Immobilization of Nuclease p1 on Chitosan Micro-spheres

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This paper presents the preparation of chitosan micro-spheres by the cross-linking method. Effects of various factors on enzyme activity were investigated, such as glutaraldehyde concentration, cross-linking time, enzyme amount, pH, and immobilized time. The immobilized conditions were optimized by orthogonal experiments. Characterizations of immobilized nuclease p1 were also evaluated. Through orthogonal optimization, optimal immobilization conditions were as follows: glutaraldehyde mass fraction w = 0.20 %, enzyme amount 6.0 mL (enzyme activity 2160 U), immobilized time 4.0 h and pH 5.8. Optimal pH of immobilized enzyme was 5.8. Optimal temperature of immobilized enzyme was 80 °C. Thermal, operational and storage stabilities of the enzyme were improved after it was immobilized on chitosan micro-spheres. Michaelis constant K_m of immobilized enzyme was 96.58 mg mL^{-1} by Lineweaver-Burk plot at 70 °C.

Key words:
Chitosan micro-spheres, nuclease p1, immobilization, characterizations

Introduction

Nuclease p1 (EC 3.1.30.1), an extracellular enzyme was first identified by Kuninaka *et al.* from *Penicillium citrinum*.[1] It can cleave 5′-nucleotides (e.g. 5′-AMP, -GMP, -CMP and -UMP) successively from the 3′-hydroxy termini of 3′-hydroxy-terminated oligonucleotides originating from RNA.[2,3] When nuclease p1 is added during the production of baker yeast, the enzyme hydrolyses the yeast RNA efficiently into 5′-GMP. An interesting feature of 5′-GMP is that it acts synergistically with monosodium glutamate and thus can largely replace monosodium glutamate in various food products. 5′-nucleotides have been widely used in the food processing and pharmaceutical industries.[4] They can be used to synthesize the antivirus and anticancer medicaments as an acridine.[5] The nucleotides' derivates have been applied in the treatment of the illness of human's central system and circulatory system.

The use of soluble enzymes has several disadvantages, e.g. instability and sensitivity to process conditions.[6] For these reasons, application of solid phase biocatalysts has become increasingly important over the last decades. Advantages of immobilized enzymes are that the biocatalysts can be used repeatedly in successive batches, or the process can eventually be carried out in a continuously operating reactor.

Chitosan, poly[β-(1–4)-linked-2-amino-2-deoxy-D-glucose], is the N-deacetylated product of chitin which is a major component of arthropod and crustacean shells such as lobsters, crabs, shrimps, and cuttlefishes.[7] This material is non-toxic, biocompatible and biodegradable.[8,9] Its reactive amino and hydroxyl groups – after chemical modifications – enable the coupling of enzymes.[10] Therefore, chitosan is often used as support material for enzyme immobilization.[11–13]

To the best of our knowledge, studies of nuclease p1 immobilized on chitosan microspheres have not been reported. In this paper, nuclease p1 was immobilized on chitosan micro-spheres. Preparation of immobilized enzyme and its characterizations were studied systematically. All the results obtained in this paper could provide a sound basis to the further exploration.

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Materials and methods

Materials

Chitosan was provided by Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang, China), the molecular mass and deacetylation degree of which was 91.000 and 95 %, respectively. Groundnut meal was a gift from Hangzhou Zhongmeihuadong Pharmaceutical Co., Ltd. (Hangzhou, China). Glucose kit was purchased from Ningbo Cicheng Reagent Factory (Ningbo, China). Nuclease p1-producing strain of *Penicillium citrinum* used throughout the study was kindly provided by Hangzhou Meiya Biotechnical Co. Ltd. (Hangzhou, China). All other reagents were of analytical grade.

