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Encapsulation of β-galactosidase from *Aspergillus oryzae* based on "fish-in-net" approach with molecular imprinting technique

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ABSTRACT

By using tetraethylorthosilicate as a silica resource and triblock copolymer P123 as a template, the encapsulations of β-galactosidase with three different models of without protection, protection of protective agent and molecular imprinting technique pretreatment were accomplished through modified "fish-in-net" route at pH 5.0. The highest enzymatic activity of β-galactosidase was gained by using pretreatment of molecular imprinting technique. Scanning electron microscopy (SEM) images showed that the matrix of encapsulated β-galactosidase was made of an aggregation of uniform microspheres of 200–300 nm, and N_2 adsorption/desorption isotherms demonstrated that the matrix of encapsulated β-galactosidase possessed average Brunauer–Emmett–Teller (BET) pore size of 27 Å and narrow pore size distribution. More importantly, compared with encapsulated β-galactosidase without protection, the hydrolytic activity of encapsulated β-galactosidase pretreated by molecular imprinting technique was about 3 times and 1.8 times, while the enzymatic activity of encapsulated β-galactosidase with the protection of protective agent increased only 1.3-fold when lactose and o-nitrophenyl-β-D-galactopyranoside (ONPG) were used as substrates, respectively. The protective effect of molecular imprinting technique pretreatment on the enzymatic activity after encapsulation was better than that by protective agent.

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1. Introduction

In the field of fundamental and applied research, the study on β-galactosidase (β-gal) (EC 3.2.1.23) has attracted many scientists' interest for a long time [1,2]. β-Gal can hydrolyze lactose to yield galactose and glucose. Nevertheless, β-gal cannot completely hydrolyze lactose due to inhibition effect of galactose produced in catalytic reaction [3], resulting in limitations in practical application. The immobilization technique has been proposed to overcome this problem to some extent. During the hydrolytic reaction, the separation between immobilized β-gal and the product has been easily undertaken in the heterogeneity solution, which would decrease the competitive inhibition of the lactose and be more favorable for forward reaction. Therefore, many researchers have been dedicated to the study of β-gal immobilization. β-Gal has been immobilized by many methods, such as sol–gel encapsulation, physical adsorption and covalent attachment [4–6]. However, the leakage of β-gal is an inevitable problem in sol–gel encapsulation and physical adsorption method. The enzymatic activity is easily weakened in covalent attachment approach. In covalent immobilization, due to multipoint covalent attachment between the enzyme and support, the leakage of enzyme can be avoided perfectly. However, the strong interaction between enzyme and support may severely alter the conformation of β-gal in the support as compared to its conformation in aqueous solution, which leads to the decrease of enzymatic activity [7]. Hence, how to enhance β-gal stability and reusability in immobilization remains a difficult problem.

In previous work, we had successfully encapsulated several kinds of enzymes (fumarase, trypsin, lipase and porcine liver esterase) in silica-based matrix using "fish-in-net" approach in which the enzyme (acting as "fish") was gradually entrapped into the "net" produced by polymerization and condensation of performed silica precursors [8]. Silica-based matrix offers a set of characteristics: good hydrothermal stability, high mechanical stability, resistance against biodegradation, biocompatibility and low toxicity [9–11]. The "fish-in-net" approach for encapsulation takes on many advantages: neutral pH, non-denaturing solvent, and mild temperature and pressure. More importantly, because the pore size of "net" is smaller than the spatial size of "fish", the entrapped "fish" cannot leach out from the "net" [8].

In the present study, the encapsulation of β-gal was carried out, based on the "fish-in-net" approach. However, the partial activity loss of β-gal still may occur during encapsulation process, owing to complexity of reagents involved in encapsulation system and interaction between β-gal and silica-based matrix. Therefore, protective agent for β-gal was adopted in the process of "fish-in-net" encapsulation in order to protect and further increase the catalytic

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activity of β-gal. Nevertheless, protective agent for β-gal cannot completely protect the active site of the enzyme from the attack of other chemical reagent. Hence, pretreatment of molecular imprinting technique (MIT) for β-gal was employed in this experiment.

