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Immobilization of β-Galactosidase onto Magnetic Beads

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Abstract A study of the cross-linking of β-galactosidase on magnetic beads is reported here. The magnetic beads were prepared from artemisia seed gum, chitosan, and magnetic fluid in the presence of a cross-linking regent (i.e., glutaraldehyde). The reactive aldehyde groups of the magnetic beads allowed the reaction of the amino groups of the enzymes. The animated magnetic beads were used for the covalent immobilization of β-galactosidase. The effect of various preparation conditions on the activity of the immobilized β-galactosidase, such as immobilizing time, amount of enzyme, and the concentration of glutaraldehyde, were investigated. The influence of pH and temperature on the activity and the stability of the enzyme, both free and immobilized, have been studied. And *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was chosen as a substrate. The β-galactosidase immobilized on the magnetic beads resulted in an increase in enzyme stability. Optimum operational temperature for immobilized enzyme was 10 °C higher than that of free enzyme and was significantly broader.

Keywords Magnetic beads · β-galactosidase · Enzyme immobilization · Properties

Introduction

During the last decade, immobilized enzymes have been mostly used in the production of food, pharmaceuticals, and other biologically important fine products. Immobilized enzymes can offer many advantages over their free forms, making this a topic of active research in the area of biotechnology. A number of methods for immobilization of enzymes have been reported in the literature and they can be divided into five basic groups: (1) covalent attachment to solid supports; (2) adsorption to solid supports; (3) entrapment within polymeric gels; (4) cross-linking to solid supports with biofunctional reagents; and

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(5) encapsulation of enzymes within membranes permeable only to substrates and products. Successful application of immobilization techniques to enzyme must satisfy the requirement of carriers having durability and high efficiency as well as low cost [1–8].

In the past decade, the application of β-galactosidase to the hydrolysis of lactose in dairy process with both the free and immobilized conditions has received great attention [9–11]. The studies have shown that milk products of which have been hydrolyzed can be consumed by the "lactose intolerance" people. The purpose of this research was focused on providing valuable information for industrial application of the immobilized β-galactosidase. In this research, β-galactosidase was immobilized on magnetic beads using glutaraldehyde as cross-linking regent. The immobilization conditions were optimized and the effect of pH and temperature on the activity and stability of free and immobilized enzyme was determined. The results show that the immobilized β-galactosidase has specific properties than the soluble one.

Experimental

Materials

The β-galactosidase used in this study was purchased from Sigma Company. ONPG, i.e. *o*-nitrophenyl-β-D-galactopyranoside was also obtained from Sigma Company. Lactose was purchased from the local market. The magnetic beads were the products of our laboratory [12, 13]. Glutaraldehyde 50% (*w*/*v*) used as a cross-linking reagent as well as other chemicals was of analytical grade.

Enzyme Immobilization

Most commonly, protein immobilization techniques through covalent coupling involve the reaction of protein amino groups with electrophilic moieties introduced on a solid support [14, 15]. In this study, the magnetic beads (2.0 g) were equilibrated in Tris–hydrochloric acid buffer solution (0.1 M, pH 7.3, 4 ml, containing 0.1 mM $MgCl_2$) for 2 h, was then transferred to the enzyme solution at 4 °C, containing β-galactosidase at a concentration ranging between 1 and 3 ml (380 U/ml). The immobilization of enzyme was carried out at 4 °C in bathing-water pot for 6 to 14 h with a stirring rate of 150 rpm to complete the cross-linking reactions among enzyme and glutaraldehyde. After this period, the enzyme immobilized magnetic beads were removed from the medium by magnetic separation. Physically bound enzyme was removed first by washing the supports with saline (1.0 M, NaCl) and then buffer solution (0.1 M, pH 7.3) and was stored at 4 °C in the same fresh buffer until use.

Quantitative Analysis of β-Galactosidase

The maximum absorbance values of β-galactosidase of 0.1 M Tris–hydrochloric acid buffer (pH 7.3) were determined at 420 nm by UV–Vis spectrometer. ONPG (1.5 ml, 6 mM), at concentrations from 0 to 6.0 mM in 0.1 M Tris–hydrochloric acid buffer solution, pH 7.3, containing 0.1 mM $MgCl_2$, was added to 0.5 ml enzyme solution (380 U/ml) and the color developed was measured at 420 nm. Then a standard curve was prepared according to the different absorbances. One unit of enzyme activity is defined as the amount of enzyme, which catalyzes the conversion of 1 mmol of ONPG to *o*-nitrophenol per minute at testing conditions.