Methods

Nuclease p1 production For nuclease p1 production, *Penicillium citrium* was grown at 30 °C in a medium (pH 7.0) consisting of (*w*): sucrose 3.0 %, potassium dihydrogen phosphate 0.10 %, ferrous sulphate 0.010 %, sodium nitrate 0.30 %, magnesium sulfate 0.050 %, potassium chloride 0.050 %, and potato extract 20 %. This 24 h grown mother culture (10 mL) was used to inoculate 50 mL of production medium containing (mass fraction *w*): glucose 6.0 %, peptone 0.20 %, groundnut meal 0.50 %, zinc sulfate 0.030 %, calcium carbonate 0.040 %, and potassium dihydrogen phosphate 0.10 %. The pH of the medium was adjusted to 5.4 by HCl. Erlenmeyer flask (500 mL) containing 50 mL of medium were incubated at 28 °C in an orbital shaker at 240 rpm for 49 h. Nuclease p1 aqueous solution was harvested by centrifugation at 3028 x g at 4 °C for 10 min, and the supernatant thus obtained was used as the crude enzyme preparation. Crude enzyme activity was 360 U mL^{-1}.

Immobilized method 2.0 g Chitosan was fully dissolved in acetic acid solution (100 mL, w = 1.0 %). Then, chitosan aqueous solution was added into the mixture of w = 10 % NaOH and φ = 95 % ethanol (volume ratio ψ = 4 : 1). Chitosan micro-spheres were washed by distilled water until neutral. Finally, chitosan microspheres with uniform size and regular shape were obtained.

Chitosan micro-spheres were reacted with glutaraldehyde (w = 0.20 %) under 30 °C for 2.0 h. Then this support was washed using distilled water to remove the excess glutaraldehyde. After that, cross-linked chitosan micro-spheres (size around 2 μm) would be obtained.

A certain amount of chitosan micro-spheres were mixed with nuclease p1 aqueous solution under 30 °C for 2.0 h. After that, immobilized enzyme support was washed using distilled water to remove the unadsorbed nuclease p1.

Determination of pH optimum and pH stability Effect of pH on free enzyme and immobilized enzyme was studied by assaying the enzymes at different pH values (4.0–7.5). pH stability was studied by pre-incubating the enzyme in buffers of different pH values (4.0–7.5), at 65 °C for 30 min. The remaining activities were determined as the method section described.

Determination of temperature optimum and thermal stability To determine the optimum temperature for the free enzyme and immobilized enzyme, the enzyme activities of nuclease p1 were measured at various temperatures using RNA as the substrate. The thermal stability was studied by incubating the enzyme at ϑ = 30, 40, 50, 60, 65, 70, 75, 80 and 90 °C for 30 min. The residual activities were determined as the method section described.

Determination of K_m and v_{max} K_m and v_{max} values of free enzyme and immobilized enzyme were determined by measuring enzyme activities with various concentrations (0.75, 1.5, 2.25, 3.00, 3.75, 4.50, 5.25, 6.00, 7.50, 9.00, 10.5 mg mL^{-1}) of RNA substrate. Kinetic constants were calculated as described by the Lineweaver–Burk method.

Nuclease p1 assay The activity of nuclease p1 was measured by the method of Fujishima *et al.*[14,15] Nuclease p1 activity was measured in terms of the amount of acid-soluble nucleotides produced by RNA hydrolysis which is catalyzed by nuclease p1. Enzyme aqueous solution (0.10 mL) or immobilized enzyme (0.10 mg) was incubated with substrate solution (w = 1.0 % RNA, c = 0.125 mol L^{-1} acetate buffer, pH 5.4, c = 3.0 mmol L^{-1} Zn^{2+}) at 69 °C for 15 min. The reaction was stopped by adding 2.0 mL of ice-cold nucleic acid precipitator (0.25 % ammonium molybdate dissolved in w = 2.5 % perchloric acid). The mixture was settled at ice-bath for more than 10 min. The precipitated RNA was removed by centrifugation at 3028 x g at 4 °C for 10 min. The supernatant fluid was diluted 50-fold with distilled water. The absorbance at 260 nm of the diluted solution was read with a blank incubation without enzymes. The activity (U) of nuclease p1 was calculated according to the following formula:

$$\text{enzyme activity (U mL}^{-1}) = $$
$$= \text{OD260} \cdot a \cdot 4.0 \cdot 50/0.1 \cdot 15 = $$
$$= \text{OD260} \cdot a \cdot 133.3$$

where a is a dilution factor of the enzyme before enzyme activity assay. One unit of enzyme activity was defined as the amount of enzyme that produced

an increase in the optical density of 1.0 in 1 min at λ = 260 nm.

