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Improving the properties of β-galactosidase from *Aspergillus oryzae* via encapsulation in aggregated silica nanoparticles

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In this study, a new immobilization method was exploited to encapsulate β-galactosidase (β-gal) from *Aspergillus oryzae* using aggregated core–shell silica nanoparticles as a matrix. Transmission electron microscopy (TEM) and Fourier transform infrared (FTIR) spectroscopy were used to characterize the material encapsulated β-gal. Compared to the free β-gal, the encapsulated β-gal shows a broader pH tolerance and thermal stability. Furthermore, the encapsulated β-gal shows better storage stability over 30 days. After nine cycles of hydrolytic reaction, the encapsulated β-gal still maintains 94.2% of its initial activity, which indicates that the β-gal exhibits excellent reusability after encapsulation.

1. Introduction

Immobilization is an important technique in the enzyme engineering field, which has been widely used to improve the enzyme performance (enzyme activity, stability, *etc.*).[1–6] Many types of immobilization technology have been developed and the sol–gel method is one of the most widely used immobilization techniques.[7–10] Especially, enzyme immobilization in silica gels has been well studied during the past few decades.[11–14] For example, Ellerby *et al.* encapsulated metalloprotein and heme proteins in porous silica glass matrices under mild conditions using a standard sol–gel process and found that the encapsulated enzymes maintained their enzymatic activity and spectroscopic properties.[15] Gill and Ballesteros carried out the entrapment of proteins in poly(glyceryl silicate) sol–gel and verified that the entrapped enzyme had high stability.[16] Ferrer *et al.* encapsulated horseradish peroxidase by an alcohol-free sol–gel route and found that the embedded enzyme retained a more native conformation than that produced by a regular sol–gel process.[17] Although great efforts have been made, some disadvantages of the sol–gel method, such as the brittleness of the sol–gel matrix, the complex pH control when preparing a biocompatible sol–gel matrix and the pore collapse during xerogel formation, have yet to be overcome.[18,19] Therefore, a simple and practical method is required to overcome these problems.

In this study, we used silica cross-linked micellar nanoparticles as the building units to create the host material for enzyme immobilization.[20,21] The silica nanoparticles consist of hydrophobic cores containing poly propylene oxide segments and hydrophilic shells formed by poly propylene oxide segments surrounded by a thin silica layer. These high-quality silica nanoparticles can be easily synthesized while possessing good dispersion in water and biocompatibility. Due to the existence of the silica shell, the nanoparticles easily assemble together due to a state of lower surface energy.[22] As a simple and irreversible process, lyophilization can promote the aggregation of the silica nanoparticles under the stress of freezing and dehydration. The interstices formed by the stacked nanoparticles provide sufficient space to encapsulate enzymes. Furthermore, the stacked nanoparticles possess abundant three dimensional mass transfer voids which will be beneficial for the diffusion of substrates. The silica encapsulated β-gal was prepared by lyophilization of a mixture of the silica nanoparticles and β-gal. The encapsulated β-gal shows good pH stability, thermal stability, storage stability and reusability.

2. Experimental

2.1 Materials

β-Galactosidase (β-gal) obtained from *Aspergillus oryzae* was purchased from Amano Enzymes Co. (Nagaya, Japan). *o*-Nitrophenol-β-D-galactoside (ONPG) was purchased from BBI Co. Ltd. (Boston, MA, USA). Tetraethyl orthosilicate (TEOS), Pluronic F127 and diethoxydimethylsilane ($Me_2Si(OEt)_2$, DEDMS) were commercially available from Sigma-Aldrich (St. Louis, Missouri, USA). Octanoic acid was purchased from Tianjin Guangfu Fine Chemical Research Institute of China. All other chemicals

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and reagents were of analytical grade. All aqueous solutions were prepared with Milli-Q water.

2.2 Synthesis of silica cross-linked micellar nanoparticles

In a typical preparation, 0.54 g of Pluronic F127 was dissolved in 10 g of HCl solution (0.57 M) at room temperature. Octanoic acid (0.8 g) was added to the mixture followed by ultrasound treatment for 1.5 h and then was stirred for 2 days. 1 g of TEOS was added to the above solution. The mixture was stirred at room temperature for 0.5 h, and 0.08 g of DEDMS was added. Stirring was continued at room temperature for 0.5 h followed by filtration using a Teflon filter (0.22 μm).

HCl and EtOH (resulting from the hydrolysis of TEOS and DEDMS) were removed by dialysis (Millipore, MW cutoff 8000–12 000) against pure water with stirring at room temperature. The water was refreshed every 2 hours. The dialysis process was monitored by measuring pH of the solution and terminated at pH 7.0. Then, the silica cross-linked micellar nanoparticles solution (40 mg silica nanoparticle per ml) was obtained.

