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Covalent immobilization of enzymes within micro-aqueous organic media

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ABSTRACT

Traditional covalent immobilization of enzymes was mostly operated within water phase. However, most of enzymes are flexible when they are in water environment, and the covalent reactions generally lead to complete or partial activity losing due to the protein conformational changes.

This paper examined enzyme covalent immobilization operated in micro-aqueous organic media, to display the differences between two environments of immobilization within water and micro-aqueous organic solvent by activity and stability determination of the resulting immobilized enzymes. Catalase, trypsin, horseradish peroxidase, laccase and glucose oxidase have been employed as model enzymes. Results showed the thermal, pH and reusable stabilities of the micro-aqueous organic covalently immobilized enzymes were improved when compared with the immobilized enzymes within water. Micro-aqueous covalent immobilization showed a remarkable advantage in remaining the enzymes catalytic activity for all the five enzymes compared with the traditional water phase immobilization. And the optimum pH values for both immobilization within water and micro-aqueous organic media shifted slightly.

1. Introduction

Enzyme immobilization is an interesting approach to obtain enzyme stabilization and consequently cost reduction [1]. Covalent immobilization of the enzyme on insoluble supports is one of the most important immobilization methods which can improve enzyme's stability besides allowing an easy recovery and re-utilization of the enzyme [2]. While covalent immobilization of enzyme may result in activity losses, there are three main reasons for this: (1) sterical hindrance (partial blocking-out of active sites); (2) diffusion limitations (reduced convection near the beads); and (3) structural changes (often caused by the immobilization process) [3].

In order to improve the activity of covalently immobilized enzyme, a considerable amount of research has been done during the last decade. Site-specific immobilization could be well ordered leading to a high number of properly oriented active sites being available for catalytic action [4], Ozturk and Kilinc recently immobilized lipase in organic solvent in the presence of fatty acid additives, in this way the active center occupied by a substrate molecular prevented a conformational change of the enzyme during covalent binding, thus preserving high activity [5]. Carrier-free immobilization, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs): enzyme protein firstly crystal or aggregated then crosslinked by a bifunctional reagent could overcome the limitations associated with conventional carrier-immobilized enzymes [6,7].

While literatures on how to overcome the problem of structural changes during covalent immobilization have rarely been reported. Klibanov [8] found enzymes apparently remain in native, enzymatic active conformations in organic solvents containing little or no water. Conformational mobility of enzymes at such low water content is generally restricted. Therefore, the proteins are more rigid in this system than in water [9]. Simon and coworkers have studied the effects of different water-miscible and water immiscible organic solvents on the conformational stabilities of some hydrolytic enzymes [10]. The reaction medium is believed to govern both the conformation of protein and the relative reactivity of their functional groups, thus influencing the immobilization pattern [11]. So, in this paper, we have investigated five enzymes, which were covalently immobilized in organic solvent or traditional water environment separately, to understand whether the organic solvent may endow the enzyme protein with resistance to conformational disorder during the chemical reaction occur (covalent immobilization) by comparing the resulting enzymes

Abbreviations: HRP, horseradish peroxidase; GOD, glucose oxidase; 4-APP, 4-amino antipyrine; ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid); BAEE, N-α-benzoyl-L-arginine ethyl ester; CLECs, crosslinked enzyme crystals; CLEAs, crosslinked enzyme aggregates.

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activity and stability. The enzyme lyophilized from aqueous solution at its optimum pH exhibits high activity in organic solvents [12,13]. This phenomenon is commonly referred to as "pH memory" in organic solvents. To induce the enzyme to form optimum conformation, enzymes were lyophilized at their optimum pH followed with transferring into organic solvents. However, complete organic media may lead enzyme denature because water participates (directly or indirectly) in all noncovalent interactions maintaining the native, catalytically active enzyme conformation [13]. So little water was added when immobilize enzyme in organic solvents to maintain enzyme activity. Since water content is important for enzymes to show sufficient activity [12,13], the activity of two environments covalently immobilized enzymes was determined under water system.