Lactose, the native substrate of β-gal, was chosen as the imprinter in the process of MIT. Before entrapment, β-gal was mixed with lactose in aqueous solution, and then the resulting solution was flash-frozen with liquid nitrogen as fast as possible and subsequently lyophilized. Pretreated β-gal powder was finally encapsulated in the silica-based matrix. Lactose bound with the β-gal in the active site during pretreatment, the active site of β-gal was blocked by lactose molecule. The conformation of enzyme in cellular environment is different from that in dilute solution due to the macromolecular crowding and molecular confinement [12]. In this experiment, the conformation of β-gal in dilute solution was used as a control to explain the ligand-induced conformational change of β-gal. When β-gal and lactose coexisted in aqueous solution, the conformation of β-gal might change due to the inducement of lactose as compared to the conformation in dilute solution, which was benefit for the hydrolytic activity in active site area. Herein, more favorable conformers could be obtained. Subsequently, the induced conformation of β-gal was locked with liquid nitrogen. Induced conformation of β-gal was favorable for the hydrolysis of lactose. After "fish-in-net" encapsulation, such induced conformation was locked to some extent in the silica-based matrix. In contrast, induced conformation cannot be locked owing to leaching phenomenon of immobilized enzyme in sol–gel encapsulation and physical adsorption methods, and "conformational memory" also cannot be accomplished due to the enzyme-support strong interaction in covalent attachment approach.

The primary goal of this study is to investigate the effect of pretreatment of MIT on the catalytic properties of encapsulated β-gal from *Aspergillus oryzae*. The specific activities of entrapped β-gal from *Aspergillus oryzae* with three different models and free β-gal in dilute solution were compared.

2. Experimental

2.1. Materials

β-Gal obtained from *Aspergillus oryzae* (not less than 14,000 U/g based on supplier's product sheet; 1 U was defined as the amount of β-gal that liberates 1.3 μmol o-nitrophenol (ONP) per 10 min at pH 4.5 and 37 °C with o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate) was purchased from Amano Enzymes Co. (Nagaya, Japan). ONPG was purchased from BBI Co. Ltd. (Boston, MA, USA). TEOS and P123 were commercially available from Sigma–Aldrich (St. Louis, Missouri, USA). KBr obtained from BDH Co. (Poole, UK) was of spectral grade. The glucose oxidase–peroxidase kit was purchased from Beijing BHKT clinical reagent Co. Ltd. (Beijing, China). All other chemicals and reagents were of analytical grade. All aqueous solutions were prepared with Milli-Q water.

2.2. Measurement of ethanol tolerance capacity

The purification of crude β-gal from Amano was firstly performed. Crude β-gal (100 g) was slowly dissolved in 1 l of the buffer (pH 7.0, 0.05 M) in ice bath and placed at 4 °C overnight. β-Gal solution was centrifuged for 30 min at 11,500 × *g*, and then the supernatant was collected. The β-gal in the supernatant was subsequently applied in the encapsulation and activity assay. β-Gal solution was incubated with ethanol solution of different concentration in the range of 0–80% at 25 °C. Aliquots of 0.1 ml of β-gal solution were periodically taken at intervals of 40 min. Then, isolated β-gal solution was added to 10 ml of 50 mM KH_2PO_4–NaOH buffer (pH 7.0) to regain activity. Unless otherwise indicated, the buffer mentioned in this study was KH_2PO_4–NaOH buffer. The assay of activity had been carried out in the buffer at 37 °C with 50 mM ONPG as a substrate for 1 min by using properly diluted β-gal to get a linear velocity.

2.3. Modified "fish-in-net" encapsulation

Lactose solution (25 ml of 37.5%, w/w) was added to 150 ml of β-gal solution. β-Gal solutions with lactose and without lactose had been flash-frozen at −80 °C in liquid nitrogen for 20 min and lyophilized soon. Lyophilized powder with and without lactose were respectively denoted as β-gal pretreated by molecular imprinting technique (β-gal-MIT) and β-gal without protection (β-gal-W). Part of β-gal-W was directly intermingled with lactose powder according to the weight ratio between the β-gal and the lactose in β-gal-MIT. This specimen was denoted as β-gal protected by protective agent (β-gal-P).

