Covalent attachment of cholesterol oxidase and horseradish peroxidase on perlite through silanization: Activity, stability and co-immobilization

Seyed-Fakhreddin Torabi a, b, Khosro Khajeh c,*, Salehe Ghasempur a, b, Nasser Ghaemi a, Seyed-Omid Ranaei Siadat b, d, *

a Department of Biotechnology, University College of Science, University of Tehran, Tehran, Iran
b New Ideas Research Group (NIRG), #11, Proshat Alley, Motahhari Street, Tehran, Iran
c Department of Biochemistry, Faculty of Science, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran
d Department of Biology, Faculty of Basic Science, University of Shahed, Tehran, Iran

Received 20 November 2006; received in revised form 1 April 2007; accepted 5 April 2007

Abstract

In the present work, co-immobilization of cholesterol oxidase (COD) and horseradish peroxidase (POD) on perlite surface was attempted. The surface of perlite were activated by 3-aminopropyltriethoxysilane and covalently bonded with COD and POD via glutaraldehyde. Enzymes activities have been assayed by spectrophotometric technique. The stabilities of immobilized COD and POD to pH were higher than those of soluble enzymes and immobilization shifted optimum pH of enzymes to the lower pH. Heat inactivation studies showed improved thermostability of the immobilized COD for more than two times, but immobilized POD was less thermostable than soluble POD. Also activity recovery of immobilized COD was about 50% since for immobilized POD was 11%. The $K_m$ of immobilized enzymes was found slightly lower than that of soluble enzymes. Immobilized COD showed inhibition in its activity at high cholesterol concentration which was not reported for soluble COD before. Co-immobilized enzymes retained 65% of its initial activity after 20 consecutive reactor batch cycles.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Cholesterol oxidase; Peroxidase; Co-immobilization; Perlite; Silanization; Thermostability

1. Introduction

Cholesterol oxidase from bacteria (COD; cholesterol:oxygen oxidoreductase, EC 1.1.3.6) is a monomeric bifunctional FAD-containing flavoprotein that catalyzes the oxidation of 3β-hydroxysteroids and the isomerization of the intermediate, Δ5-6-ene-3β-ketosteroid (cholesterol-5-en-3-one) to produce Δ3-4-ene-3β-ketosteroid (cholester-4-en-3-one) (Caldinelli et al., 2005). Horseradish peroxidase isoenzyme C (POD; hydrogen peroxide oxidoreductase donor, EC 1.11.1.7) is a classic heme enzyme containing a ferric protoporphyrin IX prosthetic group (Chakrabarti and Basak, 1996) and the prototypic class III plant peroxidase (Welinder et al., 1992). These two enzymes are important in determining free cholesterol in solution and by coupling them with cholesterol esterase; they are able to determine total cholesterol, free and esterified one, in different samples. So they are important in clinical diagnosis and food industry (Allain et al., 1974; MacLachlan et al., 2000).

Enzyme immobilization on a solid support is a technique that has been demonstrated to prevent downstream contamination of the product and make enzymes more reusable. In different applications immobilized enzymes offer the benefit of recovery and reuse of expensive enzyme catalysts. Also, immobilization of enzymes makes them more stable against thermal denaturation by decreasing their flexibility. The other benefits of immobilization of enzymes are: immobilization protected enzymes from irreversible inactivation through aggregation, proteolytic attack and action by denaturing agents (Balcao et al., 2001). COD and POD have been immobilized separately or together using different methods and supports (Huang et al., 1977; Kumar et al., 2000, 1999; Lin and Yang, 2003a; Suman and Pandir, 2003). Co-immobilized system is side to give additional advan-
tages because the proximity of the two enzymes and shorter diffusional distance create a microenvironment for the second enzyme which is rich in its substrate; this reduces the time of diffusion of the substrate to the second enzyme and make this system more sensitive towards low concentration of substrate for the enzyme than a corresponding system with the enzymes separately immobilized (Romero et al., 1989; Suman and Pundir, 2003). In the case of using porous support this effect is amplified, due to the increased diffusion limitations of the intermediate product in the sequence and intermediate product will reacts before it diffuse out of supports pores (Ge et al., 1999).