Enzyme Assays

Activities of the free and immobilized β-galactosidase were determined spectrophotometrically. β-galactosidase solution (380 U/ml) was obtained by β-galactosidase dissolved in 0.1 M Tris–hydrochloric acid buffer solution, pH 7.3, containing 0.1 mM $MgCl_2$. Then 0.5 ml of the β-galactosidase solution and 1.5 ml of ONPG (6 mM) were added to a 10 ml neat tube. After the enzymic reaction was carried out at 37 °C for 10 min with stirring, 1 ml of Na_2CO_3 (1 M) was added in to stop the reaction. The absorbance of the mixture was measured at 420 nm.

The same assay medium was used for the determination of the activity of the immobilized enzyme. The enzymatic reaction was started by the introduction of β-galactosidase immobilized magnetic beads into the assay medium and was carried out at 37 °C in a shaking water bath for 10 min. The following produce was the same as the determination of the free β-galactosidase activity.

The relative activity (%) was the ratio between the activity of every sample and the maximum activity of samples.

Selection of the Immobilization Condition

The most effective parameters determining the activity of the immobilized β-galactosidase were the concentration of glutaraldehyde, amount of enzyme and immobilizing time, and these parameters were varied in the range 0.2–2.0%, 0.5–3 ml (380 U/ml) and 6–14 h respectively.

Emzymic Assay of Free and Immobilized β-Galactosidase

The above activity assays described in 'Enzyme Assays' were also carried out over the pH range 6.3–8.8 and the temperature 32–57 °C to determine the pH and temperature in profiles for the free and immobilized enzyme. The results of pH and temperature are presented in a normalized form with the highest value of each set being assigned the value of 100% activity. The experiments were carried out in triplicate. For each set of data, the arithmetic mean values and standard deviations were calculated and the margin of error for each data set was determined according to a confidence internal of 95% using the statistical package under Excel for Windows.

Results and Discussion

Immobilization Condition

Immobilizing Time

As shown in Fig. 1, when the immobilizing time is lower than 8 h, the activity of immobilized enzyme would keep pace with the immobilizing time. This means that as the immobilized time increased, so did the activity of immobilized enzyme. But when the immobilizing time is longer than 8 h, the activity of immobilized enzyme will decrease. This is because the support has a lot of sulfonic groups; it can make the active amino groups in an acid microenvironment and the environment can make the amino groups have a better stability. So the activity of immobilized enzyme increases when the time of

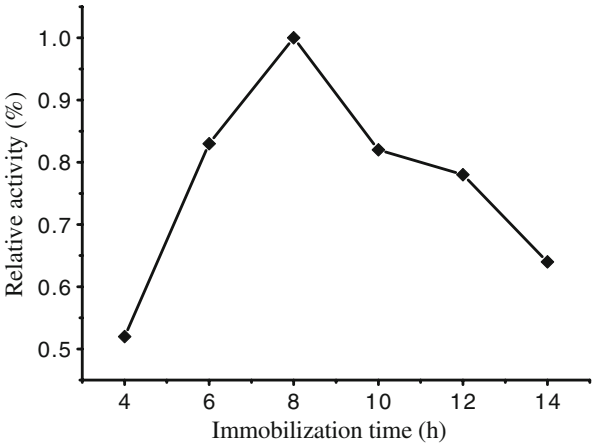

Fig. 1 The effect of immobilization time on immobilized enzyme

immobilization is raised. After immobilization, the conformation of enzyme will change; it can make the activity of immobilized enzyme decrease. So the optimal immobilizing time should be 8 h.

Amount of Enzyme

In order to maximize the immobilized β-galactosidase onto magnetic beads, initial enzyme amount was changed between 0.5 and 3.0 ml (380 U/ml) in the medium. As seen in Fig. 2, an increase in enzyme amount in the medium led to an increase in immobilization efficiency, but this declined when the amount of enzyme is higher than 2.5 ml. The reason is that the number of active groups of support is a certain datum. So if the bound position is saturated, increasing the amount of enzyme cannot raise the activity of enzyme. The reason for the declining of the activity of the enzyme when raising the amount of the enzyme may be that, when the amount of enzyme is too high, the substrate specificity will change