Protein assay Protein was measured by the method of Bradford M.[16] A rapid and sensitive method for quantitation of microgram quantities of protein was adopted using the principle of protein dye-binding, with bovine serum albumin (BSA) as standard. The concentration of protein during purification studies was calculated from the standard curve.

Data analysis Experiments were done in duplicate and samples assayed in triplicate with variations below 5 % among them in all cases.

Results and discussion

Preparation of immobilized enzyme

Chitosan was easily degraded when the aqueous solution pH was below 5.4. Therefore, chitosan micro-spheres must be cross-linked before used as immobilized enzyme support. Effect of glutaraldehyde fraction on immobilized enzyme activity is illustrated in Fig. 1.

The highest immobilized enzyme activity could be obtained when glutaraldehyde fraction was w = 0.20 % (Fig. 1). However, when glutaraldehyde mass fraction was more than 0.20 %, the enzyme activity decreased with the increase of glutaraldehyde mass fraction. Glutaraldehyde is a cross-linking agent and at the same time, a denaturant. When $-NH_2$ in enzyme molecular was excessively reacted with glutaraldehyde group, overcrowding of enzyme molecular resulted in changing of the enzyme structure. Moreover, excessive glutaraldehyde cross-linking the $-NH_2$ group in chitosan molecules would reduce the porosity of chitosan and increase the steric hindrance.

The effect of cross-linking time on immobilized enzyme activity was investigated. The results are shown in Fig. 2. With the increase of cross-linking time, immobilized enzyme activity increased. When the cross-linking time was about 2.0 h, immobilized enzyme activity reached maximum. After that, activity remained unchanged even when cross-linking time was increased (Fig. 2). This can be explained that the space of immobilized carrier is close to saturation[17] with the increase of cross-linking time. In addition, enzyme activity will decrease when free enzyme is incubated for a long time.

The effect of enzyme amount on immobilized enzyme activity was investigated. The results are shown in Figs. 3 and 4. It was found that the relative activity increased with the amount of enzyme from 1.0 mL to 6.0 mL and then remained unchanged with further increase in the amount of enzyme (Fig. 3). Before the binding sites on the surface of chitosan micro-spheres were saturated, immobilized enzyme activity increased with enzyme amount. However, when it was saturated, immobilized enzyme activity could not increase with the increase of enzyme amount. When enzyme amount was 6.0 mL, enzyme activity recovery was up to 50 % (Fig. 4).

The effect of pH on immobilized enzyme activity was studied. The results are shown in Fig. 5. The maximum activity of immobilized enzyme was around from pH 5.4 to 5.8 (Fig. 5). It may be relevant to optimum pH or pH stability of immobilized enzyme.

The effect of immobilized time on enzyme activity was investigated. The results are shown in Fig. 6. Immobilized enzyme activity increased with the increase of immobilized time. When the time reached 4.0 h, the maximum enzyme activity was obtained (Fig. 6). This meant that the immobilized reaction was complete within 4.0 h. Immobilized enzyme activity was unchanged when the time was prolonged from 4.0 to 5.5 h. When immobilized time was more than 5.5 h, immobilized enzyme activity began to decrease. The reason being that the structure of the enzyme immobilized on chitosan microspheres had changed.