Perlites are amorphous aluminum silicates with high content of silica more than 70%. Commercially, the term perlite is used describe either natural or expanded perlite which formed by heating and stirring, until the solution became clear and avoided from boiling or becoming yellow. Then 90.0 ml of double distilled water added slowly. The solution allowed boil for 1 min which made milky solution, then immediately cooled in mixture of ice and water to become clear solution. Finally 2.07 g of cholic acid was added and solved by gently stirring. The solution was stored in amber colored bottle at 4 °C until use.

2.3. Preparation of color reagents

The color reagent was prepared for COD and POD separately. Color reagent of COD prepared by solving 32 mg 4-aminooantipyrine (4-AAP) and 200 mg phenol in 0.1 M sodium phosphate buffer (pH 7.0) until total volume reached 100 ml. In the case of POD color reagent prepared by solving 50 mg 4-AAP and 1620 mg phenol in 0.2 M sodium phosphate buffer (pH 7.0) until total volume reached 100 ml. The color reagents were stored in amber colored bottle at 4 °C until use. The color reagent older than a week was discarded.

2.4. Enzyme activity assay and kinetic parameters determination

Peroxidase activity was assayed by colorimetric method with slight modification (Wagner and Nicell, 2001). The assay uses phenol, 4-AAP and H$_2$O$_2$ as color-generating substrates in the saturating concentrations. With phenol, H$_2$O$_2$ and 4-AAP concentrations present in sufficient quantity, the rate of color generation at 505 nm is proportional to the rate of H$_2$O$_2$ consumption. The reaction mixture (1 ml) contained 80 mmol l$^{-1}$ phenol, 1.14 mmol l$^{-1}$ 4-AAP, 0.75 mmol l$^{-1}$ H$_2$O$_2$, 10 μl POD (5.18 × 10$^{-2}$ μg) and total volume set by adding buffer. Under such conditions, the rate of formation of colored product which absorbs light at a peak wavelength of 505 nm was calculated using a molar extinction coefficient of 7100 M$^{-1}$ cm$^{-1}$ at 25 °C.

Cholesterol oxidase activity was assayed by a modification of the method of Allain et al. (1974). The amount of hydrogen peroxide which was produced by COD during cholesterol oxidation was measured from the amount of quinonemine formed by the oxidation of 4-AAP and phenol by hydrogen peroxide in presence of POD. The reaction mixture (1 ml) contained 20 mmol l$^{-1}$ phenol, 1.6 mmol l$^{-1}$ 4-AAP, 0.9 mmol l$^{-1}$ cholesterol, 2 μl POD (1.036 μg), 20 μl diluted COD (2.64 μg) and total volume set by adding buffer. The rate of formation colored product was measured like POD activity assay. In the case of immobilized enzyme activity assay after addition of 50 μl suspension of immobilized enzyme to the each mixture reaction in vials, they was sealed and then vortexed for 1 min and then rapidly vials were centrifuged at 16,000 × g for 30 s. The supernatants were used for determining of the product absorbance like free enzyme. All reactions and measurements were carried out at room temperature.

For free COD kinetic parameters, the results obtained can be treated by using the Michaelis–Menten equation to yield estimates of $V_{\text{max}}$ and $K_m$. Several concentration of cholesterol were used ranging from 8 to 3200 μM. In addition, the effect of substrate concentration on POD activity was studied to determine the kinetic parameters ($V_{\text{max}}$ and $K_m$). Several concentration of

2.2. Preparation of cholesterol solution

Cholesterol was used as a substrate for COD. 500 mg of cholesterol was dissolved in 5.0 ml of Triton X-100 by slowly heating and stirring, until the solution became clear and avoided

2. Materials and methods

2.1. Materials

Cholesterol oxidase (COD) (E.C. 1.1.3.6) with a specific activity of 27 U mg$^{-1}$ solid and horseradish peroxidase (POD) (E.C. 1.11.1.7) with a specific activity of 502 U mg$^{-1}$ solid were procured from Sigma and Fluka, respectively. Glutaraldehyde 25% was purchased from TAAB (Laboratories, England, UK). 3-Aminopropyltriethoxysilane (APTS) (99%), Triton X-100 and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (MO, USA). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Aqueous solutions were prepared in doubly distilled deionized water. Perlite obtained from Tabriz, Iran sources.

2.2. Preparation of cholesterol solution

Cholesterol was used as a substrate for COD. 500 mg of cholesterol was dissolved in 5.0 ml of Triton X-100 by slowly heating and stirring, until the solution became clear and avoided
hydrogen peroxide were used ranging from 5 to 5000 μM. Values of \( V_0 \) were determined at short reaction time in the initial linear phase of the reaction, from triplicate measurements for both immobilized and soluble enzymes.