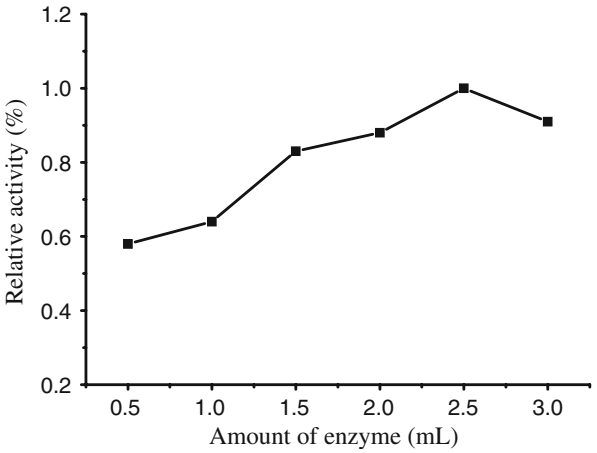

Fig. 2 The effect of amount of enzyme on immobilized enzyme

because of steric hindrances and diffusional effects. So in this study, the optimal amount of enzyme for 2 g of support should be 2.5 ml.

Concentration of Glutaraldehyde

As far as enzyme is concerned, glutaraldehyde is not only the cross-linking reagent but also the denaturing reagent. So the concentration of glutaraldehyde can affect the activity of immobilized enzyme directly. As shown in Fig. 3, when the concentration of glutaraldehyde is lower than 1.2%, the activity of immobilized enzyme would keep pace with the concentration of glutaraldehyde. But when the concentration of glutaraldehyde is higher than 1.2%, the activity of immobilized enzyme would decrease. The reason for this is that when the concentration of glutaraldehyde is lower, there would be not enough glutaraldehyde joining the cross-linking reaction. But when the concentration is too high, glutaraldehyde will experience aldol condensation, which can affect the construction of the holes on the surface of beads. This effect will not only make the immobilization harder but will also likely change the conformation of enzyme, making the activity of enzyme decline. As a result, the optimal concentration of glutaraldehyde will be 1.2%.

Enzymic Assay of Free and Immobilized β-Galactosidase

The effect of temperature on the activity of enzyme has two aspects: raising the temperature can speed up the enzymatic reaction; raising the temperature can also make the enzyme denatured. The effect of temperature on the free and immobilized β-galactosidase activities were investigated by using ONPG as substrate as shown in Fig. 4. The temperature profile of the immobilized β-galactosidase was broader at higher temperature compared to the free counterpart. At low temperature section, the catalytic activity of free and immobilized β-galactosidase increased with the rise of the temperature at first and, after a maximum for each enzyme at 37 and 47 °C, decreased at higher temperature, respectively. And the optimal temperature of β-galactosidase was enhanced by 10 °C after immobilization. As was evident from the data, the immobilized enzyme possessed better heat resistance than free enzyme. Gluay Bayramoglu et al., who immobilized β-galactosidase onto magnetic poly (GMA-MMA) beads, have obtained the same results [16]. This may be explained by stating that the immobilization procedure could protect the enzyme active conformation

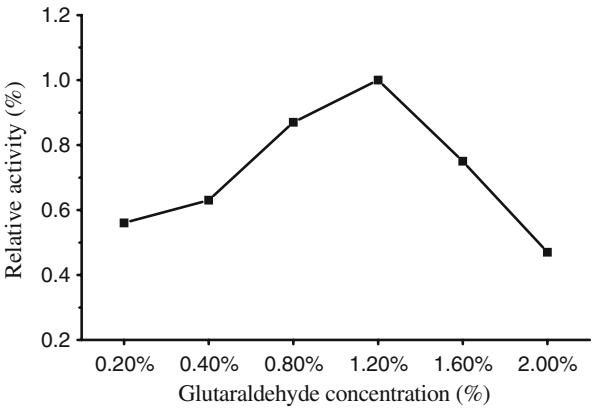

Fig. 3 The effect of glutaraldehyde concentration on immobilized enzyme

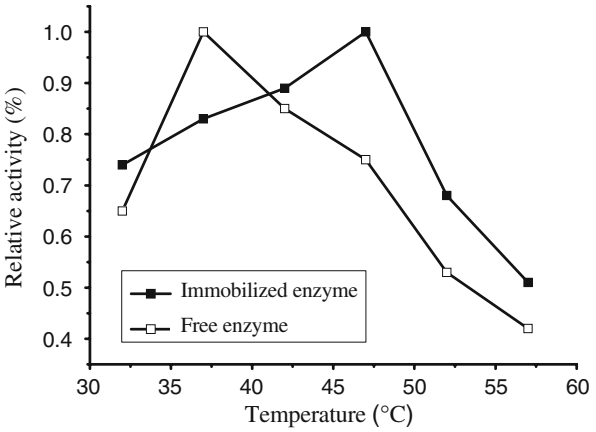

Fig. 4 Temperature profiles of the free and immobilized enzyme

from distortion or damage by heat exchange. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating forces due to restricted conformational mobility of the molecules following immobilization. Therefore, the immobilized enzyme could work in harsh environmental conditions with less activity loss compared to the free counterpart [1, 17–19].