In the present study, water content of various organic solvents for micro-aqueous organic media covalent immobilization was optimized. Temperature and pH profiles, thermal, pH and reusability of micro-aqueous organic immobilized enzymes were determined and the results were compared with that of the water-phase immobilized enzymes.

2. Materials and methods

2.1. Enzymes and chemicals

Glucose oxidase (GOD) (EC 1.1.3.4), trypsin (EC 3.4.21.4), laccase (EC 1.10.3.2), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and o-dianisidine were obtained from Sigma–Aldrich Co. (Shanghai, China). Horseradish peroxidase (HRP) (EC 1.11.1.7), N-α-benzoyl-L-arginine ethyl ester (BAEE) and catalase (EC 1.11.1.6) were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Chitosan, hydrogen peroxide, glutaraldehyde and 4-amino antipyrine were from local chemical and biochemical agent companies. All of the other chemicals and reagents used were analytical grade.

2.2. Preparation and activation of chitosan microspheres

Three grams of chitosan flakes were added into 99 ml of distilled water and suspended by magnetic stirring for 10 min. One milliliter of glacial acetic acid was then added and mixing continued for 3 h at room temperature and filtrated through a gauze pad to remove impurities (small particles, residues of the chitin deacetylation process). The solution thus obtained was stored at room temperature and was used within 1 week of preparation [14]. After that, the filtrate was dispersed through a pipettor into a coagulation solution [2.5 M NaOH and 16.7% (v/v) ethanol] to form chitosan microspheres about 2 mm in diameter. The system was kept for about 2 h until complete coagulation of particles. The resulting particles were washed with distilled water until pH 7.0 was reached and classified using stainless steel sieves. This methodology modified the protocol described by Arruda and Santana [15].

Chitosan microspheres were activated under glutaraldehyde with different concentrations according to Table 1 at 25 °C for 2 h followed by washing with distilled water till pH 7.0 before immobilization.

2.3. Immobilization of enzymes onto the activated chitosan microspheres

Catalase (0.2 mg/ml), trypsin (0.2 mg/ml), HRP (0.1 mg/ml), laccase (2 mg/ml) and GOD (10 mg/ml) solution were prepared in corresponding buffer according to Table 1 following with lyophilizing.

The lyophilized enzymes were put into the buffers according to Table 1 and mixed with the activated chitosan beads for water phase immobilization. For micro-aqueous organic immobilization, organic solvents with little water were employed as coupling media. The reactions performed at 0 °C for 2 h in a rotary shaker, and the immobilized enzymes were stored in 4 °C for further use.

2.4. Assay of enzyme activity

The activity of catalase was determined spectrophotometrically by the direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme [16]. Activity determination was carried out according to a modification of method described by Çetinus and Öztop [14] under optimum conditions. One unit of catalase activity is defined as the decomposition of 1 μmol hydrogen peroxide per min at 25 °C and pH 7.0 [17]. Esterolytic activity of trypsin was determined at 25 °C in 67 mM Tris–HCl buffer, pH 8.0 using BAEE as substrate [18]. One unit of esterolytic activity was defined as the amount of enzyme that hydrolyses 1.0 μmol of BAEE per minute at 25 °C. The activity test for HRP was carried out using the 4-APP method [19]. One unit of HRP activity is defined as the amount of enzyme that decomposed 1 μmol hydrogen peroxide per min at 25 °C and pH 7.0. Laccase activity measurement was operated employing ABTS as substrate [20]. One unit of activity is the amount of enzyme that catalysed the formation of 1 μmol of ABTS radical/min [21]. The enzyme activity of the free and immobilized GOD was measured using the method stated in the Sigma Technical Bulletin. The method involved spectrophotometric determination of the amount of the hydrogen peroxide formed from glucose oxidation [22]. One unit causes the oxidation of 1 μmol of o-dianisidine per minute under the conditions specified. All of the measurements have been slightly adjusted to meet the solid enzymes analysis. A certain weight of chitosan beads (immobilized with enzymes) were mixed with substrate solutions to react. After 5 min, the reaction was terminated by removal of the chitosan beads from the reaction mixture. The absorbance of the reaction mixture was determined and the immobilized enzymatic activity was calculated.

