorption resin NKA-9, which is crosslinked polystyrene with macroporous structure, was purchased from San Xing Resin Technology (Anhui, China). Polyethylenimine (M.W:1800) and glutaraldehyde were purchased from Macklin (Shanghai, China) and Sinopharm Chemical RCo., Ltd. (Shanghai, China), respectively. Glucose Assay Kit was purchased from Shanghai Rongsheng Biological Technology Co., Ltd. (Shanghai, China).

2.2. Preparation and treatment of macroporous resins

To more effectively maximize the amount of the adsorbed enzyme, the macroporous resin was pretreated to remove impurities and contaminants. The macroporous resin (20 g) was soaked in ethanol (100 mL) for 24 h and then washed repeatedly with deionized water three times. The macroporous resin was soaked with 5% hydrochloric acid (100 mL), and then, the resin was washed with deionized water until it reached a neutral pH. The resin was soaked in 1 M sodium hydroxide (100 mL) for 4 h, and then, the resin was washed with

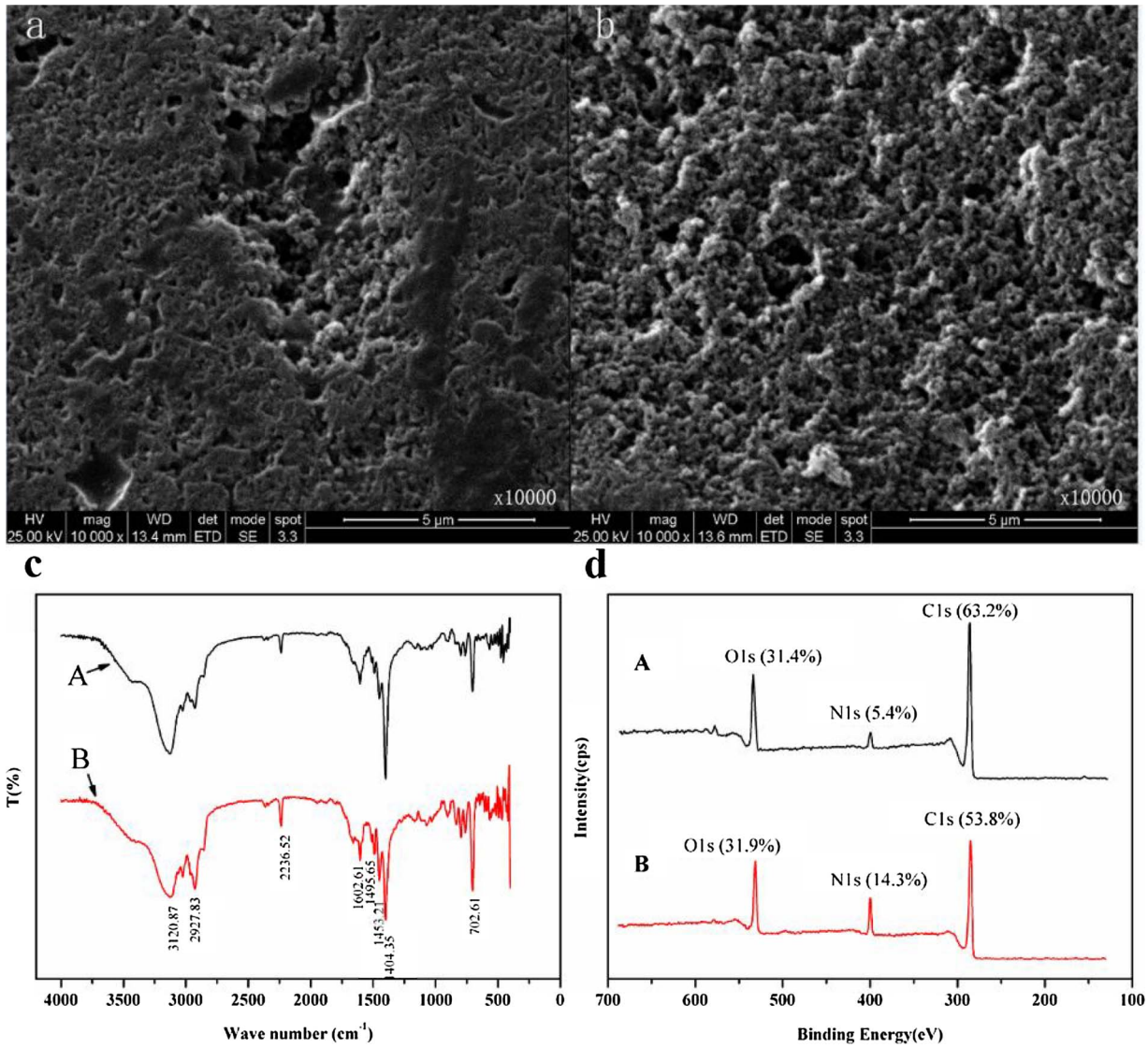

Fig. 2. The morphology and structure were characterized by ESEM, FT-IR, and XPS (a: NKA-9 without modification, amplification X10000; b: NKA-9 with 6% PEI treatment, amplification X10000; c: results of FITR, (A) NKA-9 modified by PEI, (B) NKA-9I, IR KBr (cm^{-1}): 3121 (aliphatic, C—H), 2928 (aromatic, C—H), 2237 (C═C), 1603, 1496, 1453, 1404,703 (aromatic frame); d: results of XPS, (A) NKA-9 modified by PEI, (B) NKA-9I).