2.3 Encapsulation of β-gal

Crude β-gal was purified as follows: 2 g of crude β-gal was dissolved in 10 ml of HAC–NaAC buffer (50 mM, pH 4.5) in an ice bath. The β-gal solution was centrifuged at 3500*g* for 3 hours using an ultrafiltration device (Millipore, MW cutoff 10 000). The suspension was lyophilized. The β-gal solution (8 mg ml^{-1}) was prepared by re-dissolving the lyophilized β-gal in the buffer (pH 4.5) at 4 °C.

Purified β-gal solution (5 ml, 8 mg ml^{-1}) was added to 10 ml of the silica cross-linked micellar nanoparticle solution with slow stirring at 4 °C. The resulting solution was frozen at −20 °C for 30 min and then lyophilized. The lyophilized β-gal was repeatedly rinsed with Milli-Q water at room temperature, until no absorbance of the protein in the supernatant could be detected. The wet sample was lyophilized again. Finally, the lyophilized powder was stored at 4 °C.

The protein content of the enzyme solution was determined using the Bradford method.[23] Bovine serum albumin was used as standard. The encapsulation yield of β-gal is 89.2% in this experiment.

2.4 Characterization of silica cross-linked micellar nanoparticles

Transmission electron microscopy was performed using an FEI Tecnai G2 F20 s-twin (FEI, American) at 200 keV. The particle size of the silica nanoparticles was measured by a Malvern Zetasizer Nano-S instrument at room temperature using the Dynamic Light Scattering (DLS) principle with a HeNe laser (633 nm). The FTIR spectrum was recorded on a Nicolet 5700 FTIR spectrometer with a resolution of 4 cm^{-1} through the KBr method.

2.5 Enzyme activity assay (ONPG assay)

The enzyme activity of β-gal was measured using ONPG (*O*-nitrophenyl-β-D-galactopyranoside) as a substrate according to our previous method with a slight modification.[24] The reaction system contained 100 μl of ONPG (50 mM) and 800 μl of HAc–NaAc buffer (50 mM, pH 4.5). The hydrolytic reaction was triggered by adding the enzyme. And then, the reaction mixture was incubated in a water bath at 37 °C for 10 min. Thereafter, the reaction was stopped by adding 2 ml of Na_2CO_3 solution (0.5 M) followed by centrifugation at 3500*g* for 3 min. The amount of produced *ortho*-nitro-phenol (ONP) was determined at 410 nm. The enzyme activity (1 U) was defined as the amount of β-gal that liberates 1 μmol ONP per min under the above experimental conditions.

2.6 Stability of encapsulated β-gal

pH stability. The pH stability of the free β-gal and the encapsulated β-gal was determined by incubating the enzyme in different buffers (pH 2.5–10.8) for 1 h at 25 °C. 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). The encapsulated β-gal was collected by centrifugation (3500*g* for 3 min) and air dried. And then the residual β-gal activity was determined at its optimal pH according to the assay in Section 2.5.

Temperature stability. Free or the encapsulated β-gal was added to HAC–NaAC buffer (50 mM, pH 4.5), followed by incubation at different temperatures (40–70 °C) for 120 min. The specific activity of free or the encapsulated β-gal was assayed according to the description in Section 2.5.

Storage stability. The encapsulated β-gal was kept at room temperature under dry conditions (air-dried) for 30 days. The residual activity was checked from time to time according to the description in Section 2.5.

Reusability. The encapsulated β-gal was used in successive batches. The relative activity of the encapsulated β-gal was measured according to the assay in Section 2.5. After each cycle, the reaction mixture was centrifuged at 3500*g* for 3 min. The obtained precipitate was washed with HAC–NaAC buffer (50 mM, pH 4.5) to remove any residual substrate or product and then kept overnight in a vacuum oven at room temperature for complete drying. After drying, the powder was used in the next cycle under otherwise equivalent conditions.

3. Results and discussion

3.1 Synthesis and assembly of silica nanoparticles

We designed a novel method for encapsulating β-galactosidase (β-gal) from *Aspergillus oryzae* through assembly of the silica nanoparticles (Fig. 1).

Uniform spherical particles (90 nm) can be observed in Fig. 2a, which is consistent with the results of light scattering measurements (Fig. 2c). The inset of Fig. 2a demonstrates that an individual silica nanoparticle is a solid sphere and its surface does not possess any large porous structure. From Fig. 2b, the aggregation of the silica nanoparticles can be found after lyophilization and the shape of the silica nanoparticles during the aggregation does not change. Furthermore, the aggregated silica nanoparticles can form many interstices (about 20 nm × 20 nm × 20 nm, calculated by the formulation of the interstice (hole) in closest-packing of spheres[25]), which

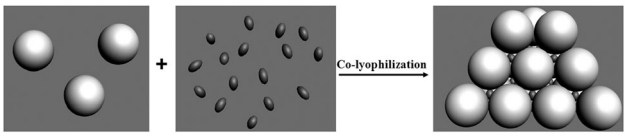

Fig. 1 The new immobilization method for encapsulating β-gal in the aggregated silica nanoparticles. The gray sphere and the black ellipsoid represent the silica nanoparticles and β-gal, respectively.