According to the results of *One-factor-at-a-time method*, four factors that mainly affected the enzyme activity were selected to determine which were the significant factors. The factors were glutaraldehyde mass fraction, immobilized time, enzyme amount, and pH. Orthogonal design is shown in Table 1. Orthogonal experimental[18] results and and their analysis are shown in Tables 2 and 3, respectively.

The factors could be ranked according to R (contribution percentage), namely, A > D > C > B (Tables 1 and 2). The optimal condition was $A_2B_2C_2D_3$. That was glutaraldehyde fraction w = 0.20 %, enzyme volume 6.0 mL (enzyme activity 2160 U), immobilized time 4.0 h, and pH 5.8.

The significant factors affecting enzyme activity were glutaraldehyde fraction and immobilized time (Table 3). Therefore, during preparation of immobilized enzyme, the most important thing was to control glutaraldehyde fraction and immobilized time.

To confirm the optimal conditions, a set of four replicate experiments with the optimal conditions were carried out. The average relative enzyme activity was 100.9 %.

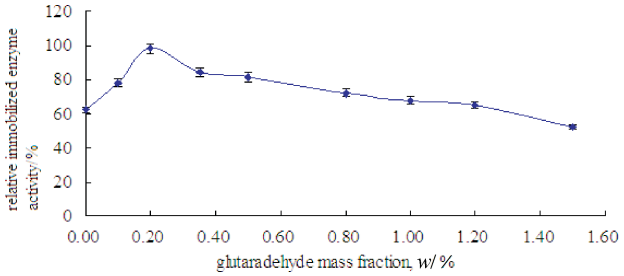

Fig. 1 – *Effect of glutaraldehyde mass fraction on immobilized enzyme activity*

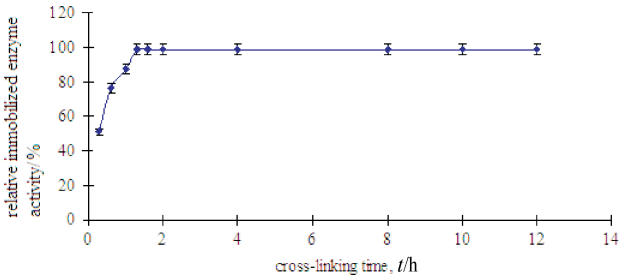

Fig. 2 – *Effect of cross-linking time on immobilized enzyme activity*

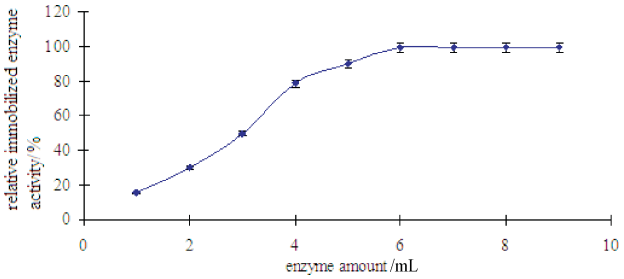

Fig. 3 – *Effect of enzyme amount on immobilized enzyme activity*

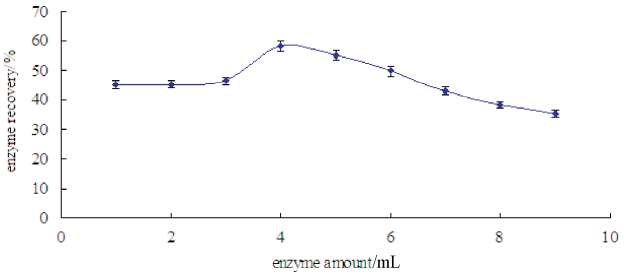

Fig. 4 – *Effect of enzyme amount on enzyme activity recovery*

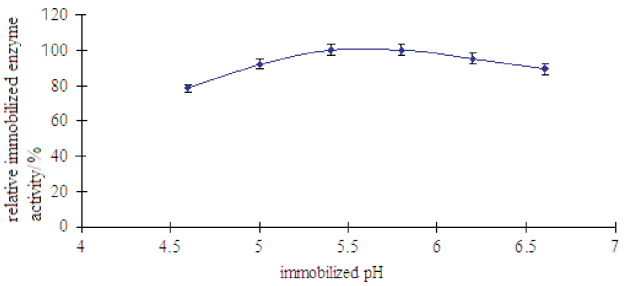

Fig. 5 – *Effect of pH on immobilized enzyme activity*

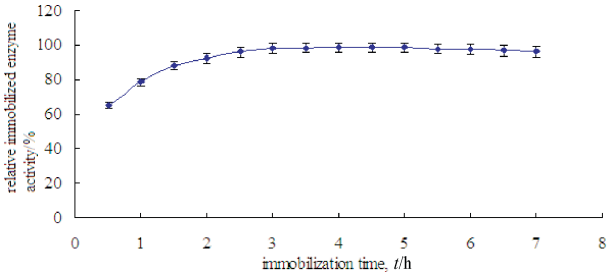

Fig. 6 – *Effect of immobilization time on immobilized enzyme activity*

Table 1 – *Levels of different factors*

Factors	Level 1	Level 2	Level 3
A glutaraldehyde mass fraction/%	0.15	0.20	0.25
B enzyme amount/mL	5.0	6.0	7.0
C immobilized time/h	3.0	4.0	5.0
D pH	5.0	5.4	5.8

Table 2 – *The result of orthogonal array*