The preparation of encapsulated β-gal was based on "fish-in-net" method [7]. The synthesis mixture had a molar composition of TEOS/P123/H_2O/ethanol/HCl of 1:0.015:5.3:18.1:0.3, and the pH value in mixture was 5.0. After removal of ethanol by evacuation under vacuum for 72 h at ambient temperature, preformed precursors were assembled in the glycerol solution of an equivalent amount at room temperature. The weight ratio between the enzyme and lactose was 0.34:0.26 in β-gal-MIT and β-gal-P. To make the equal amount of β-gal applied in three different models, β-gal-MIT (0.6 g), β-gal-P (0.6 g) and β-gal-W (0.34 g) were respectively added to 20 g of the precursors at 4 °C, and 5 ml of the buffer was added to this system simultaneously with magnetic stirring. The systems had been aged at 4 °C for 72 h to sufficiently form hydrogel that would be preserved at 4 °C. After entrapment, encapsulated β-gal-MIT, β-gal-P and β-gal-W were denoted as E-β-gal-MIT, E-β-gal-P and E-β-gal-W, respectively.

Aged precursors without β-gal were prepared according to above procedure for determination of removal condition of template P123.

2.4. Characterization of the matrix

Only if the template P123 as structure-directing agent was removed, can all the characteristics of immobilized β-gal be subsequently measured. The removal of the template P123 was performed by washing. Hence, the best washing conditions were screened. The best washing conditions were as follows: the concentration of ethanol solution was 20% (w/w) and the washing operation was repeated with three times.

After degassing at 100 °C for 12 h, N_2 adsorption–desorption isotherms of immobilized β-gal were measured using ASAP 2020 at 77 K. The specific surface area of the samples was evaluated by using the multiple-point Brunauer–Emmett–Teller (BET) method in the relative pressure range P/P_0 = 0.05–0.3. The pore size distribution curve was computed by using the Barrett–Joyner–Halenda (BJH) method, and pore size came from analysis of the peak position of the distribution curve. SEM experiment was performed on a JSM-6700F electron microscope (JEOL, Japan) with an acceleration voltage of 150 kV [13].

2.5. Enzymatic activity assay

In this experiment, enzymatic assay was performed according to the modified methods [14,15]. Before measurement, the gel of immobilized β-gal was washed thoroughly by washing reagent.

The precipitate was suspended in buffer and allowed to strand for 5 min.

2.5.1. Encapsulation yield in different models

The total amount of encapsulated β-gal was quantified according to the difference between the total amount of β-gal added to the encapsulated system and that recovered in the pooled supernatant and washing solutions. The protein content of the enzyme solutions was determined using Bradford method [16]. Bovine serum albumin was used as standard.

The encapsulation yield was calculated as follows:

$$Y = (W_1 - W_2) \times 100/W_1$$

Y = encapsulation yield (%), W_1 = the total amount of β-gal added to the encapsulated system, and W_2 = the total amount of β-gal recovered in the pooled supernatant and washing fractions.

2.5.2. Lactose assay

With lactose as a substrate, the system including 100 μl of 37.5% lactose solution, 100 μl of properly dilute immobilized β-gal with different models and 900 μl of buffer (pH 7.0, 0.05 M) had been placed in boiling water for 5 min to stop the reaction, after catalysis reaction had been carried out for 10 min at 37 °C. After centrifugation, the activity of β-gal was evaluated through detecting the amount of glucose in supernatant by glucose oxidase–peroxidase kit. The precipitate had been placed in oven at 60 °C for 24 h and then was quantified. The obtained precipitate was silica matrix with encapsulated β-gal in reaction system. For free β-gal, the activity was measured according to above mentioned process except centrifugation step. One unit of β-gal activity was defined as the amount of β-gal that liberates 1 μmol glucose per min in the defined conditions. After washing and drying, the weight of dry aged precursors was obtained. The content of β-gal in silica matrix was calculated as follows:

$$C = \frac{W_3}{W_3 + W_4}$$

C = the content of β-gal in silica matrix, W_3 = weight of total encapsulated β-gal, and W_4 = weight of total obtained dry aged precursor.

Then, the specific activities of encapsulated β-gal with different models were calculated as follows:

$$S_1 = \frac{M_1}{W_5 \times C}$$

S_1 = specific activity of encapsulated β-gal, M_1 = molar amount of glucose produced per min, and W_5 = weight of dry silica matrix with encapsulated β-gal in reaction system.