deionized water until it reached a neutral pH. After filtration, the resins were stored at 4 °C.

2.3. Assays of enzyme activity and protein concentration

The activity of the free and immobilized enzyme Tpebgl3 was measured. One method comprised a mixture (200 μL) containing 5 mM cellobiose, citric acid Na_2HPO_4 buffer (100 mM, pH 5.0), and an appropriate diluted ratio of free Tpebgl3 or immobilized enzyme instead of free enzyme (10 mg). After incubation at 90 °C in a shaking water bath for 20 min, the reaction was ended through cooling with an ice bath. Then, the concentration of glucose in the reaction sample was detected with a Glucose Assay Kit, and it was measured at wavelength 505 nm by using an absorbance microplate reader (SpectraMax190, Molecular Devices, LLC, Sunnyvale, CA). Another method was a mixture (200 μL) that contained 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), citric acid Na_2HPO_4 buffer (100 mM, pH5.0), and an appropriate diluted ratio of free Tpebgl3 or 10 mg immobilized Tpebgl3. After incubation at 90 °C in a shaking water bath for 20 min, the reaction was terminated by the addition of 600 μL of 1 M Na_2CO_3 and measured at wavelength 405 nm by using an absorbance microplate reader. One unit of enzyme activity [U] was defined as the amount of enzyme required for the liberation of 1 μmol glucose or *p*NP per minute under the assay conditions. And free enzyme and immobilized were marked with U/mL and U/g, respectively. The protein concentration was examined with the Bradford protein Assay Kit (Sangon Biotech, Shanghai, China), and the reaction mixture contained 200 μL Bradford protein Assay Kit and 6 μL detected sample. Their calibration curves are given in the Supplementary material S1, S2, and S3.

2.4. Surface modification and characterization of immobilized enzyme

The macroporous resin (1 g) was modified with 0, 2%, 4%, 6%, 8%, 10%, or 12% PEI. The reaction, kept at 28 °C, pH 4.5 and shaken at 150 rpm, was inoculated in 100 mL mixture for 4 h, and then, it was washed with deionized water. The modified resin was incubated in a mixture (10 mL) kept at 28 °C and 150 rpm for 12 h, and the mixture contained 10 mM pH 4.5 citric acid Na_2HPO_4 buffer and 0.5 mg/mL Tpebgl3. After reaction, the mixture was directly coated with 0, 0.25%, 0.50%, 0.75%, 1.00%, 1.25%, 1.50%, or 2.00% glutaraldehyde at the same reaction condition for 2 h. Each step was washed with deionized water to remove unbound enzyme and other excess components,