2.5. Water content of organic solvents effects on immobilized enzymes activity

To find the optimum immobilization conditions, we tried different concentrations of glutaradehyde for activation and ratio of enzyme to carrier for immobilization (data unpublished), under the found optimum concentrations and ratio (see Table 1), corresponding water content (0.1–2.0%) of various organic solvents (chloroform, acetic ether, ethanol, 1,4-dioxane and ether) were studied.

2.6. Determinations of activity and stability of the covalent immobilized enzymes

2.6.1. The active temperature and pH ranges of immobilized enzymes

The activity of pH profile was determined at various pH under optimal temperature, and the activity of temperature profile was determined at indicated temperatures at optimum pH.

2.6.2. The thermal and pH stability of immobilized enzymes

The thermal and pH stability of both environments immobilized enzymes were ascertained by measuring the residual activity of enzymes exposed to a given temperature in corresponding buffer for various times, or exposed to buffers with different pH values for 5 h, and the residual activity of samples was performed under optimum conditions.

Table 1
Parameters for immobilization.

Enzyme	Buffer for lyophilization and water-phase immobilization	Conc. of glutaradehyde (%, w/v)	Ratio of enzyme to carrier (mg enzyme/g beads)
Catalase	0.05 M, pH 7.0 PBS	0.05	5.00
Trypsin	0.05 M, pH 7.6 Tris–HCl	0.50	3.00
HRP	0.2 M, pH 7.0 PBS	0.30	0.50
Laccase	0.1 M citric acid–0.2 M Na_2HPO_4, pH 4.2	0.80	11.3
GOD	0.1 M, pH 6.0 PBS	0.10	80.0

2.6.3. The reusable stability of immobilized enzymes

The residual activities of the immobilized enzymes were tested as described in assay of enzyme activity. After each reaction run, the enzyme–chitosan beads were removed and washed with corresponding buffer to remove any residual substrate on the chitosan beads. They were then reintroduced into fresh reaction medium and enzymes activities were detected.

3. Results and discussion

3.1. Water of organic solvent effects on the covalently immobilized enzyme activity

Removal of water should drastically distort that conformation and inactivate the enzyme, so to the enzyme activity the real question is not whether water is indeed required but how much water [13]. A small amount of water was essential to catalytic activity, but too much water may lead the enzyme protein "flexible", which could make the enzyme lose its rigidity and easier denature during covalent immobilization. Optimization of water content was shown in Table 2 which ranged between 0.4% and 1.6% (v/v). Either too much or too little water will disturb the function of micro-aqueous organic immobilized enzymes.

3.2. Determination of activity of the covalently immobilized enzymes

The active characteristics of five immobilized enzymes which covalently immobilized within the corresponding media have been compared.

All the results shown in figures (Figs. 1–5) are just only the remarkable group of micro-aqueous organic immobilized one: micro-aqueous chloroform immobilized catalase and laccase, micro-aqueous acetic ether immobilized trypsin, micro-aqueous 1,4-dioxane immobilized HRP and GOD. The data of rest micro-aqueous organic immobilized enzymes are not shown.

Temperature profiles of water and micro-aqueous organic immobilized enzymes are shown in Fig. 1. The optimum reaction temperatures for both water and micro-aqueous organic immobilized catalase were not shown significantly different, since both were 10 °C higher than that of free catalase. This phenomenon may due to the slightly protecting effect at the high temperatures at which enzyme deactivation takes place [23]. At the same time, the micro-aqueous organic immobilized catalase showed much higher activity than that of water-phase immobilized one. The optimum temperature for micro-aqueous organic immobilized trypsin was 55 °C, which was higher than that of water-phase immobilized trypsin (45 °C). And similar results have been obtained from the rest enzymes: micro-aqueous organic immobilized enzymes showed some extent higher activity than water-phase immobilized enzymes with similar optimum temperatures for both type of immobilization. When the covalent modification occurred in micro-aqueous organic phase, that more enzyme molecules maintained the correct conformation due to protein "rigid" in organic solvents with "pH memory" might be a possible explanation for this phenomenon [24,25].

