Covalent Immobilization of Organophosphorus Hydrolase onto Insoluble Bovine Collagen Fibers

by

Yanhong Li,[1,3] Xinju Jia[3] and Biyu Peng[1,2]*

[1]*Key Lab. of Leather Chemistry and Engineering of Ministry of Education, Sichuan University,* Chengdu, Sichuan 610065, P. R. China

[2]*National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University,* Chengdu 610065, P. R. China

[3]*College of Light Industry, Textile and Food Engineering, Sichuan University,* Chengdu 610065, P. R. China

Abstract

An organophosphorous hydrolase (OPH) was prepared and partially purified from *Flavobacterium sp*. The crude enzyme, with an activity of 1030U/g, was immobilized onto insoluble bovine collagen fibers (hide powder), instead of collagen membrane, through glutaraldehyde coupling. Optimal conditions of enzyme immobilization and properties of the immobilized enzyme preparation were investigated. Compared to glutaraldehyde tanned and chrome tanned hide powders, non-tanned hide powder showed higher immobilized enzyme activity. The optimal enzyme immobilization conditions are as follows: 10mg of enzyme was immobilized onto 500mg of hide powder in a 50mM phosphate buffer of pH 7.5 at 20°C, and 20% glutaraldehyde offer (based on the hide powder weight) was used to couple the enzyme and collagen fibers. The enzyme activity yield was about 35% and the hide powder immobilized OPH has an enzyme activity of about 7U/g. The immobilized enzyme showed the same temperature and pH profiles as the free enzyme, and it performed at much higher pH and with better thermal stability. The K_m value of the immobilized enzyme was a somewhat higher (0.388mM) than that of the free enzyme (0.215mM). The reusability test showed that about 85% activity was retained after 10 use cycles. After storing for eight months at 20°C, the residual activity of the immobilized enzyme preparation was 98%.

Introduction

Organophosphorus compounds are extensively used as pesticides and chemical warfare agents. These compounds function by inhibiting acetylcholinesterase of many living organisms, including humans. Repeated or prolonged exposure to organophosphates can cause delayed cholinergic toxicity and neurotoxicity.[1] The high toxicity of organophosphorus neurotoxics and their widespread use in modern agriculture practices have increased public concerns. Due to the environmental concern associated with the accumulation of these compounds in food products and water supplies, there is a growing requirement to develop safe, convenient, and economically feasible methods for their detoxification.[2] But, current techniques for detoxifying organophosphate pesticides mainly depend on harsh chemical treatment, incineration, and landfills. These methods are unsafe, inefficient and unsatisfactory.

Enzymatic degradation of organophosphorus compounds has been a subject of considerable attention in the past decade. Organophosphorus hydrolase (OPH) isolated from soil microorganisms, such as *Pseudomonas* and *Flavobacterium sp*., has been shown to effectively degrade organophosphates.[3-7] OPH is a metalloenzyme that has broad substrate specificity.[5] It degrades organophosphates ranging from pesticides to chemical warfare toxins. It has potential application in both demilitarization and decontamination. However, practical applications of large-scale enzymatic degradation have always been limited by the prohibitive costs of isolation, purification and the limited stability of OPH. In addition, it is laborious to recover pure active enzyme from the reaction mixture. So, immobilization of enzyme onto carriers i.e. for very large scale bioreactors would be a valuable technique for increasing the life span of the biocatalyst. As another cost-effective alternative, whole cells (either growing or non-growing), especially the cells with surface-expressed OPH, can be immobilized on a support (such as in an immobilized-cell bioreactor).[2,8,9]

*Corresponding author e-mail: pengbiyu@scu.edu.cn; Tel. +86-28-85401208; fax: +86-28-85401208.
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Immobilized enzymes are currently the object of considerable interest. There are several reasons for the preparation and use of immobilized enzymes. In addition to a more convenient handing of enzyme preparations, the two main targeted benefits are easy separation of the enzyme from the product, and reuse of the enzyme. Many kinds of materials can be used as carriers for the immobilized enzymes. Reports of materials such as activated alumina particles,[10] polyethylene glycol-based hydrogels,[11] nylon,[12] trityl agarose,[13] collagen hydrolysate films[14] and mesoporous silica[15] as supports for immobilized OPH have been published. The goal was to develop enzyme reactors for detoxification of organophosphorous pesticides and chemical warfare toxins.