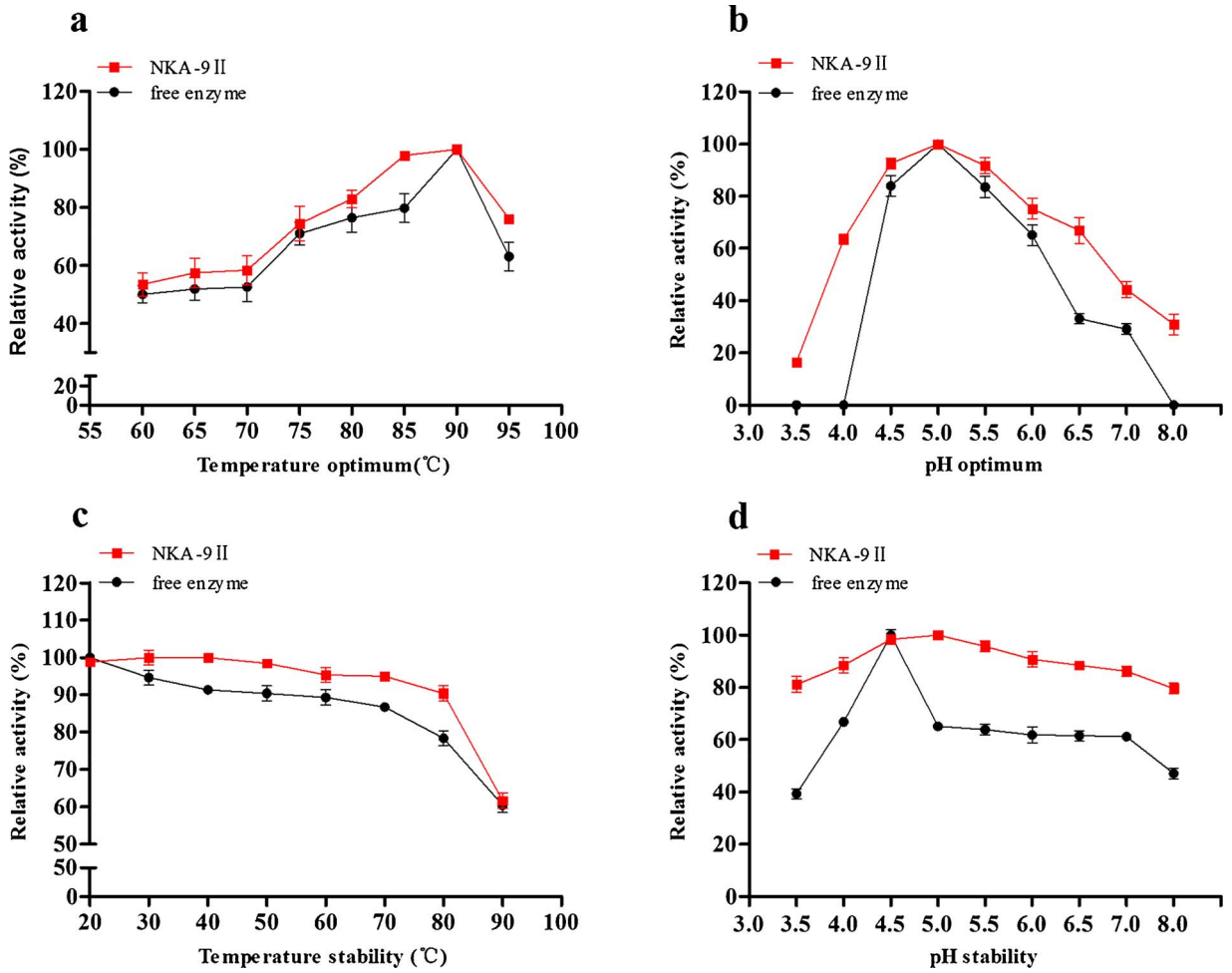

Fig. 3. The properties of free Tpebgl3 and NKA-9II (optimal temperature, optimal pH, temperature stability, and pH stability are shown in panels a, b, c, and d, respectively; the initial activity of NKA-9II at the optimal condition was defined as 100%, and these activities were expressed as relative values).

Table 1
Effects of metal cations on the activity of free Tpebgl3 and NKA-9II.

Cation of reagent	Substrates		Fe^{2+}	Li^{+}	Al^{3+}	K^{+}	Ni^{2+}	Mn^{2+}	Ca^{2+}	Zn^{2+}	Mg^{2+}
Relative activity (%)	Cellobiose	free	88.4 ± 1.0	88.1 ± 1.0	90.6 ± 1.0	110.2 ± 1.2	97.1 ± 1.1	113.5 ± 1.2	100.4 ± 1.1	108.4 ± 1.2	98.6 ± 1.1
		NkA-9II	141.1 ± 1.1	109.0 ± 0.9	94.2 ± 0.7	106.7 ± 0.8	91.0 ± 0.7	166.3 ± 1.3	145.3 ± 1.1	192.3 ± 1.4	93.7 ± 0.7
	*p*NPG	free	102.7 ± 0.3	86.4 ± 0.4	89.2 ± 0.5	101.8 ± 0.2	77.3 ± 0.2	109.3 ± 0.4	99.1 ± 0.6	95.7 ± 0.4	114.0 ± 0.1
		NkA-9II	87.9 ± 0.7	66.8 ± 0.6	78.7 ± 0.4	80.2 ± 0.3	81.2 ± 0.7	153.4 ± 0.5	104.4 ± 0.3	199.1 ± 0.6	121.0 ± 0.8

including the glutaraldehyde solution, through vacuum filtration. The properties of the modified macroporous resin were investigated via Quanta 200 environmental scanning Electron microscopy (ESEM), Fourier transform infrared spectroscopy (FTIR) and X-ray Photoelectron Spectroscopy (XPS); they were from FEI Company (USA), Bruker, (Germany), and KRATOS (UK), respectively.