2.5. Preparation of the support and enzyme immobilization

Different procedures have been used for support preparation and enzyme immobilization on silica supports by using 3-APTS and glutaraldehyde (Blasi et al., 2005; Ettalibi and Baratti, 2001; Ferreira et al., 2003; Kim et al., 2004; Singh et al., 1999). In this study our method is slightly different with previous studies. After separating fine perlite powder with special mesh size with sieve, 25 g of dry perlite were washed by stirring overnight in methanol to remove any organic contaminant and then were washed by distilled water. After that 5 N NaOH was added to the cleaned perlite and the solution was heated for 30 min in a boiling water bath. Precipitated perlite were filtered and rinsed with excess water until NaOH removed and the washed water reached pH 7. Perlite powder was suspended in freshly prepared 3-APTS 10% (v/v) in 50 mM acetate buffer, pH 4.0. The suspension was incubated at 75 °C for 4 h with constant mixing then washed thoroughly with abundant water in order to remove 3-APTS molecules not linked to the surface of support. Alkylamine-derivatized perlite powder was soaked in freshly prepared 10% (v/v) glutaraldehyde, as an activating agent, in deionized water. Support activation was carried out at 25 °C, with stirring for 2 h. The activated perlite were then removed by filtration and thoroughly rinsed with distilled water after glutaraldehyde treatment to remove any adsorbed cross-linker. The activated support was spread onto a clean petridish and dried in the air. The free terminal aldehyde groups were subsequently cross-linked to amines groups on the enzyme surface through Schiff’s base formation by incubating support in the enzyme solutions.

Five hundred milligram of dried activated support was used for each enzyme immobilization. 130 μg COD and different amount of POD separately were used for COD and POD immobilization. Also 100 μg COD and 112.5 μg POD together were used for co-immobilization (excess amount of POD was used to saturate support surface). After adding enzymes solution, suspensions were gently agitated overnight at 4 °C. The mixtures were then centrifuged and the supernatants were removed.

Supports were rinsed with phosphate buffer (0.1 M and pH 7.0) then incubated in 100 mM glycin for 30 min to block any unreacted aldehyde groups. After centrifuging and removing supernatants supports were soaked with intermittent shaking in 0.05% Tween-20 and 0.5 M NaCl in phosphate buffer to remove non-specifically adsorbed enzymes (all steps were carried out on ice). The resulting immobilized enzymes were held at 4 °C prior to use. The amount of immobilized enzymes on perlite was determined by measuring the residual enzyme present in all the supernatants by the Bradford method using coomassie brilliant blue G-250 and bovine serum albumin as the standard protein (Sedmak and Grossberg, 1977). The activities of the resulting immobilized COD and POD and the elution solutions were evaluated by the assay of the activity recovery and relative activity.

2.6. Thermal stability and reusable stability

The thermal stability of the soluble and immobilized enzymes was studied by measuring the residual activity after incubation of the soluble or immobilized enzymes in plastic eppendorf in heat block in 0.1 and 0.2 M phosphate buffer pH 7.0 for COD and POD, respectively, in the absence of substrates, at various temperatures for different incubation times. After incubation of enzymes in different temperatures, samples were removed and cooled on ice at least for 30 min and subsequently, the remaining activity was determined. The activity of the same enzyme solution, kept on ice, was considered as the control (100%). Moreover, thermostability of soluble POD and immobilized POD was studied in 80 °C in presence of trehalose 20% (w/v). Heat inactivation rate constants (\( k_i \)) were calculated from a first-order exponential approach:

\[
\frac{A_{\text{cat}}}{A_{\text{cat},0}} = \exp(-k_it).
\]

where \( k_i \) is heat inactivation rate constant, \( A_{\text{cat}} \) and \( A_{\text{cat},0} \) the activities of the enzymes before and after incubation and \( t \) is the time of incubation (min). All data used in this formula are the average of triplicate of experiments.

In the reusable stability studies, co-immobilized enzymes were used. After determining the enzyme activity of the co-immobilized enzymes, the supernatant from the suspension was decanted and the supports were washed for three times with 0.1 M phosphate buffer, pH 7.0. The supports were then centrifuged, the supernatant was decanted and the recycled supports subjected to the activity assay for the second cycle and so on.