2.5.3. ONPG assay

With ONPG as a substrate, the system containing 25 μl of ONPG (50 mM), 100 μl of properly dilute immobilized β-gal and 875 μl of the buffer was added to 2 ml of Na_2CO_3 (0.5 M) solution to quench the reaction, after hydrolysis reaction had been carried out for 3 min in water bath at 37 °C. After centrifuging for 3 min at 12,000 rpm, the absorbance of supernatant at 410 nm was measured by UV 2550 (Shimadzu, Japan). As above depicted, the precipitate was disposed. For free β-gal, the activity was measured according to above mentioned process except centrifugation step. One unit of β-gal activity was defined as the amount of β-gal that liberates 1 μmol o-nitrophenol (ONP, $\varepsilon = 1385\ M^{-1}\ cm^{-1}$) per min in the defined conditions. Subsequently, the specific activities of encapsulated β-gal with different models were calculated as follows:

$$S_2 = \frac{M_2}{W_6 \times C}$$

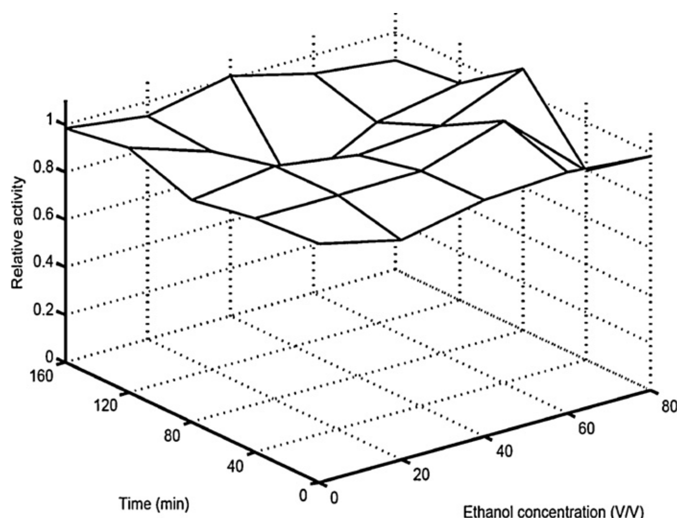

Fig. 1. Ethanol tolerance capacity of free β-gal as a function of time and ethanol concentration (%).

S_2 = specific activity of encapsulated β-gal, M_2 = molar amount of ONP produced per min, and W_6 = weight of dry silica matrix with encapsulated β-gal in reaction system.

2.6. Measurement of pH stability

For the soluble β-gal, 100 μl of β-gal solution had been mixed with 900 μl of different buffers (pH 2.5–10.8) at 25 °C for 30 min. The buffers used were citric acid–sodium citrate (pH 2.5–4.6), K_2HPO_4–KH_2PO_4 (pH 5.8–8.0) and Gly–NaOH (pH 9.3–10.8), respectively. The solution (0.1 ml) was taken from above system, and then had been placed in 900 μl of the buffer (pH 7.0, 0.05 M) for 2 h so that the hydrolytic activity of β-gal recovered. Then, the specific activity was determined according to the assay of activity depicted in Section 2.5.3.

For the encapsulated β-gal, suitable amount of encapsulated β-gal had been immersed in different buffers (pH 2.5–10.8) at 25 °C for 30 min. After centrifuging, the precipitate had been placed in the buffer (pH 7.0, 0.05 M) for 2 h. Based on ONPG assay of activity depicted in Section 2.5.3, the specific activity was identified.

2.7. Assessment of operational stability

The operation of reaction was the same as that of above depicted procedure in ONPG assay, except that reaction time prolonged to 24 h to ensure that the hydrolytic reaction was sufficiently completed. Then, the precipitate was washed by the buffer to avoid the effect of residual ONP on subsequent assay. After centrifuging for 3 min at 12,000 rpm, the precipitate was applied in the next duplicate assay. The assay was completed after nine cycles.

3. Results and discussion

3.1. Identification of properties of free β-gal

3.1.1. Ethanol tolerance capacity

Because the system of encapsulation contained the ethanol, ethanol tolerance capacity of β-gal were investigated. Results demonstrated that residual activity of free β-gal almost did not lose with increase of reaction time and ethanol concentration, and that residual activity still approached to 100% for 160 min in ethanol concentration of 80% (Fig. 1).