2.5. The characteristics of free and immobilized enzyme

The effects of temperature on the immobilized and free enzyme activity were observed. The mixture (200 μL) was measured at 60, 65, 70, 75, 80, 90 and 95 °C; the influence of pH on the immobilized enzyme (10 mg) was tested by the one pH gradient method (pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0) at 90 °C, and cellulose was used as a substrate. The temperature and pH stability of the immobilized and free enzyme activity was observed. The immobilized enzyme and free enzyme were incubated at 20, 30, 40, 50, 60, 70, 80, and 90 °C in a water bath for 3 h, and then, the thermal stability of the immobilized or free enzyme was detected with cellulose as the substrate. The pH stability of the immobilized and free enzyme was investigated after they were kept in 100 mM citric acid Na_2HPO_4 buffer (pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0) for 3 h at room temperature.

2.6. Effect of different metal cations on the catalytic activity of the free and immobilized enzyme

In the reaction mixture, the effects of metal cations on the catalytic activity of immobilized enzyme were determined with cellobiose and *p*NPG as the substrate; the final concentrations of metals were all 2 mM. The metal cations included Fe^{2+}, Li^{2+}, Al^{3+}, K^{+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, and Mg^{2+}.

2.7. Effect of Zn^{2+} on the reusability and thermostability of the immobilized enzyme

The activity of the modified immobilized enzyme was measured at

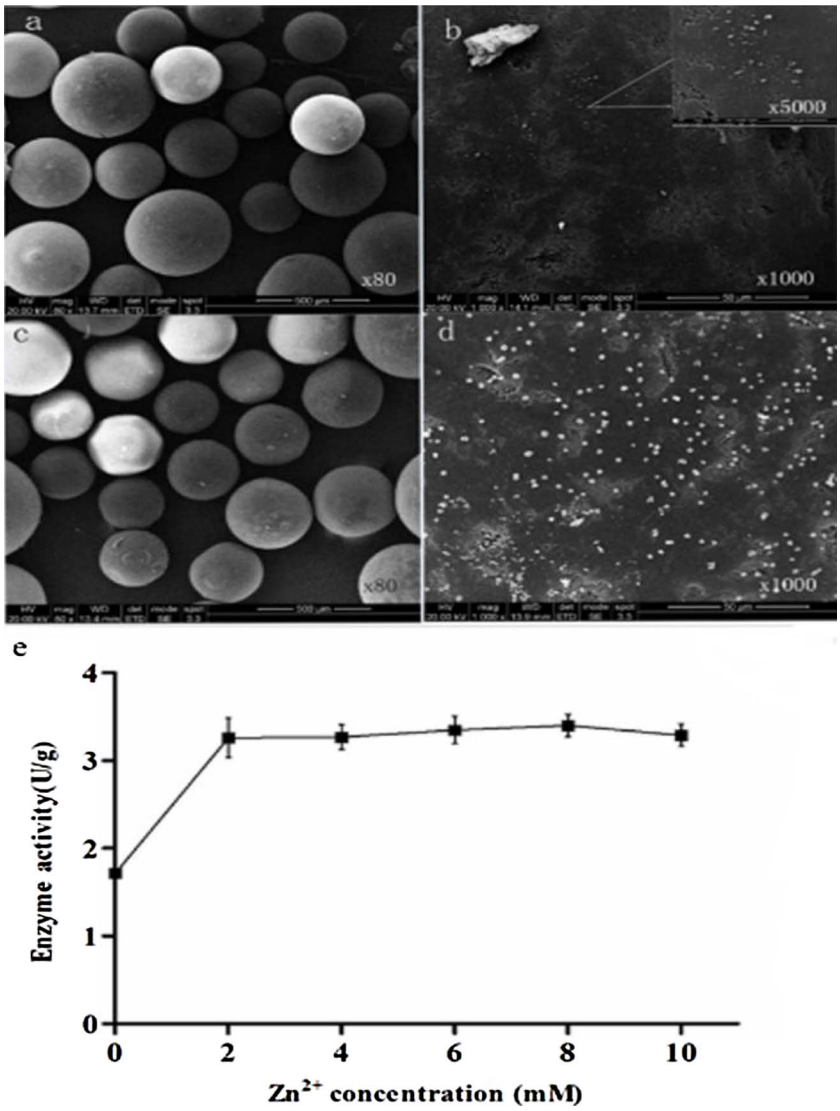

Fig. 4. ESEM of the surface of resin NKA-9II and effect of Zn^{2+} concentration on the activity of NKA-II (a, b: the surface of NKA-9II in absence of cations c, d: the surface of NKA-9II with metal cation ion Zn^{2+}; e: the effect of Zn^{2+} concentration on the activity of NKA-II; a, c, amplification X80; b, d, amplification X1000).

Table 2
Kinetic parameters of NKA-9II in the presence and absence of Zn^{2+} in the reaction mixture.