3. Result and discussion

3.1. Enzyme immobilization and co-immobilization

Enzymes were immobilized on porous silica support, perlite, which its porosity parameters were analyzed by scanning electron microscopy (SEM). It is obvious that the support is a porous one with pore size of about 25 μm (Fig. 1). After immobilization procedure almost all COD molecules had disappeared from the solution and it was assumed they were bounded to the support surface and no activity and protein concentration was detected in the supernatant at the end of the immobilization procedure. Furthermore, blank activated perlite had no detectable activity. The immobilized COD appeared to lose half of its activity compared to soluble enzyme during immobilization procedure. For POD, the yield of protein immobilization was less in compare with COD (Table 1) in the similar ratio of the enzyme to perlite. According to the COD (Lario et al., 2003) and POD (Gajhede et al., 1997) crystal structure, there is more available lysine residues for reaction with glutaraldehyde in COD surface (Fig. 2) and so this may be one of the reasons that POD has lower immobilization yield in the same condition in compare with COD. Moreover, the yield of POD immobilization was differed from 80 to 100% by increasing ratio of POD to perlite (Fig. 3). The relative immobilized POD activity (activity recovery) was not measured more than 11% (when we used
Table 1
Enzyme loading and the kinetic properties of the soluble and immobilized COD and POD

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(K_M) (mmol/L)</th>
<th>Yield of immobilization (%)</th>
<th>(P) ((\mu g/g) support)(^b)</th>
<th>(R) (%)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble COD</td>
<td>0.095</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Immobilized COD</td>
<td>0.070</td>
<td>100</td>
<td>260</td>
<td>50</td>
</tr>
<tr>
<td>Soluble POD</td>
<td>0.531</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Immobilized POD</td>
<td>0.462</td>
<td>97</td>
<td>218</td>
<td>3.7</td>
</tr>
</tbody>
</table>

\(^a\) All data in the table are the averages of the triplicate of experiments (standard deviation was measured less than 7\% for soluble enzymes and less than 11\% for immobilized enzymes).

\(^b\) Protein content per gram support.

\(^c\) \(R\) is the activity recovery of the immobilized enzyme.

Fig. 1. Scanning electron microscopic (SEM) images of perlite surface before (A) and after chemical treatment and surface activation (B).

Fig. 2. Three-dimensional structure of COD (A) and POD (B) with the accessible lysine residues (red) (Rasmol pictures using the coordinates from PDB). There are more than 15 lysines available on COD surface respect to three lysines on the surface of POD. In POD structure Lys232 located near one of the two calciums (white sphere) and Lys174 is close to iron ion (yellow sphere) of heme group.
the highest ratio of POD per perlite in immobilization procedure). As it was shown in Fig. 3 in higher POD concentration used for immobilization activity recovery is higher and it is in agreement with previously reported that activity recovery of the immobilized POD increased with the concentration of enzyme solution (Lai and Lin, 2005). When ratio of the enzyme to support increased, it has been suggested that POD molecules arrange to gathered to each other since its $p_f$ is 7.2 and pH of immobilization procedure in previous and our works was neutral. Therefore, because of this phenomenon and according to the polymeric nature of glutaraldehyde on the support surface which provide multi attachment site (explained afterwards in the text), POD will conjugate through lower lysine residues and fewer bonds formed. This may lead to less distortion in the three-dimensional architecture and less decreasing in the enzyme flexibility, and then enzyme molecules could retain more of their activity (Balcao et al., 2001; Castillo et al., 2003).

Comparing with the soluble enzyme and according to the yield of enzyme immobilization, it was shown that the procedure of immobilization lead to more reduction in POD activity than COD. It must be pointed out that enzyme immobilization via covalent binding will change enzyme conformation and reduce its flexibility and may cause reduction in enzyme mobility to induce fit to a substrate (Zhao, 2004) that may lead to the reduction of COD and POD activities after immobilization. These alterations result from structural changes introduced to the enzyme by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution and also mass transfer limitation which commonly occurred (Krajewska, 2004). It is already accepted that some alteration in properties of the immobilized enzyme, such as a reduced specific catalytic activity and/or increased thermal stability, in comparison with its soluble counterpart, are the result of conformational effects inside the pores, electrostatic and partitioning effects in the immobilized enzyme microenvironment, and external and internal mass transfer limitations (Tardioli et al., 2006) besides other effects such as non-productive orientation (Gottschalk and Jaenicke, 1991), decreased protein flexibility resulting from multi-point attachment and/or enzyme overcrowding on the surface of the support (Clark, 1994) might negatively affect the performances of this immobilized system.