Table 2
The optimum water content (v/v) of organic solvents for enzyme immobilization.

Organic solvents	Enzymes				
	Catalase	Trypsin	HRP	Laccase	GOD
Chloroform	0.6%	1.0%	–	0.8%	–
Acetic ether	0.6%	0.4%	–	0.4%	–
Ethanol	0.8%	–	1.0%	–	1.0%
1,4-Dioxane	–	0.4%	1.0%	–	1.0%
Ether	–	–	1.0%	1.0%	1.6%

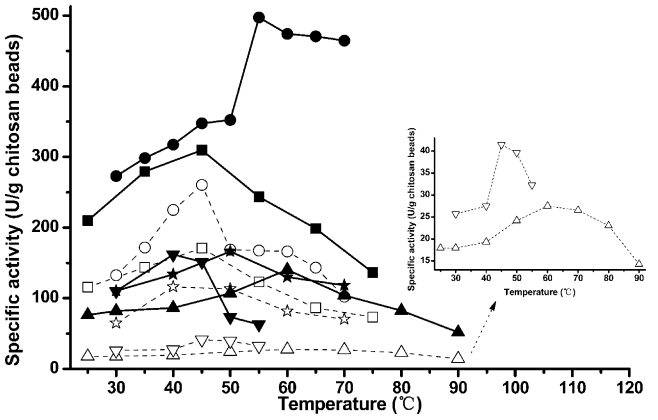

Fig. 1. Temperature profiles of immobilized enzymes: the solid line (—) with solid symbols and the dash line (- - - -) with open symbols represent micro-aqueous organic media immobilized enzymes and aqueous-phase immobilized ones, respectively. Catalase (■, □), trypsin (●, ○), HRP (▲, △), laccase (★, ☆), GOD (▼, ▽).

To examine the pH property of the immobilized enzymes' activity, a same or similar optimum pH value can be found for water or micro-aqueous organic phase immobilization (Fig. 2). However, micro-aqueous organic immobilized enzymes showed a broader pH range with clearly increased specific activity. The improved

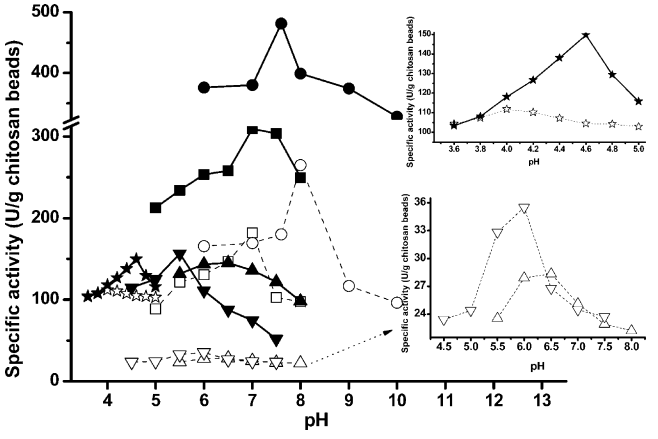

Fig. 2. pH profiles of immobilized enzymes: the solid line (—) with solid symbols and the dash line (- - - -) with open symbols represent micro-aqueous organic media immobilized enzymes and aqueous-phase immobilized ones, respectively. Catalase (■, □), trypsin (●, ○), HRP (▲, △), laccase (★, ☆), GOD (▼, ▽).