3.1.2. The size of enzyme molecule and substrate

β-Gal from *Aspergillus oryzae* without crystal structures data (molecular mass = 110 kDa [17]) showed amino acid sequence homology of 73.5% to β-gal from *Penicillium* sp. (molecular mass = 120 kDa, 11.1 nm × 11.1 nm × 16.1 nm and PDB code is 1XC6) [18] through "blast two sequence" program [19]. Hence, we assumed that three-dimensional size of β-gal from *Aspergillus oryzae* was similar to β-gal from *Penicillium* sp. For the substrate, the spatial size of ONPG (9 Å × 4 Å × 7 Å) and lactose (10 Å × 4 Å × 7 Å) was much smaller than that of β-gal.

3.1.3. The design of the diameter of the mass transfer channel

In order to avoid the leakage of β-gal after encapsulation and enhance diffusion of substrate and product molecules from the matrix during hydrolysis reaction, we presumed that the entrance size of the matrix must be not only smaller than spatial size of β-gal but also enough larger to the substrate molecule. Hence, the pore size must be controlled in the range of 2–11 nm. The pore size of mesoporous molecular sieves depends on the core size of the micelle obtained by surfactant self-assembly pathway during synthesis process [8,20]. The micellization of PEO–PPO–PEO copolymers is mainly attributed to hydrophobic property of PO block [21]. In this experiment, the whole aging process should be carried out at 4 °C to protect the enzymatic activity, after the β-gal was added to the performed precursor system. However, PO block becomes more hydrophilic at low temperature, which leads to unfavorable micellization [22]. In order to overcome this problem, the high concentration of PEO–PPO–PEO copolymer should be adopted for the formation of micelle. If and only if the concentration of block copolymer was controlled in the range of 0.5–6% (w/w), ordered mesoporous materials could be yielded, as reported by Stucky and co-workers [23]. Hence, in this experiment, 6% (w/w) was adopted as the upper limit of concentration. In classical synthesis of mesoporous material, operation of hydrothermal treatment and recrystallization was favorable to enhance the thermal stability and regularity of mesoporous silica [8]. For the hydrothermal treatment, the reaction solution was placed at high temperature environment (in general, 100–130 °C) after the synthesis reaction of mesoporous silica. As for the recrystallization, the as-synthesized powder samples have been immersed into deionized water at high temperature (100–150 °C) for several days (sometimes even 1 week) [8]. However, β-gal could not undergo higher temperature treatment, hydrothermal treatment and recrystallization could not be used in this experiment. Herein, high concentration block copolymer and long aging time were used to compensate the above mentioned deficiencies to some extent for yielding well-ordered mesoporous silica in which substrates and products could easily diffuse.

3.2. Characteristic of the mesoporous material after encapsulation

In fact, TEOS, as silica resource, was relatively slowly hydrolyzed during synthesis at room temperature in this work. After hydrolysis and condensation of silicate, SEM of E-β-gal-MIT was presented after completely washing (Fig. 2). The results demonstrated that condensation of silica species was formed by the aggregation of microspheres with a particle size of 200–300 nm.

After rinsing, N_2 adsorption/desorption isotherms of aged precursor and E-β-gal-MIT were measured. The pore size distribution of aged precursor and E-β-gal-MIT was very sharp (Fig. 3). N_2 adsorption/desorption isotherms exhibited narrow hysteresis loops of H_3 types classified by IUPAC [24]. Pore size, total pore volume and BET surface area of aged precursor were all higher than that of E-β-gal-MIT, as shown in Table 1. This data implied that encapsulated β-gal took up the space in channel. Taking into consideration that pore size of 47 Å in mesoporous silica can be