In the case of co-immobilization of COD and POD, excess amount of POD was used to saturate support surface. Measurements were shown that POD immobilization yield decreased in co-immobilization procedure (to 78%) while COD immobilization yield was not changed. Activity recovery of both enzymes in co-immobilization increased in compare with single enzyme immobilization (from 50 to 70% and 11 to 22% for COD and POD, respectively). This fact can be one of the advantages of co-immobilized enzymes. The lower yield of POD immobilization may be due to the competition of COD with POD in immobilization. Also higher activity of immobilized COD may be due to the mass transfer effects and proximity of two enzymes which immobilized (Ge et al., 1999).

### 3.2. Effect of pH on the activity of free and immobilized enzymes

The bell-shaped pH profiles were observed for different types of the enzymes (Fig. 4). The results showed the optimum pH of soluble COD, POD and immobilized POD are around 7, which was similar to that reported for COD and POD. However, activity

---

**Fig. 3.** Yield of POD immobilization (cross-hatched column) and remaining activity yield (black column) of enzyme after immobilization procedure. All immobilizations were carried out at pH 7.0 (0.2 M phosphate buffer) with gently agitation overnight at 4°C.

**Fig. 4.** The influence of pH on the activity of free and immobilized COD and POD. All reactions were carried out at room temperature under the variety of pH (0.1 and 0.2 M phosphate buffer for COD and POD, respectively) and enzymes were added to the mixture reactions before relative activity determination. Free COD, (○) immobilized COD, (▲) free POD, (△) immobilized POD.
of soluble COD decreased sharply in basic media and as Fig. 4 shown decreasing in the activity of soluble POD in acidic pH is higher, conversely (Lin and Yang, 2003a,b; Nishiya et al., 1997). The optimal pH of immobilized COD was changed and shifted to the lower pH in compare to the soluble COD while optimal pH of immobilized POD was not changed significantly.

Immobilized COD and POD showed better stability at different pH values and have wider pH range than native enzymes. But this effect was more significant at lower pH for both enzymes. While enzymes were immobilized on siliceous activated support the presence of protonated, un-reacted amino groups and also Si–OH on the surface of the support might repel protons from the region in the vicinity of the surface and create a higher pH at the boundary layer between the support and the bulk solution and this microenvironment with a pH slightly higher than bulk solution lead to the lowering of apparent optimal pH which occurred in the case of immobilized enzymes. This phenomenon may be one of the reasons of better stability of immobilized enzymes at lower pH especially for COD. The stability of the secondary structure of the POD was also found to decrease at pH below 4.5 (Chattopadhyay and Mazumdar, 2000). Significant increasing in activity of immobilized POD in compare with soluble POD in acidic condition may due to stabilization of secondary structure of POD which covalent attachment caused.

3.3. Kinetic parameters of soluble and immobilized COD and POD

The kinetic parameter values of the enzymes were determined from Michaelis–Menten equation (Fig. 5). Unlike soluble COD, immobilized COD showed inhibition of enzyme activity at high substrate concentrations (Fig. 5A), so called “substrate inhibition”, which is one of the most common deviations from Michaelis–Menten kinetics. The substrate inhibition of COD was occurred in the concentrations more than 400 μM of cholesterol. There is not any report in the literature about substrate inhibition of COD (free or immobilized) but it could be because of conformational change in enzyme structure which may be result of covalent attachment of enzyme to the support. Regarding to POD, by increasing in the hydrogen peroxide concentration, the activity reached to the maximum and then suicide effect of hydrogen peroxide cause decreases in enzyme activity. It is known that excess hydrogen peroxide inhibits POD activity (Arnao et al., 1990; Hiner et al., 1995). Concentration of hydrogen peroxide which inhibit the activity of POD in both immobilized and soluble form determined 1 mM and immobilization had not shown any affect in protection of enzyme from suicide effect of hydrogen peroxide (Fig. 5B). In determination the values of \( V_0 \), the initial linear phase lasted approximately 30 s; departure from linearity at later times indicated that enzyme inactivation was being observed, so these later data were not used in calculations. For both immobilized and soluble enzymes initial estimates of \( V_{\text{max}} \) and \( K_m \) were obtained by using the Hanes equation, a linear transformation of the Michaelis–Menten equation as were shown in Fig. 5, insets (without considering substrate inhibition range) (Palmer, 1991).