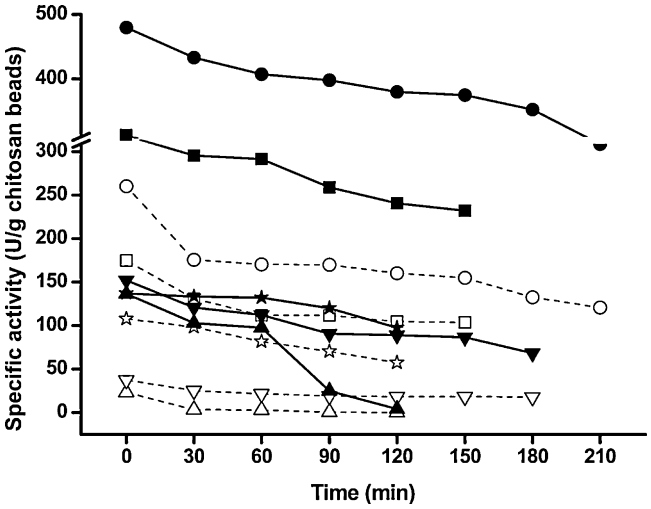

Fig. 3. Thermal stability of immobilized enzymes: the solid line (—) with solid symbols and the dash line (- - - -) with open symbols represent micro-aqueous organic media immobilized enzymes and aqueous-phase immobilized ones, respectively. Catalase (■, □), preincubate at 50 °C; trypsin (●, ○), preincubate at 65 °C; HRP (▲, △), preincubate at 70 °C; laccase (★, ☆), preincubate at 60 °C; GOD (▼, ▽), preincubate at 60 °C.

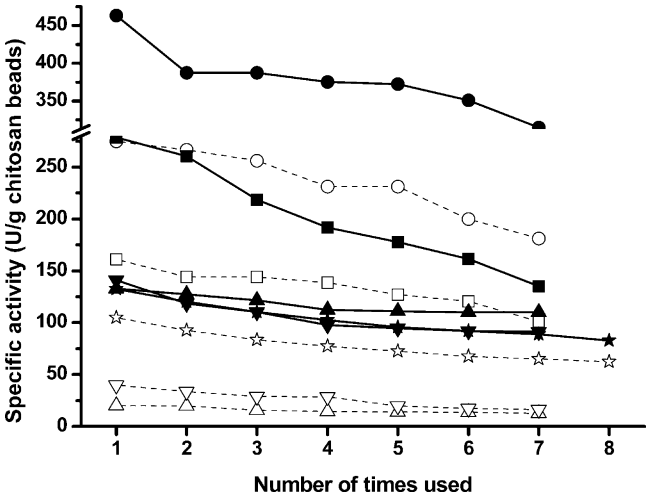

Fig. 5. Reusability of immobilized enzymes: the solid line (—) with solid symbols and the dash line (- - - -) with open symbols represent micro-aqueous organic media immobilized enzymes and aqueous-phase immobilized ones, respectively. Catalase (■, □), trypsin (●, ○), HRP (▲, △), laccase (★, ☆), GOD (▼, ▽).

activity of micro-aqueous organic immobilized enzymes appeared to be due to the structural rigidity of proteins in organic solvents resulting in high kinetic barriers that prevent the native-like conformation from unfolding [12] during covalent reaction occurring.

3.3. Thermal, pH, reusable stability of the covalently immobilized enzymes

For practical use, it is important to consider the enzyme stability with respect to various parameters like heat-resistance, reusability and storability those which are shown in Figs. 3–5, respectively. As Fig. 3 shows, water phase immobilized catalase lost about 40% of its activity whereas the micro-aqueous immobilized one only lost about 23% activity after 2 h incubation at 50 °C. The higher residual activities which referred as better thermo-stability of the micro-aqueous organic immobilization can also be found from the rest four enzymes.