Concentrations of Zn^{2+}	k_m (mM)	k_{cat} (s^{-1})	k_{cat}/k_m ($mM^{-1}s^{-1}$)
0 mM	2.32 ± 0.3	26.89 ± 2.1	11.6
2 mM	3.03 ± 0.3	51.43 ± 3.3	17.0

90 °C for 20 min in the presence and absence of 2 mM Zn^{2+}, and cellulose was used as the substrate. When the reaction ended, the resin was washed with 100 mM citric acid Na_2HPO_4 buffer. Then, this reaction process was repeated 12 times to measure the reusability and deactivation stability of the immobilized enzyme.

The effect of Zn^{2+} on the thermostability of the immobilized enzyme was also observed. The immobilized enzyme in the presence and absence of 2 mM Zn^{2+} was incubated at 85 and 90 °C,in a water bath for 1–7 h, and then, the thermal stability of the immobilized enzyme was detected with cellulose as the substrate.

2.8. Effect of metal ion Zn^{2+} on the catalytic activity of the immobilized enzyme inhibited by high concentration of glucose

The effect of the glucose concentration on enzyme activity was assessed, and the initial rate of β-glucosidase hydrolysis was sensitive to glucose [24,25]. The reaction, in the presence or absence of Zn^{2+}, was carried out with the initial concentration of glucose ranging from 10 to 400 mM, at a temperature of 90 °C and pH of 5.0 for the free and immobilized enzyme (2 mM Zn^{2+}). *p*NPG was used as the substrate, thereby avoiding the effect of the glucose produced by hydrolysis of cellobiose.

3. Results and discussion

3.1. Modification and characterization of NKA-9

Polar NKA-9 is crosslinked polystyrene. The pore size of NKA-9 is in the range of 20–22 nm, and the specific surface area of NKA-9 is 570–590 m^2/g. The degree of adsorption of protein to the resin is very weak, and enzyme protein is easily lost in aqueous medium. The amount of adsorbed protein can be increased and the interaction between the resin and protein can be strengthened by modifying the structure of NKA-9 [26]. As seen in Fig. 1a, to enhance the loading of the immobilized enzyme, the resin was modified with different concentrations of PEI for 4 h, and then, prepared Tpebgl3 was adsorbed by the modified NKA-9 for 12 h. With the increase of PEI concentration, the activity of the immobilized enzyme and adsorbed protein were significantly increased. The highest activity and adsorption rate were 1.31 U/mL and 84.7%, respectively. It was named NKA-9I, and the