Runs	A	B	C	D	Relative enzyme activity/%
1	1	1	1	1	84.0
2	1	2	2	2	78.2
3	1	3	3	3	80.2
4	2	1	2	3	100.0
5	2	2	3	1	85.2
6	2	3	1	2	83.6
7	3	1	3	2	55.8
8	3	2	1	3	80.8
9	3	3	2	1	72.8
K_1	80.80	79.933	82.80	80.667	
K_2	89.60	81.40	83.667	72.533	
K_3	69.80	78.867	73.733	87.00	
R	19.80	2.533	9.934	14.467	

Table 3 – *Results of ANOVA and optimal factor levels by Taguchi method. DF degree of freedom, MS mean square, R contribution percentage, *Significance < 0.05.*

Factors	MS	DF	F	Significance
glutaraldehyde mass fraction	590.480	2	60.83	*
enzyme amount	9.707	2	1.00	
immobilized time	181.627	2	18.711	
pH	315.547	2	32.507	*
error	30.75	2		

Characterizations of immobilized enzyme

Compared with free enzyme, optimum pH of immobilized enzyme increased 0.5 units (Fig. 7). The pH optimum value of immobilized enzyme shifted to a higher or lower pH, depending on surface charges of the supports.

pH stability of the enzyme had been enhanced after enzyme was immobilized (Fig. 8). Enzyme may be in the micro-environment formed by the supports. The change of aqueous solution pH did not affect the protective layer of the supports. Alternatively, maybe there is an interaction between the supports and nuclease p1, making enzyme molecules conform more firmly.

The effect of temperature on enzyme activity is shown in Fig. 9. Optimum temperature of immobilized enzyme had been increased $\vartheta = 10$ °C (Fig. 9). This means that immobilized enzyme had higher resistance to the change of temperature that that of free enzyme. At low temperature level, the enzyme activity of free enzyme was higher than that of immobilized enzyme. Perhaps the molecular diffusion resistance and the conformation change of immobilized enzyme cause the decrease of the reaction rate. At high temperature level, the enzyme activity of both free enzyme and immobilized enzyme decreased sharply with the increase in temperature. A possible explanation is that with the increase in temperature the enzyme molecules spread out acutely and ultimately lost their biocatalytic activity.