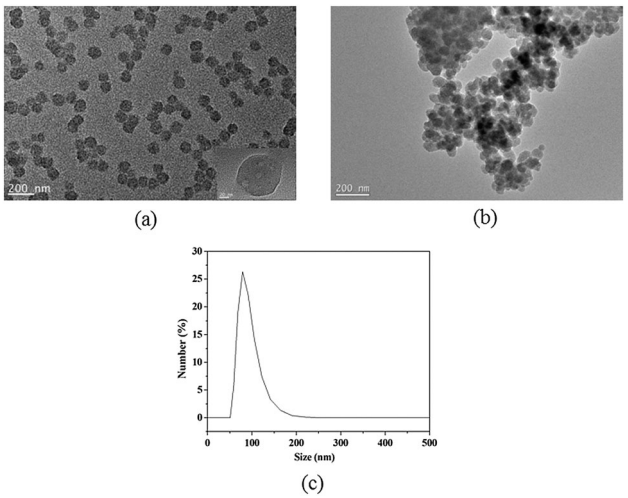

Fig. 2 (a) TEM images of the silica nanoparticles. Inset: individual silica nanoparticle. (b) The matrix loaded with β-gal. (c) Light scattering measurement of the silica nanoparticles.

might provide enough room to accommodate β-gal (16 nm × 11 nm × 11 nm).[26]

It's known that the lyophilisation process can induce the aggregation of nanoparticles.[27] In this study, lyophilisation accelerates water removal so as to promote the formation of the silica nanoparticle matrices. Furthermore, freezing and dehydration stresses generated by lyophilisation also help to form the silica nanoparticles matrices. Moreover, no obvious difference between the aggregated silica nanoparticles with β-gal and the counterpart without β-gal was found in TEM images.

To confirm the encapsulation of β-gal in the matrix, the FT-IR spectra of the silica nanoparticles, β-gal and the matrix loaded with β-gal were studied. The characteristic bands of Si–O–Si could be observed at 808 and 1100 cm^{-1} in Fig. 3a[28,29] and the characteristic bands of the β-gal can be observed at 1644 and 1552 cm^{-1} in Fig. 3c.[30] After immobilization, all these characteristic bands for both β-gal and Si–O–Si can be found in Fig. 3b, which verifies that β-gal is successfully entrapped in the aggregated silica nanoparticles.

3.2 Stability

pH stability is an important parameter for an immobilized enzyme. We investigated the pH stability of the encapsulated β-gal by first incubating the encapsulated β-gal at different pH values (2.5–10.6) for 30 min and then measuring the activity at its optimal pH. The free β-gal is stable between pH values

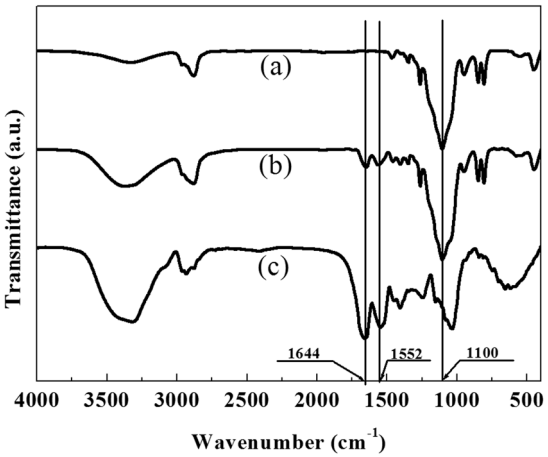

Fig. 3 FTIR spectrum of: (a) the aggregated silica nanoparticles, (b) the aggregated silica nanoparticles loaded with β-gal, and (c) pure β-gal.

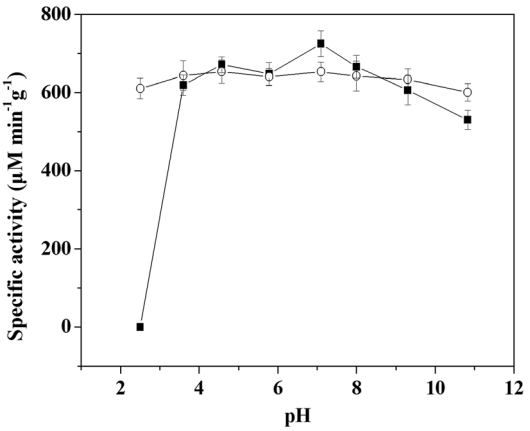

Fig. 4 pH stability of free β-gal (■) and encapsulated β-gal (○) at different pH media.