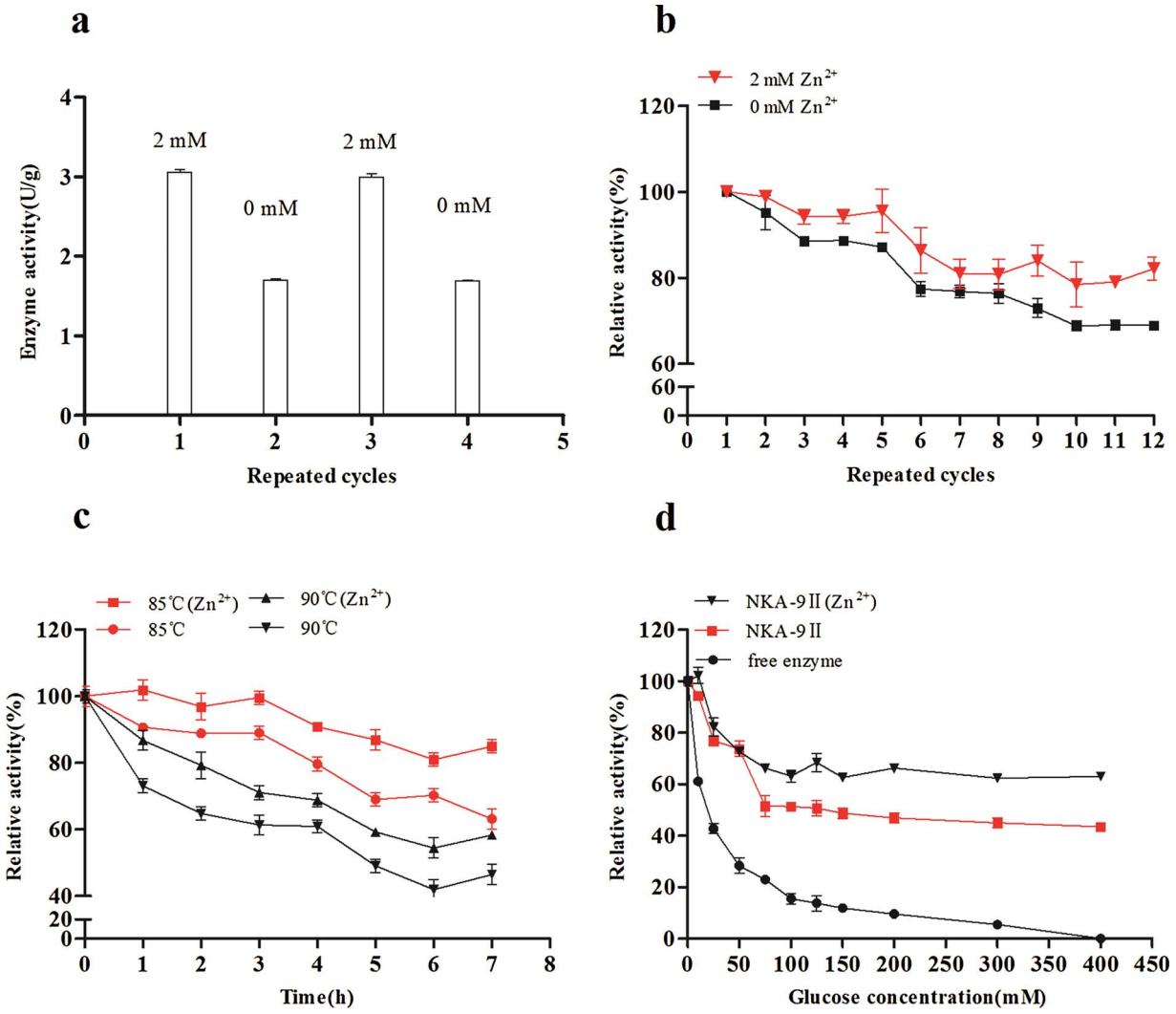

Fig. 5. The effect of Zn^{2+} on the reusability, thermostability, and glucose tolerance of immobilized enzyme (a, b: the effect of Zn^{2+} on the reusability of NKA-9II with 2 mM Zn^{2+} addition or not; the initial activity of immobilized enzyme was defined as 100% and the residual activity was expressed as relative values; c, d: the effect of Zn^{2+} on thermostability and glucose tolerance, respectively; the initial activity of NKA-9II before treatment was defined as 100%, and the activity after treatment was expressed as relative values).

activity of NKA-9I was 2.4-fold greater than that of its original support without PEI (Fig. 1a). As shown in Fig. 2a and Fig. 2b, an image (magnified 10 000 times) of NKA-9 resin surfaces before and after modification by PEI was provided by environmental scanning electron microscopy (ESEM). The result of ESEM suggested that a certain amount of PEI could significantly increase the porosity of NKA-9. FT-IR (Fig. 2c) and XPS (Fig. 2d) results just indicated the increase of nitrogen content and the presence of protein on resin NKA-9 modified by PEI [27]. Further, when NKA-9I was crosslinked with glutaraldehyde (named NKA-9II), the highest activity of NKA-9II was 1.7 U/g with 0.25% glutaraldehyde, which was 3.1-fold that of NKA-9 (0.55 U/g) without modification (Fig. 1b). Although the high concentration of glutaraldehyde has a great influence on free enzyme activity (Fig. 1c), glutaraldehyde treatment was given to the crosslinked polystyrene to enable covalent coupling between free amino groups of Tpebgl3 and the free aldehyde group of glutaraldehyde [28,29]. The results reflected that the structure of the resin was improved by the treatment with PEI and glutaraldehyde, resulting in NKA-9II with high enzyme activity [30].

3.2. The optimal conditions for free and immobilized Tpebgl3

The optimal reaction conditions for NKA-9II were measured, and the reaction temperature of NKA-9II was between 60 and 95 °C because of the thermal properties of free Tpebgl3. The pH was set with the increasing gradient method (pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0). The results are given in Fig. 3a and 3b. The optimal temperature and pH of NKA-9II were 90 °C and pH 5.0, respectively. The optimal reaction conditions for the immobilized enzyme were the same as that for the free enzyme. The activity of NKA-9II remained higher than that of the free enzyme across a larger application range of temperature and pH. Notably, the free enzyme was fully inactivated under a relatively acidic condition (pH 4.0); however, NKA-9II had 63.5% of the optimal enzyme activity. The effect of temperature and pH on the immobilized enzymes was much weaker than that of the free enzyme in cellulose hydrolysis. Moreover, the activity of NKA-9II was more than 30% higher than that of the free enzyme at a pH level of 8.