The effect of pH on the activity of the free and immobilized enzyme was examined in the pH range from 6.3 to 8.8 and the results are presented in Fig. 5. The pH value for optimum activity for the free β-galactosidase was found to be 7.7. On the other hand, the optimal pH for the immobilized β-galactosidase was found to be 8.3. The shift to alkaline region for the immobilized enzyme can be due to two reasons. First, it is the electro-loading property of the support. As the beads bear the negative electricity, it can attract the H^+ in the reactive solution to its nearby surroundings, which may lead to the effect of lowering pH of immobilized enzyme area than that of other areas. Thus, the pH of this area should be raised to a certain amount so as to enable the enzyme to function properly. As a result, the pH of the immobilized enzyme appears to be higher than that of free enzymes.

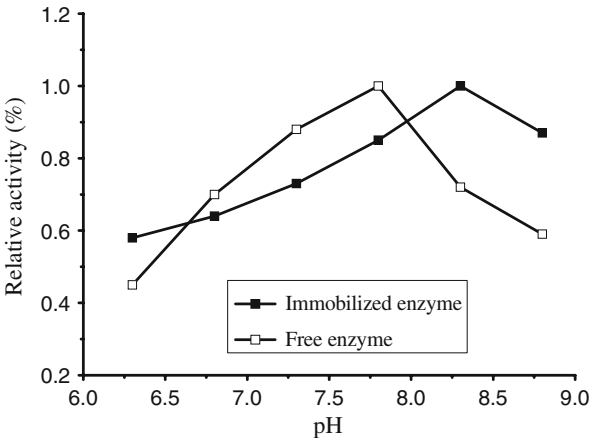

Fig. 5 pH profiles of the free and immobilized enzyme

Second, it is the property of the products. When the products are acid, they would have to pile up in the enzyme reacting area due to its spreading being prevented. Thus, the pH of this area increases accordingly and should be raised to a certain amount so as to enable the enzyme to function properly. In other words, the immobilized β-galactosidase exhibited good adaptability to environmental alkalinity.

Reusability of the Immobilized β-Galactosidase

To determine the reusability of immobilized β-galactosidase, the activity was assayed in several batches. Residual activity of immobilized β-galactosidase on reuse is shown in Fig. 6. The activity of the first batch was taken as 100%. The assay condition remained the same for all batches. The immobilized β-galactosidase had a better reusability, as it retained 53% residual activity after nine times. There is a possible reason that the conformation of immobilized enzymes can be adjusted to the optimal state during reuses, and the kind of conformation adjustment is suitable for further activation of the immobilized enzymes. Due to the deactivation of enzyme, however, the relative activity of the immobilized enzyme decreased gradually again when it was reused more than five times, as shown in Fig. 6. It was demonstrated that immobilized β-galactosidase could be used over three times.

Conclusion

The objective of the present paper is to develop techniques for immobilization of β-galactosidase and to study different properties. In this study, the enzyme "β-galactosidase" was covalently immobilized on the magnetic beads which were products of our laboratory. The immobilized β-galactosidase have been characterized and compared with its soluble counterpart in terms of temperature and pH. The results showed that the immobilized β-galactosidase retained much of their activity in wider ranges of temperature and pH than that of the free form. And the immobilized β-galactosidase could retain 53% residual activity after using nine times.

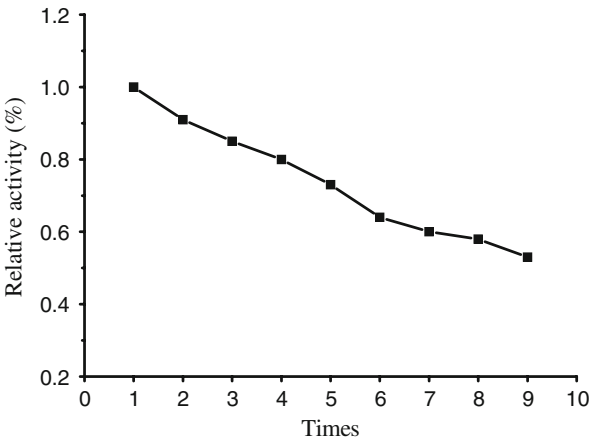

Fig. 6 Reuse of immobilized enzyme

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