As in the Table 1 was shown \( K_m \) of both immobilized COD and POD decreased slightly in compare with soluble enzymes, but changing in \( K_m \) of immobilized COD was more significant.

According to the literature and previous researches there are two different mechanisms in immobilization of proteins with glutaraldehyde and 3-APTS. In the first one glutaraldehyde acts as a simple linker and one molecule link amine groups of protein and 3-APTS as shown in Fig. 6 (left) (Blasi et al., 2005; Facci et al., 2002; Ferreira et al., 2003; Paolo Facci et al., 2002; Zhao, 2004). In the second mechanism glutaraldehyde polymerized on the surface and attached to the 3-APTS (Fig. 6, right) (Kim et al., 2004; Singh et al., 1999). It is not clear yet that in which condition glutaraldehyde polymerization occurred in glutaraldehyde attachment step (Migneault et al., 2004). But in all works that one glutaraldehyde participates in attachment of proteins and 3-APTS to each other, the concentration of this compound were under 2.5% (v/v). Therefore, it seems that in our work (which the concentration of glutaraldehyde was 10%), enzymes
Fig. 6. The two different mechanisms of protein immobilization by glutaraldehyde and 3-APTS (left: single glutaraldehyde, right: polymerized glutaraldehyde).

attached to the surface by polymerized form of glutaraldehyde (Singh et al., 1999). In Fig. 1, SEM images of perlite before and after chemical treatment step (before enzyme immobilization) was shown. According to the size of component (about \( \mu \text{m} \)) which was introduced to the perlite surface after chemical treatment step it seems that glutaraldehyde polymerized on the surface of perlite and then act as an enzyme linker. By this way multipoint of attachment introduce on the surface (especially for COD which has spread distribution of lysine residues on its surface). Long polymer chains might prevent enzyme interaction with the substrate and decrease in the molecular mobility due to the multipoint attachment or change in the microenvironment of the enzyme molecules (Cao, 2005). These events may lead in decreasing in the enzyme activity or substrate inhibition which occurred in the case of immobilized COD on perlite.

3.4. Thermal stability and reusability of soluble and immobilized COD and POD

Thermoinactivation results showed that soluble POD is more thermostable than soluble COD in the same temperature. COD lost totally its activity after 10 min incubation at 70 °C while POD retains about 30% of its initial activity after 20 min incubation in 80 °C. As shown in Table 2 by increasing the temperature of incubation, the rate of heat inactivation \( (k_i) \) increased for both enzymes. Immobilization of COD had positive effect on stabilization of the enzyme against heat inactivation (Fig. 7). Immobilized COD had about 20% more activity after 20 min incubation in 60 °C than soluble COD and the rate of heat inactivation after immobilization decreased about 1.8 times. More stability of immobilized COD than its soluble form is usual character of immobilized enzymes. This may be due to the protection of the immobilized enzymes from structural rearrangement and lower flexibility of immobilized form, due to the likely multipoint attachments to the support (Ferreira et al., 2003; Zhao, 2004). On the other hand, as it is clear the immobilization has negative effect on POD thermostabilization and the immobilized POD is less thermostable than soluble one (Table 2 and Fig. 8). This event was occurred for immobilized POD which incubated at 60, 70 and 80 °C. Therefore, less stability of immobilized POD was assumed to be independent of temperature (Fig. 8).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temperature (°C)</th>
<th>( k_i, \text{soluble} \times 10^3 ) (min(^{-1}))</th>
<th>( k_i, \text{immobilized} \times 10^3 ) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>50</td>
<td>(-10.4 \pm 0.6)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>(-94.7 \pm 8.0)</td>
<td>(-43.4 \pm 1.3)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>(-825 \pm 99)</td>
<td>(-)</td>
</tr>
<tr>
<td>POD</td>
<td>60</td>
<td>(-11.2 \pm 1.1)</td>
<td>(-37.6 \pm 2.4)</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>(-27.4 \pm 1.3)</td>
<td>(-107 \pm 7.4)</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>(-74.8 \pm 3.2)</td>
<td>(-246 \pm 27)</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>(-674 \pm 85)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td>80(^b)</td>
<td>(-38.5 \pm 1.6)</td>
<td>(-245 \pm 16)</td>
</tr>
</tbody>
</table>

Incubation of soluble and immobilized POD in presence of trehalose 20% (w/v) in 80 °C.