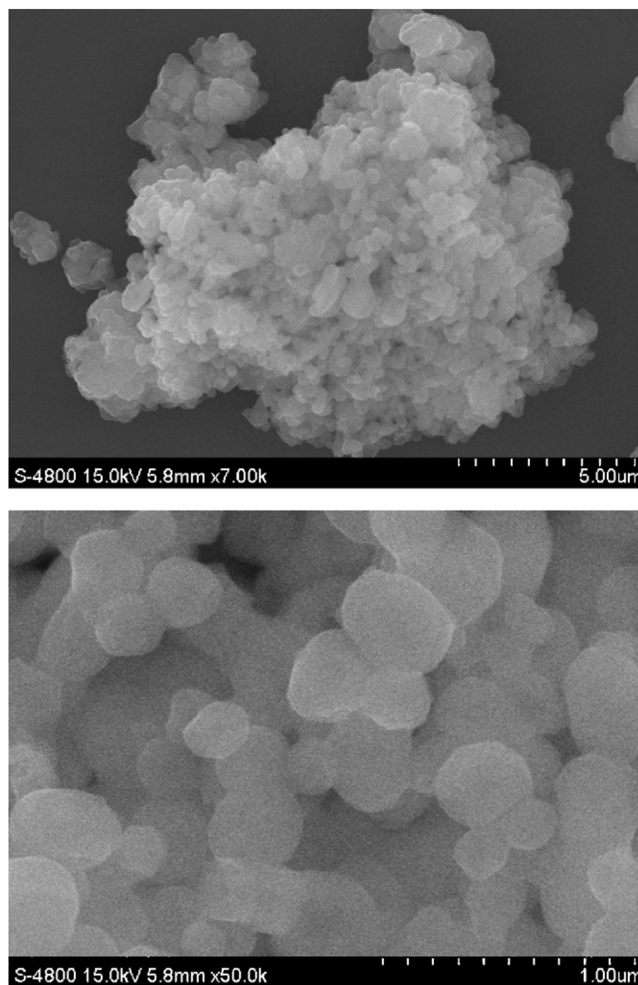

Fig. 2. SEM image of E-β-gal-MIT.

obtained by using P123 as a template at 35 °C [23], pore size of 27 Å in this experiment may be attributed to the relative low aging temperature (4 °C). At 4 °C, the core size of micelle becomes smaller due to hydrophilic trend of PO block [21]. The experimental data demonstrated that the narrow and long channel in the matrix was enveloped with thick wall. As a result, the matrix must be provided with good hydrothermal stability. The diffusion of substrate and product may be effected in narrow channel to a certain extent. Nevertheless, the leaching rate of product might be higher than the diffusion rate of substrate, due to the spatial size difference between these two molecules. It is favorable to facilitate forward reaction in hydrolytic reaction.

3.3. Comparison of the specific activity of free β-gal, E-β-gal-MIT, E-β-gal-P and E-β-gal-W and protective mechanism of pretreatment of MIT

With lactose as a substrate, the specific activity of E-β-gal-MIT, E-β-gal-P and free β-gal was 3 times, 1.3 times and 1.9 times higher than that of E-β-gal-W, respectively. With ONPG as a substrate, the specific activity of E-β-gal-MIT, E-β-gal-P and free β-gal was 1.8-fold, 1.3-fold and 1.4-fold compared with E-β-gal-W, respectively. The specific activities of E-β-gal-W were 198 U/mg (protein) and 476 U/mg (protein), when lactose and ONPG acted as substrate, respectively (Table 2). In E-β-gal-MIT, E-β-gal-P and E-β-gal-W model, 94%, 88% and 96% of initially applied β-gal were encapsulated.