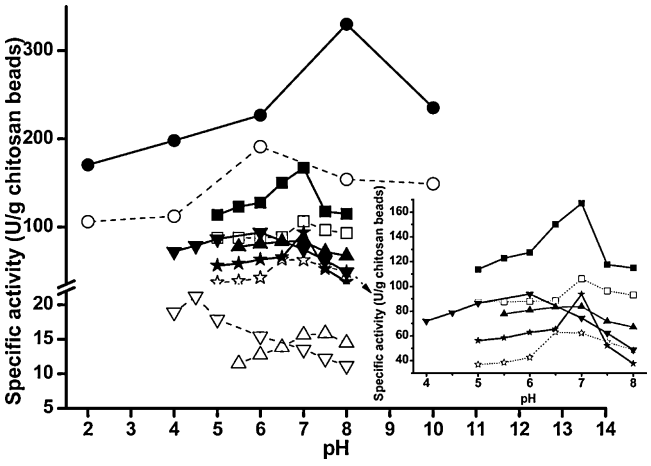

Fig. 4. pH stability of immobilized enzymes, treated by buffer with different pH values for 5 h: the solid line (—) with solid symbols and the dash line (- - - -) with open symbols represent micro-aqueous organic media immobilized enzymes and aqueous-phase immobilized ones, respectively. Catalase (■, □), treated by pH 5.0–8.0; trypsin (●, ○), treated by pH 2.0–10.0; HRP (▲, △), treated by pH 5.5–8.0; laccase (★, ☆) treated by pH 5.0–8.0; GOD (▼, ▽), treated by pH 4.0–8.0.

Fig. 4 shows pH stability of the covalently immobilized enzymes: after dealing with various pH for 5 h, micro-aqueous organic covalently immobilized enzymes performed a higher specific activity and better pH tolerance than water immobilized enzymes. Both the temperature and pH stability have been improved for the micro-aqueous organic immobilized enzymes.

The reusability of the covalently immobilized enzymes, shown in Fig. 5, indicates that after 7 times of using, micro-aqueous organic covalently immobilized catalase retained 34% more activities than that of water phase covalently immobilized one. After 7 times usage, micro-aqueous organic media immobilized HRP, GOD, laccase and trypsin showed 7.7 times, 4.5 times, 90% and 74% more active than water phase immobilized ones, respectively.

In such low water content of micro-aqueous organic media, conformational mobility of enzymes is generally restricted, and the proteins are more rigid in organic solvent than in water [8]. When the covalent reaction occurred in organic solvent environment, a higher proportion of enzyme molecules may "remember" and keep the "correct" conformation (the active conformation before lyophilized) without or with less conformational changes, and this might be an explanation for the significant improved enzymes activity for micro-aqueous organic phase covalent immobilization than the traditional water phase covalent immobilization. In addition, this "correct" conformation may some how give the immobilized enzyme proteins a better state to resistant with higher temperature and wider pH range. The key to protein function is the maintenance of an appropriate balance between the molecular stability on one hand and the structural flexibility on the other. Stability is needed to ensure the appropriate geometry for ligand binding, as well as to avoid denaturation, while flexibility is necessary to allow catalysis at a metabolically appropriate rate [26]. The "correct" conformation might maintain the appropriate balance between molecular stability and structural flexibility (the sufficient water for the enzymatic reaction) which is obtained by micro-aqueous organic phase covalent immobilization.

Considering the dramatic high activity of CLECs and CLEAs enzymes [7], we would suppose another explanation that when enzyme molecules are crystallized or aggregated, the molecules are more "rigid" (than those in water environment), and the "rigid" proteins have better resistance with the conformational changing during the further cross-linking reaction. Since crystallization adjusts the protein molecules to their "correct" conformation (the

active conformation), and aggregation, if with a very careful operation, may also obtain the correct conformation in some extent.

4. Conclusion

The present study compared the enzymatic characteristics of five enzymes covalently immobilized in micro-aqueous organic solvents with the enzymatic characteristics of ones covalently immobilized in water. The results have shown that micro-aqueous covalent immobilization affords the immobilized enzymes markedly ameliorated activities and an improved stability to resistant with higher temperature and wider pH range. In micro-aqueous organic environment, enzyme proteins (lyophilized) become "rigid" with the correct conformation (refer to "pH memory"), this "rigid" structure contributes further to protecting the protein molecules from conformational changes during the covalent reaction occurring.

However, whether micro-aqueous immobilization could be widely applied for other more enzymes, it needs further researches. In addition, there are a numerous organic solvents. Are there a simple principle and a convenient progress for solvents choosing towards a certain protein or enzyme is also a key problem for the further widely use of micro-aqueous covalent immobilization.

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