3.6–9.3 (Fig. 4). As for the encapsulated β-gal, the pH stability curve exhibits a plateau over a broad pH range of 2.5–10.6 (Fig. 4). The experimental data demonstrate that the pH stability has been improved by encapsulation of β-gal in aggregated silica nanoparticles.

Wang *et al.* found catalase encapsulated in nanoporous silica spheres to have improved stability against pH change compared with the free enzyme by the physical adsorption method, which was consistent with our results.[31]

The thermal inactivation of β-gal often limits its application. In this study, the thermal-stability of free and the encapsulated β-gal in the range of 40–70 °C has been investigated to assess the potential advantage of the encapsulated β-gal and the results are shown in Fig. 5. After 120 min incubation, the free and the encapsulated β-gal were stable at lower temperature (40–50 °C) and became vulnerable to inactivation at higher temperature (50–70 °C) at which the specific activity of encapsulated β-gal decreased at a slower rate than that of the free one. These results suggested that the encapsulated β-gal could exhibit a better thermo-stability at higher temperatures. The encapsulation of β-gal in aggregated silica nanoparticles might

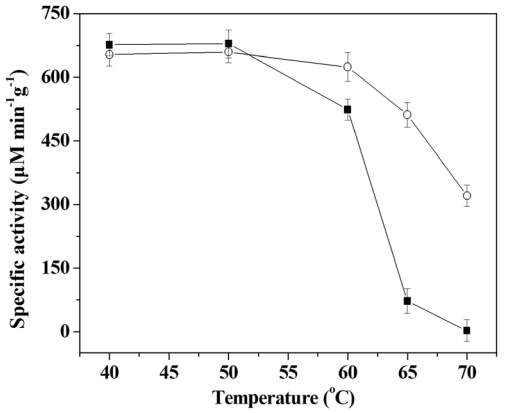

Fig. 5 Thermal stability of free β-gal (■) and encapsulated β-gal (○).

Table 1 Storage stability of the encapsulated β-gal for hydrolytic activity towards ONPG

Storage time (day)	Activity (U mg^{-1})	Residual activity (%)
0	653.0	100.0
5	649.7	99.5
10	633.5	97.1
15	626.7	96.0
20	602.0	92.2
25	591.0	90.5
30	571.0	87.4

preserve the tertiary structure of the protein and protect β-gal from disassembling of the active center caused by the diminution of non-covalent forces at higher temperatures.[32,33] The encapsulated β-gal with higher thermal stability is beneficial to the hydrolysis of the lactose in milk at higher temperature, reducing bacterium contamination and accelerating reaction rates in a continuous-flow system.

The practical application of enzymes requires that they could be maintained for long periods. In this study, the storage stability of the encapsulated β-gal has been investigated and the results are shown in Table 1. We found that the enzyme activity of the encapsulated β-gal gradually decreases with the increasing storage time and the encapsulated β-gal could maintain a higher enzyme activity (87.4% of its initial activity) even after 30 days of storage. The high storage stability of the encapsulated β-gal may be attributed to a reduction in the rate of inactivation of β-gal as a result of encapsulation in aggregated silica nanoparticles.

Table 2 Reusability of the encapsulated β-gal for hydrolytic activity towards ONPG

Cycle no.	Activity (U mg^{-1})	Residual activity (%)
1	653.0	100.0
2	636.7	97.5
3	635.4	97.3
4	633.4	97.0
5	628.2	96.3
6	626.9	96.1
7	625.6	95.9
8	621.0	95.1
9	615.1	94.2

It's known that the immobilized enzyme can be reused to decrease the cost of enzymes for an enzymatic reaction. We investigated the reusability of the encapsulated β-gal and found that the enzyme activity remained at almost 94.2% of its initial activity even after nine reaction cycles (Table 2).

4. Conclusions

In this study, a novel encapsulation method was exploited to immobilize β-galactosidase (β-gal) in aggregated silica nanoparticles through a simple lyophilization step. Once the assembly of the silica nanoparticles occurred, the matrix formed by the building units exhibited perfect stability in aqueous solution. The interstice formed by the aggregated silica nanoparticles provided a comfortable microenvironment for β-gal. The encapsulated β-gal exhibited good pH stability, temperature stability, storage stability and reusability. In the future, core–shell nanoparticles modified by organically modified silicates or loaded with metal ions could be employed to entrap enzyme molecules, providing multifunctional materials.

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