3.3. The temperature and pH stability of free and immobilized Tpebgl3

The effect of temperature variations on free enzyme and immobilized Tpebgl3 activity was measured. NKA-9II was incubated at different temperatures (20, 30, 40, 50, 60, 70, 80, 90 °C) for 3 h. It can be clearly observed in Fig. 3c that the temperature stability of NKA-9II was better than that of the free enzyme at a high temperature, using cellulose as the substrate. However, the stability of NKA-9II at 90 °C

rapidly decreased; the literatures about temperature stability showed this decrease might be due to leaching of the enzyme from the swollen polymer matrix at a high reaction temperature [14,31]. The influence of different values of pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0) is given in Fig. 3d; NKA-9II retained 79.6% of the initial activity under alkaline conditions (pH 8.0), which was higher by 32% compared with free enzyme. Forty-two percent of the initial activity for NKA-9II under an acidic condition (pH 3.5) remained, but the activity of free enzyme was completely lost at the same condition. The results showed that the loss of free activity at extreme pH values can be attributed to the denaturation of some enzyme molecules. The enzyme immobilized by the resin most likely may follow a specific mechanism, perhaps involving covalent coupling between free amino groups and the free aldehyde group of glutaraldehyde on some specific places of the enzyme surface, and maintain the three-dimensional structure and saturation of the Tpebgl3 [32]. The results showed that the immobilization support could resist the interference of high temperature and extreme pH, and this is consistent with the previous reports [3,33].

3.4. Effect of different metal cations on the catalytic efficiency of the free and immobilized enzymes

The influences of various metal cations on the catalytic efficiency were determined with metal ion solutions at a final concentration of 2 mM (Table 1). When cellobiose was the enzymatic substrate, the activity of free Tpebgl3 was easily inhibited by Fe^{2+}, Li^{+}, and Al^{3+}. Only three metal cations, K^{+}, Mn^{2+} and Zn^{2+}, had a weak positive effect on the activity of free Tpebgl3. Although the divalent metal cations Fe^{2+}, Mn^{2+}, and Ca^{2+} could improve and enhance the catalytic efficiency of the NKA-9II immobilization, the catalytic efficiency of NKA-9II with Zn^{2+} (2 mM), which was nearly 1.92 times higher than that of the free metal ion, was the highest among all the metal cations tested. Al^{3+}, Ni^{2+} and Mg^{2+} have been shown to inhibit the activity of NKA-9II. Moreover, with *p*NPG as the substrate, the relative activity of NKA-9II with metal cations Zn^{2+} and Mn^{2+} increased significantly to 199% and 153%, respectively. It was found that the coordinating metal cation Zn^{2+} could significantly increase the catalytic efficiency of NKA-9II with different substrates. However, high concentration of Zn^{2+}, given in Fig. 4e, had little influence on the activity of NKA-9II. Meanwhile, the values of k_m and k_{cat} in the presence and absence of Zn^{2+} were measured and the results are given in Table 2. K_m reflects structural changes at the active site of the enzyme, and the k_{cat} values reflect specific ion effects [26]. The k_m value is 2.32 mM for immobilized Tpebgl3 without the addition of Zn^{2+}, and the k_m value is 3.03 mM for immobilized Tpebgl3 with 2 mM Zn^{2+}. The slightly higher k_m value with 2 mM Zn^{2+} added was suggestive of alteration in affinity of the active site towards substrate binding, possibly because of diffusion limitation [31]. The k_{cat} of immobilized Tpebgl3 with 2 mM Zn^{2+} increased significantly to 51.43 s^{-1}, which is 1.91 times that of immobilized Tpebgl3 without addition of Zn^{2+}. The catalytic efficiency k_{cat}/k_m could reflect the most complete information about the substrate-enzyme interactions[34], and the value of k_{cat}/k_m in presence of Zn^{2+} was 17.0 mM^{-1} min^{-1}, which is 1.46 times that of the metal cation ion-free control (Table 2). The results indicated that Zn^{2+} could be an optimal activator for the catalytic activity of the immobilized enzyme. Compared to the absence of metal ions (Fig. 4a and b), the results of the ESEM showed there are more protein groups on the surface of the resin activated by metal ion Zn^{2+} (Fig. 4c and d), and the Tpebgl3 was evenly distributed on the surface of the resin, which made the protein be exposed on the surface of the resin, increasing the contact area between the enzyme and the substrate. One possibility was that Tpebgl3 immobilized on NKA-9, showing specific affinity and selectivity toward metal cation Zn^{2+}, was negatively charged in the pH 5.0 buffer, leading to the interaction between Zn^{2+} and the protein [35]. However, the predicted pI of Tpebgl3 is 5.28 [1], and thus, the most likely reason for this finding was that glutaraldehyde cross-linking altered the structure of Tpebgl3 on NKA-9II, leading to shifts in the isoelectric point of the protein. In addition, adding Zn^{2+} changed the binding between the resin and Tpebgl3, which made the protein groups exposed on the surface [36]. This fact also confirms the previously published finding that metal cations are good activators for the free enzyme and provides the first report that the catalytic efficiency of an immobilized enzyme was enhanced by adding metal cations.