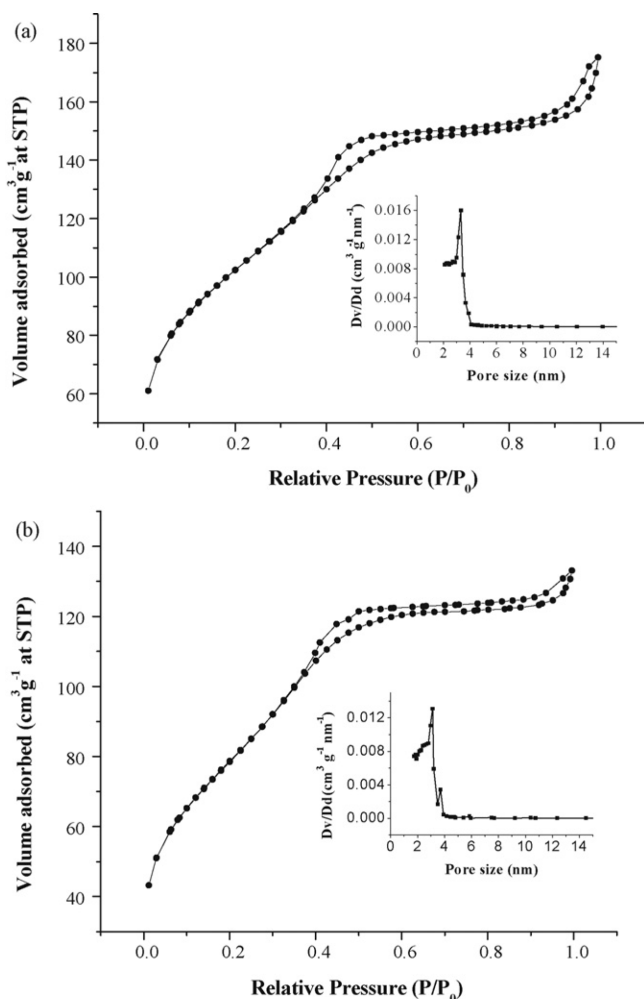

Fig. 3. Nitrogen adsorption–desorption isotherms and BJH pore size distributions of aged precursor (a) and E-β-gal-MIT (b).

Experimental data demonstrated that the specific activity of E-β-gal-P was higher than E-β-gal-W, and the specific activity of E-β-gal-MIT was higher than E-β-gal-P (Table 2). Above mentioned results suggested that the lactose molecular provide effective protection for E-β-gal-MIT, and that the protective effect of pretreatment of MIT was better than protective agent. The specific activity of free β-gal was higher than that of E-β-gal-P and E-β-gal-W, indicating that β-gal molecule may be attacked by some reagents during encapsulation. On the other hand, the specific activity of free β-gal was lower than that of E-β-gal-MIT, confirming that pretreatment of MIT not only protected β-gal molecule from harmful effects resulted from encapsulation system but also enhanced the catalytic activity of β-gal due to favorable conformational change. With both lactose and ONPG as substrates, respectively, the activity of E-β-gal-MIT was enhanced about 3-fold and 1.8-fold compared with E-β-gal-W. This 1.2-fold difference demonstrated that E-β-gal-MIT displayed higher recognition specificity for native substrate owing to the subtle conformational change induced by lactose.

Table 1
Pore size, pore volume and BET surface area of aged precursors and E-β-gal-MIT after washing.

Sample	BET surface area ($m^2 g^{-1}$)	Pore volume ($cm^3 g^{-1}$)	Average pore diameter (Å)
Aged precursors	368	0.250	27.2
E-β-gal-MIT	289	0.196	27.1

Table 2
Comparison of specific activity of immobilized and free β-gal.

Substrate	Specific activity (U/mg)			
	E-β-gal-W	E-β-gal-P	E-β-gal-MIT	Free β-gal
Lactose	198	257	566	390
ONPG	476	612	879	671

Generally, the function of enzyme depends on its structure. The enzymatic activity is essentially related to its conformation. In this experiment, lactose entered active site of β-gal upon MIT process and induced conformational change in β-gal. Such subtle change contributed to the formation of the accessible conformational space in active site. During succeeding encapsulation, β-gal was gradually confined in limited space of inorganic silica skeleton so that the induced conformation was "fixed" to some extent. Even though lactose was removed through washing, the induced conformation might not revert to the former conformation. As a result, the catalytic activity of encapsulated β-gal was reinforced. In "E-β-gal-MIT" encapsulation, lactose might play the role of dual protection for β-gal. Firstly, the lactose molecular binding in the active site pocket could avoid the attack of other reagents to key amino acid residues. Secondly, β-gal was surrounded by a hydration layer that played a primary role in maintaining the catalytic activity of β-gal under physiological conditions. Hydration layer of β-gal might be destroyed by chemical reagents such as HCl, ethanol and the silicate during entrapment. Added lactose as polyol, forming "sugar shell" around the β-gal, was responsible for the maintenance of three-dimensional structure of β-gal and protection of hydration layer. Furthermore, owing to the presence of "sugar shell", the direct contact between β-gal and inorganic skeleton was avoided. After removal of "sugar shell" through washing, the inorganic skeleton provided enough space for β-gal to maintain its flexibility in catalytic reaction. The existence of such space was essential to the presence of catalytic behavior of enzyme [25,26].