The free and immobilized enzyme were incubated in a water bath at different temperatures for half an hour, respectively (Fig. 10). The activity of both free enzyme and immobilized enzyme decreased sharply with increasing temperature up to 70 °C. The activity of immobilized enzyme on paper cellulose remained more than 90 % after it had been incubated at 70 °C for 9.0 h (Fig. 11). However, only 63 % activity of free enzyme was maintained under the same condition. Compared with free enzyme, the thermal stability of immobilized enzyme on chitosan microspheres increased greatly, probably because more stable three-dimensional structures of immobilized enzyme enhanced the denaturation of heat-resistant enzyme. The improvement of optimum temperature and thermal stability of the enzyme is favorable for industrial application.

Storage stability of immobilized enzyme was quite good (Fig. 12). The activity remained unchanged after 90 days. However, more than 10 % activity of free enzyme was lost after 90 days. Generally, the combination of immobilized carrier with enzyme enhances enzyme stability. The increase of storage stability of immobilized enzyme is very favourable for industrial application.

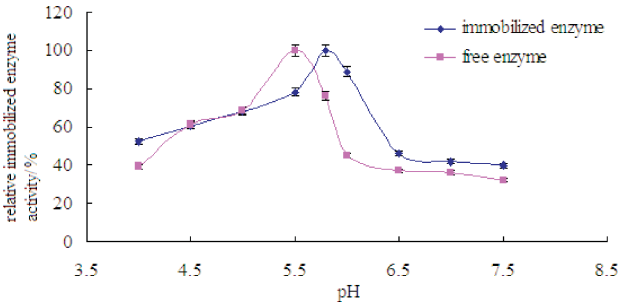

Fig. 7 – *Optimum pH of free enzyme and immobilized enzyme*

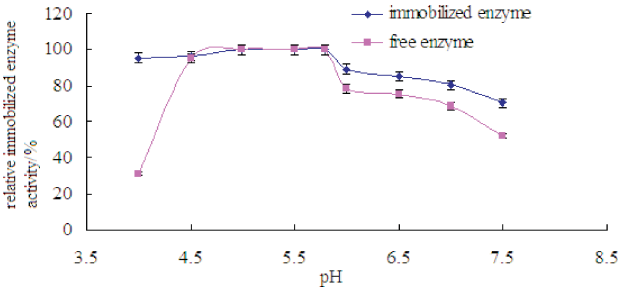

Fig. 8 – *pH stability of free and immobilized enzyme*

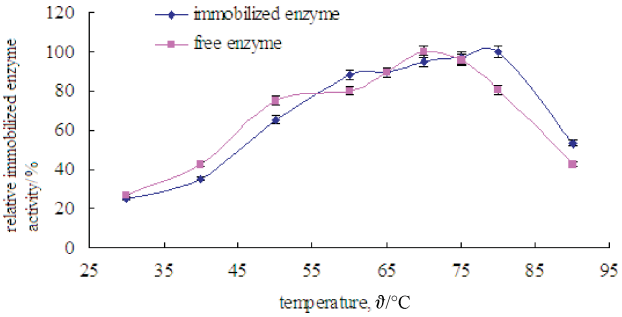

Fig. 9 – *Optimum temperature of free and immobilized enzyme*

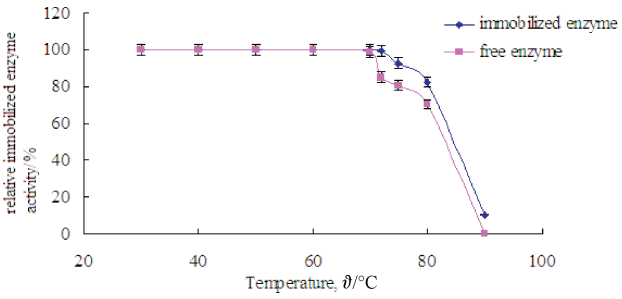

Fig. 10 – *Temperature stability of free and immobilized enzyme*

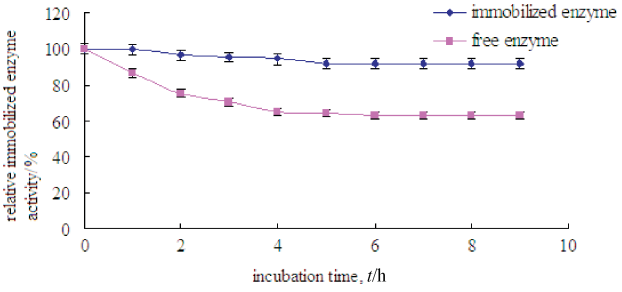

Fig. 11 – *Temperature stability of free and immobilized enzyme after a long time storage*