Collagen is the most abundant protein constituent of higher vertebrates, mainly acting as a structural component to support and hold individual cells together in tissues. The basic molecular unit of collagen is a triple helix composed of three similar, but not identical, polypeptide chains. A collagen molecule (so-called tropocollagen) has telopeptides at each end at which collagen molecules are linked to each other to form a three-dimensional peptide fibril structure, i.e. collagen fibril. As the degree of crosslinking of collagen through telopetides increases, the collagen fibril remains somewhat soluble in salt or acidic solution, but is otherwise insoluble. The biomaterial collagen offers a number of advantages as a carrier material for enzyme and whole microbial cell immobilization. Collagen has a large number of potential binding sites for enzyme attachment. Its proteinaceous nature contributes polar and nonpolar amino acid residuals for strong, cooperative non-covalent interactions with enzyme molecules. It has high hydrophilicity, biocompatibility and biodegradability. Hence, many kinds of enzymes have been successfully immobilized onto the collagen matrix.[16-18] But, in previous research, enzymes were immobilized in collagen membranes made from collagen fibril paste or collagen hydrolysate by three different methods: impregnation, entrapment, and electrolytic co-deposition.

Hide powder (ground collagen fibers from bovine hide) is a kind of collagen fibrous material, which is insoluble in water. The tanned hide power shows good chemical and thermal stability, mechanical properties and anti-microbial character. The dried tanned powder is loose and porous. It is easily rehydrated, but does not swell significantly in acidic or alkaline solution, unlike untanned collagen. Hence, when an enzyme is coupled to hide powder using a tanning agent such as glutaraldehyde, the tanned hide powder immobilized enzyme will be easily stored and highly-efficient in use, because it can be stored in a dry state; it has high hydrophilicity and a relatively large specific surface area for efficient mass transfer of substrate from the bulk. Its loose and porous structure also facilitates mass transport during reaction in both tank and column reactors. By contrast, collagen membrane is rather compact; hence, transport of substrate across the membrane immobilized enzyme presents a major problem, especially for a thick membrane. Most importantly, performance and physical parameters make tanned hide powder an ideal filler for column-type reactors. The substrate solution can easily pass through the column and it has a low pressure drop.

In the present study, OPH is immobilized onto insoluble collagen fibers (hide powder) by covalent crosslinking. Glutaraldehyde was chosen as the coupling agent, because it is an effective, primary coupling agent for enzyme immobilization, and it is also a very effective tanning agent in leather making. The optimal conditions of enzyme immobilization and the properties of the immobilized enzyme are investigated.

MATERIALS AND METHODS

Materials

Flavobacterium sp. strain 27551 was obtained from the American Type Culture Collection (ATCC). Streptomycin sulfate, glutaraldehyde and paraoxon were purchased from Sigma Chemical Company. Standard bovine hide powder (insoluble collagen fibers, 100-150 meshes) was obtained from Chinese Academy of Forestry. All other reagents used in this study were analytical grade.

Methods

Preparation and Partial Purification of Organophosphorus Hydrolase (OPH) from Flavobacterium sp.

Organophosphorus hydrolase was prepared and partially purified from *Flavobacterium sp.* (ATCC 27551). The cultures were grown in a nutrient broth supplemented with 5g of glucose and 0.5g of calcium nitrate per liter of both.[14] Liquid cultures were incubated at 30°C in a rotary shaker at 190 rpm. The cells were harvested after 72 hrs, and centrifuged at 6000×G for 20min at 4°C. The cell pellets were suspended in 25mL of 10mM potassium phosphate buffer of pH 7.2 with 10mM NaCl. The cell suspension was passed twice through a chilled French pressure cell (1200PSI) and centrifuged at 10,000×G for 20min at 4°C. The supernatant solution was subjected to 1% streptomycin sulfate precipitation to remove nucleic acids. Then the supernatant solution was precipitated with 45% ammonium sulfate. The precipitated protein was suspended in 20mL of 10 mM potassium phosphate buffer (pH 7.2), dialyzed against the same buffer for 24 hours, and finally the dialysate was lyophilized. This crude enzyme solid was used for immobilization.