\(^a\) All data in the table are the averages of the triplicate of experiments.
Incubation of soluble POD in presence of 20% of trehalose caused more than 1.5 times decreasing the rate of heat inactivation of soluble POD in 80°C. Trehalose is known as a biomacromolecule stabilizing agent which acts through preferentially raising the free energy of the denatured state, shifting the equilibrium in favor of the native state (Bolen and Baskakov, 2001). So it seems that the stabilizing effect of trehalose on soluble POD due to making POD denaturation process unfavorable. As the results of thermostability assays of soluble and immobilized POD in presence and absence of trehalose showed that trehalose had stabilizing effect on soluble POD but it had no effect on stabilization of the immobilized form of this enzyme (Fig. 9). According to the above mentioned results, it seems that thermostabilization of the immobilized form of POD which occurred in all examined temperature, is through another mechanism than thermal denaturation which trehalose can suppress it commonly (Auton and Bolen, 2004; Kaushik and Bhat, 2003).

Moreover, Ugarova et al. report decreasing in glutaraldehyde modified POD thermostability in compare with native enzyme (Ugarova et al., 1977). At 0°C, only three lysines modified. From all six lysine residues of POD, in lysine modification reaction, it has been shown that Lys232 react completely; Lys174 and Lys241 partially react and Lys65, Lys84 and Lys149 participate very little in reaction with modifying agents (O’Brien et al., 2001). These results are in agreement with accessibility of the lysine side chain and amino acid surface area which shows that Lys232, Lys174 and Lys241 are the most accessible between all lysines. The POD crystal structure (Gajhede et al., 1997) shows that very little of Lys84 is exposed and nitrogens of Lys149 and Lys65, which are moderately exposed, engaged in salt pairing with carboxylate side chains of Glu64 and Asp258. Lys232 located near one of the two calciums and Lys174 is close to heme group as shown in Fig. 2. Consequently because of the vicinity of covalently bounded residue of POD to the support to one of the calcium ions (Lys232) and the prosthetic heme group (Lys174)
Fig. 10. The influence of the number of reuse on the activity of co-immobilized COD and POD with repeated cycles. All cycles were carried out at room temperature under 0.4 mM cholesterol concentration.

and alteration in conformation which may occurred in structure around that groups by covalent attachment, it seems that lower thermostability of immobilized POD may be due to calcium ion and/or heme group (or iron ion) loss during incubation in high temperature (Huang et al., 2005).

Reusability is a crucial feature of immobilized enzyme preparations in most practical applications. Inactivation and enzyme bleed are the most prominent problems. The dependence of relative activity on the number of reuses of co-immobilized COD and POD is illustrated in Fig. 10. The relative activity of co-immobilized system decreased with the increase of the number of reuses. Supports were assayed for cholesterol activity at 0.4 mM substrate (the concentration of cholesterol was chosen under activity inhibition region) in 20 sequential cycles. As shown in Fig. 10, co-immobilized system can still keep its relative activity at 85% after 10 reuses and 65% after twenty reuses. Enzymes bleed from the perlite was found to be below detection limits. This data show better reusability than previous study (Lin and Yang, 2003b).

4. Conclusions

A cheap porous silica material, perlite, was activated and used for enzyme immobilization. COD and POD can be covalent-immobilized on to the activated perlite surface with glutaraldehyde and also co-immobilization of these two enzymes was investigated. Immobilized COD retains higher activity after immobilization in compare with POD. K_m of both enzymes was not increased after immobilization and in addition, immobilized COD and POD can retain most of the activity in wider ranges of pH than native COD and POD. Immobilized form of COD had better thermostability than the soluble form of this enzyme. Co-immobilized system had good reusable stability and it was shown to have better reusable stability than previous studies. Thus, the co-immobilized system may have medical or industrial application for cholesterol determination or clearance.

Acknowledgements

This work was supported by a grant from New Ideas Research Institute (NIRI). The authors also wish to acknowledge S. Hashemi, M. Barshan, S.E. Ranaei Siadat, K. Niknam and Sh. Barzegar for their helpful comments.

References