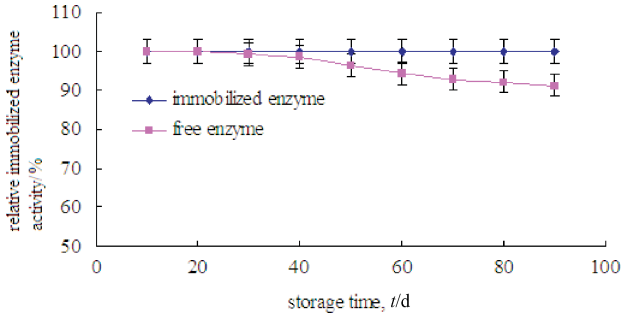

Fig. 12 – *Storage stability of free and immobilized enzyme*

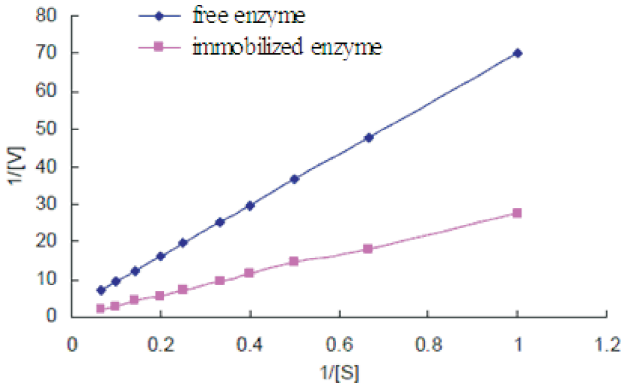

Fig. 13 – *Lineweaver-Burk plots of nuclease p1. The enzyme activity was determined under standard assay conditions. Symbols: S: RNA concentration (mg mL^{-1}); v reaction rate (mg mL^{-1} min^{-1}).*

Table 4 – *Michaelis constant of soluble and immobilized enzyme*

Category	K_m/mg mL^{-1}	v_{max}/mg mL^{-1} min^{-1}
free enzyme	24.28	0.54
immobilized enzyme	96.58	3.57

Determination of free and immobilized nuclease p1 K_m and v_{max} was studied. The results are shown in Fig. 13 and Table 4. Michaelis constant of immobilized enzyme were three times higher than that of free enzyme (Table 4 and Fig. 13). This increase may have resulted from conformational and steric modifications introduced by the attachment of the enzyme to the support and to mass transfer resistances inherent in the morphology of the support used, thus reducing the affinity of the substrate for the active site of the enzyme.

Conclusions

Nuclease p1 immobilized on chitosan micro-spheres was studied in this paper. Optimal immobilization conditions were as follows: glutaraldehyde mass fraction 0.20 %, enzyme amount 6.0 mL (enzyme activity 2160 U), immobilized time 4.0 h and pH 5.8. Characterizations of immobilized enzyme such as optimum pH, optimum temperature and storage stability were significantly higher than those of the free enzyme. This made immobilized enzyme better adapted to the change of pH and temperature, more conducive to industrial production.

List of symbols

a – dilution factor
c – molar concentration, mmol L^{-1}, mol L^{-1}
F – F distribution test
k – constant, mg mL^{-1}
K_m – Michaelis constant, mg mL^{-1}
K_1, K_2, K_3 – average result of each factor
R – contribution percentage, %
t – time, h, d
w – mass fraction, %
ϑ – temperature, °C
λ – wavelength, nm
φ – volume fraction, %
ψ – volume ratio

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