Preparation of Tanned Hide Powder

Hide powder, which had been tanned (crosslinked) by either glutaraldehyde or chromium sulfate, was used as substrates for OPH immobilization. A "slightly" chromium-tanned hide powder was prepared as follows: 6g of white hide powder (untreated hide powder) was soaked overnight in a conical

flask with 30mL of distilled water in a rotary shaker. 30mL of 3 g/L chromium sulfate solution was then added. After shaking for 2 hours, the chromium bath was slowly basified to pH 4.0 with 100g/L $NaHCO_3$. Then, the conical flask was continuously stirred for another 6 hours at pH 4.0 and 35°C. The chromium-tanned hide powder was washed three times with distilled water. This tanned hide power was air dried and analyzed for chromium content as per the standard ASTM method (ASTM D2807). The Cr_2O_3 content in the dried tanned hide powder was 0.57%.

White hide powder was also "tanned" by glutaraldehyde of different dosages according to the following procedure. 2g of white hide powder was soaked overnight in 20mL of 50mM potassium phosphate buffer of pH 7.2. 4, 8 or 12mL of 25g/L glutaraldehyde solution (5%, 10% or 15% glutaraldehyde offer level based on the weight of hide powder) was then added individually to the hide powder suspension. The suspension was continuously stirred for 8 hours and then centrifuged to separate the supernatant solution. These tanned hide powders were washed thoroughly to remove salts and unreacted glutaraldehyde.

OPH Activity Assay

Paraoxon was used as the substrate for OPH activity assays. Enzyme activity is defined in units where 1 U is the amount of enzyme required to catalyze the hydrolysis of 1 μmol of paraoxon in 1 min. Enzyme activity was determined photometrically at 401nm using a spectrophotometer (Milton Roy Spectronic 20D) based on the absorbance of p-nitrophenol, the hydrolysis product of paraoxon. 5mL of 0.5mM paraoxon solution in 50mM pH 8.5 Tris buffer containing 10% (w/w) methanol was added to a test tube and incubated for 10 min at 30°C. 50 μL of suitably-diluted enzyme solution (in pH 8.5 Tris buffer) was also added to the test tube. The well-mixed solution was incubated for 10min at 30°C. The absorbance was read at 401nm, immediately. Enzyme activity was calculated by comparing the absorbance from a standard (calibration) curve of p-nitrophenol. The obtained crude OPH had an activity of 1.03U/mg.

OPH Activity Stability Against Glutaraldehyde

0.5 mM paraoxon solutions, each with varying glutaraldehyde concentrations, in 50 mM sodium borate buffers of pH 7.5, 8.5 and 9.5, containing 10% methanol, were prepared. The paraoxon solutions with different glutaraldehyde concentrations and pH were used as the substrates for assaying OPH activity. Because Tris reacts with glutaraldehyde, sodium borate buffer was used here. 50 μL of suitably-diluted OPH solution was added to 5 mL of one of the above paraoxon solutions, and incubated for 10 minutes at 30°C. The absorbance was then read at 401nm immediately and the OPH activities in different glutaraldehyde concentration solutions were obtained.

Tolerances of OPH to glutaraldehyde at varying pH were also tested. OPH solutions were diluted with pH 6.5, 7.5 (potassium phosphate) and pH 8.5 (sodium borate) buffers, individually; then, different amounts of glutaraldehyde were added to these OPH solutions. These OPH solutions containing glutaraldehyde were kept in a 25°C water bath for 24 hours. Residual OPH activity was then analyzed at regular time intervals.

Immobilization of OPH on Hide Powders

Glutaraldehyde tanned hide powders, slightly chromium-tanned hide powder and white hide powder were used as the carriers for OPH immobilization. 500mg of each of the above (2.2.2) hide powders (dry) was soaked in 8 mL of 50 mM potassium phosphate buffer with different pH (from 6.5 to 8.0) in a vial and stirred for 4 hours. 5-20 mg quantities of OPH were dissolved in 2 mL of the corresponding buffer. The OPH solutions were then added to the hide powder suspensions. After stirring for 60 min, varying dosages of 12.5g/L glutaraldehyde (10%-25% of hide powder weight), were added in two to five sequential additions over a one hour interval, in 2 mL increments. Enzyme immobilization was carried out in 25mL vials at 4-20°C in a Glas-Col tilting plate rotator at 80 rpm. Variables during immobilization, including hide powder type, glutaraldehyde dosage, immobilization pH and amount of added OPH were investigated. The hide powders with immobilized OPH were thoroughly washed with distilled water and air dried at room temperature (25°C).