Thus, it is feasible that the enzyme is stabilized using pretreatment of MIT, and then its induced conformation is "frozen" using entrapment. Gill has also proposed the similar conclusion that the enzyme should be stabilized before encapsulation [27]. It is assumed that pretreatment of molecular imprinting technique can be applied to the immobilization of many enzymes. In pretreatment of MIT, induced favorable conformation of β-gal was locked using liquid nitrogen. After "fish-in-net" entrapment, β-gal in silica-based matrix possessed more population of favorable conformer, and thus encapsulated β-gal could exhibit higher enzymatic activity than that in dilute solution. It is suggested that even if the locked favorable conformation of encapsulated β-gal was unlocked after the removal of lactose molecule, β-gal in silica-based matrix still could maintain favorable conformation to some extent. Due to the effect of macromolecular crowding and molecular confinement, induced favorable conformers of β-gal in silica-based matrix could be retained and enriched [12,28]. Ackerman and co-workers had also obtained the immobilized organophosphorus hydrolase with higher specific activity as compare to enzymes in stock solution using physical adsorption method [29], which was consistent with our results. Certainly, all descriptions on protective mechanism in this manuscript are some hypotheses, and future work should be performed to further ascertain why this happens.

3.4. pH stability

The activity of β-gal from *Aspergillus oryzae* before and after immobilization was less susceptible to pH changes in the range of 3.6–10.8 (Fig. 4). At pH 2.5, both soluble and immobilized β-gal

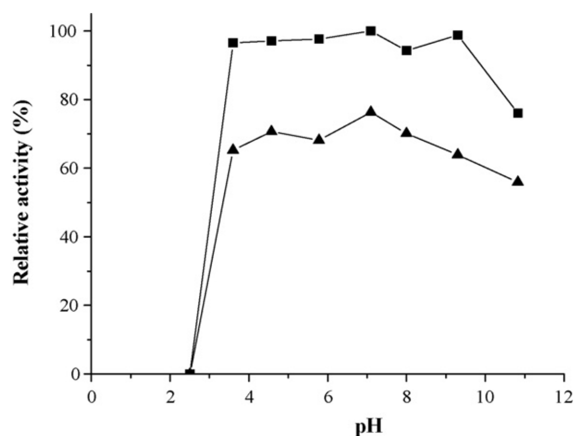

Fig. 4. The pH dependence of stability of free β-gal (▲) and E-β-gal-MIT (■). The specific activity of E-β-gal-MIT (879 U/mg) at pH 7.0 and 37 °C with ONPG as substrate was taken as control (100%). Other specific activity was assayed at various pH (2.5–10.8).

lost the activity completely. The stability of encapsulated β-gal was slightly better than free β-gal when pH changed from 3.6 to 10.8.

3.5. Operational stability

As above mentioned in Section 3.1.2, three-dimensional size of β-gal from *Aspergillus oryzae* is so big that cannot escape from the matrix that possesses mean pore size of 2.7 nm. No β-gal leached out from the matrix. Moreover, E-β-gal-MIT displayed a perfect reusability owing to no leakage. After nine runs, the hydrolysis activity still maintained almost 100% of its initial activity. The results sufficiently demonstrated that E-β-gal-MIT was suitable for the system of successive operations.

4. Summary and conclusions

In this experiment, encapsulated β-gal was successfully prepared, based on "fish-in-net" encapsulation route and molecular imprinting technique. In pretreatment of molecular imprinting technique, lactose was used as the ligand of β-gal, and could efficiently induce "enzyme memory". In "fish-in-net" entrapment, lactose was used as the protective agent for β-gal. Therefore, the whole encapsulation process was different from the immobilization with simple substrate protection. The encapsulated β-gal pretreated by molecular imprinting technique possessed higher activity than β-gal without protection. Compared with the encapsulated β-gal without protection, the specific activity of encapsulated β-gal pretreated by molecular imprinting technique was increased 3-fold and 1.8-fold when lactose and ONPG were used as substrate, respectively. Moreover, the immobilized β-gal pretreated by molecular imprinting technique exhibited good reusability during nine cycles and even higher specific activity than free β-gal. Hence, through modified approach which combined "fish-in-net" encapsulation with molecular imprinting technique, the enzymatic behavior of encapsulated β-gal was well improved.

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