3.5. Effect of metal ion Zn^{2+} on the reusability of the immobilized enzyme

Regeneration of carrier is a key step in the reversible enzyme immobilization technique. Thus, it is necessary to estimate the regeneration efficiency of NKA-9II. As given in Fig. 5a, the effect of Zn^{2+} on NKA-9II was investigated with cellobiose as substrate. NKA-9II started with 2 mM Zn^{2+} in the reaction mixture and then 2 repeated cycles in the absence of free metal ion. The activity of NKA-9II with 2 mM Zn^{2+} was 1.80 times higher than the activity after 2 repeated cycles. Moreover, the activity after 3 repeated cycles with the addition of 2 mM Zn^{2+} was 1.76-fold that after 2 repeated cycles and 1.77-fold that after 4 repeated cycles without the addition of Zn^{2+}. These findings might be due to the Zn^{2+} activating the catalytic activity of NKA-9II, but the cation Zn^{2+} was not absorbed by the support and did not form a stable metal-ion bond with protein. Therefore, the reusability of NKA-9II required the participation of Zn^{2+}, and the activity of NKA-9II would be enhanced. The effect of Zn^{2+} on reusability is shown in Fig. 5b. The results showed the activity of NKA-9II with 2 mM Zn^{2+} was maintained at 78% of the initial activity at the end of 10 repeated cycles, which is 10% higher than that with free metal ion as control. The presence of metal ions did not destroy the interaction between the resin and the enzyme protein. The leakage of Tpebgl3 and the denaturation of the resin do not constitute a major problem; the result indicated that using NKA-9II with Zn^{2+} provides great benefits for industrial applications and reduces the high cost of free enzymes with non-repeatability.

3.6. Effect of metal ion Zn^{2+} on the thermostability of the immobilized enzyme

Due to the thermal characteristics of Tpebgl3, the thermostability of the immobilized Tpebgl3 will be critical for enzymatic hydrolysis of pharmacologically active components and industrial-scale applications. The effect of Zn^{2+} on the thermostability of immobilized Tpebgl3 was measured. NKA-9II was incubated at different temperatures (85 °C, 90 °C) for 7 h in the presence and absence of Zn^{2+}. It could be clearly observed in Fig. 5c that the high temperature stability of NKA-9II was improved with cellulose as the substrate. Thermostability assays of the immobilized Tpebgl3 with 2 mM Zn^{2+} showed that 84% of its residual activity was retained, which is 21% higher than that with free metal ion after incubation at 85 °C for 7 h; the residual activity of NKA-9II retained in the presence of 2 mM Zn^{2+} was more than 58% of its initial activity after incubation at 90 °C for 7 h, which is 12% higher than that with free metal ion at a high temperature (90 °C). The results showed the stabilization effect of metal cation Zn^{2+} may be attributed to stabilizing the enzyme surface ionic net by forming some ionic cross-linking or ionic bridges stabilizing the concrete area [32,33,37], mainly due to multimeric proteins where the metal cation plays a critical role in stabilizing the multimeric structure.

3.7. Effect of metal cation Zn^{2+} on the catalytic efficiency of the free and immobilized enzymes inhibited by a high concentration of glucose

Glycosidase, in the degradation of glycosides, will produce a large amount of glucose; glucose inhibits the enzyme's hydrolysis, leading to decrease of the conversion rate and output of the substrate. The glucose tolerance of free Tpebgl3 and the immobilized enzyme was investigated between 10 and 400 mM glucose, and *p*NPG was used as the enzymatic substrate to avoid the effect of the glucose produced by hydrolysis of

cellobiose. The results are given in Fig. 5d. The free enzyme was rapidly inactivated with increasing glucose concentration to 400 mM, and only 15% of the initial activity was retained with 100 mM glucose. NKA-9II with 400 mM glucose concentration maintained 44% of its initial activity. However, metal cation Zn^{2+} could enhance the glucose tolerance and retain 63% of the initial activity of NKA-9II, which is 19.5% higher than the activity of NKA-9II in the absence of Zn^{2+}. On the one hand, it may be that the structure of the resin protected the active site of the enzyme and avoided the influence of the high glucose concentration in the enzyme molecules. On the other hand, the results also showed that the interaction between metal cation Zn^{2+} and Tpebgl3 immobilized on the resin may block the effect of glucose on enzyme activity, resulting in an increase in the glucose tolerance of the immobilized enzyme.

4. Conclusions

The immobilization of thermostable GH3 β-glycosidase (Tpebgl3) was obtained by using NKA-9 macroporous resin modified with PEI and glutaraldehyde. The properties of NKA-9II, including thermal stability, pH stability, reusability, and tolerance of glucose, were greatly improved compared with free Tpebgl3. Moreover, this manuscript provides the first report that the coordinating metal cation Zn^{2+} enabled an immobilized enzyme to exhibit higher catalytic efficiency. Meanwhile, the addition of Zn^{2+} could greatly advance the reusability, thermostability, and glucose tolerance of NKA-9II compared with that with free metal ion, which is extremely beneficial for industrial applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2018.01.004.

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