Activity Assay and Characterization of Immobilized OPH

5 mg of hide powder with immobilized OPH was enclosed in a small filter paper packet. Immobilized enzyme activity was assayed in a similar way to that of the soluble enzyme, except a filter paper packet with immobilized enzyme was used instead of the soluble enzyme. Absorbance was immediately read at 401nm after removal of the filter packet. Enzyme activity yield was calculated as the ratio of determined activity of an aliquot of immobilized enzyme to the same aliquot of initial active solution, expressed as percent. The filter packet was washed with distilled water and air dried. The same procedure was repeated 10 times to assess the reusability of the immobilized enzyme preparation.

The immobilized enzyme on hide powder was characterized by evaluating the potential effects of immobilization on its thermal stability, pH stability and reaction kinetics, especially in comparison with free enzyme. Activities of immobilized enzyme and free enzyme were assayed at different temperatures (20-70°C) and varying pH (6-12), and the pH and temperature-activity profiles of immobilized and free enzymes were obtained. The thermal and pH stabilities of immobilized and free enzymes were also assessed. Free enzyme was dissolved and immobilized enzyme was soaked in varying pH buffers, pH from 6 to 11, and kept in a 32°C water bath. Residual activities of the free enzyme and immobilized enzyme were determined after 14h incubation. Free enzyme solution and immobilized enzyme suspension, both at pH 9, were incubated for one hour in a water bath with different temperatures, from 30 to 70°C; after which the residual enzyme activity was assayed.

Measurements of reaction kinetics for immobilized and free enzymes were also made. Immobilized enzyme and free enzyme reacted with different concentration paraoxon solutions at 30°C and pH 9.0 for 10 min, and the hydrolysis rates of paraoxon was determined by monitoring the concentration of p-nitophenol. Lineweaver-Burk plots were drawn, which indicate the relationship between the hydrolysis rates and concentration of paraoxon. From these, the values of Michaelis constant (K_m) and maximum reaction rate (V_{max}) were obtained.

Results and Discussion

The Influence of Glutaraldehyde on OPH Activity

Glutaraldehyde is a good crosslinking agent for proteins. It has been extensively used as an enzyme immobilizing agent for many years, and it is also one of the most effective tanning agents for leather making. But, the intermolecular and intramolecular crosslinking in enzyme molecules, especially excessive crosslinking, probably results in denaturation and impaired enzyme activity. Hence, the influence of glutaraldehyde on OPH activity was investigated first. Paraoxon solutions with different glutaraldehyde concentrations were used as the substrate for OPH activity assays, and the relative activities of OPH in these solutions are shown in Figure 1. The relative activity of OPH in the paraoxon solution without glutaraldehyde is regarded as 100%.

The presence of glutaraldehyde in the substrate solution impairs the ability of OPH to catalyze paraoxon hydrolysis. And the OPH activity decreases with increasing glutaraldehyde concentration and pH, as shown in Figure 1. The curves of OPH activity with glutaraldehyde concentration at pH 7.5 and 8.5 are very similar, but OPH activity decreases remarkably at pH 9.5. When the glutaraldehyde concentration increased to 5 g/L, the enzyme activity was about 85% at both pH 7.5 and 8.5, but the residual activity at pH 9.5 was only 73%.

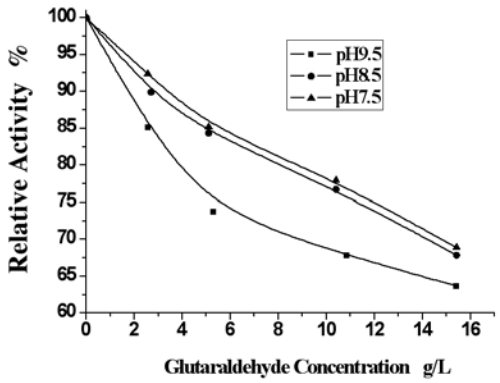

Figure 1. OPH activity against glutaraldehyde concentrations in the substrate solution.

Generally, several hours are required to immobilize an enzyme on a carrier using glutaraldehyde covalent crosslinking. Hence, the tolerance of OPH to the glutaraldehyde solutions of different concentration and pH was also investigated. OPH solutions were suitably diluted (2 mg/mL) with buffers containing varying glutaraldehyde concentration and pH (6.5, 7.5 or 8.5). The effects of aging time on enzyme activity are shown in Figure 2.

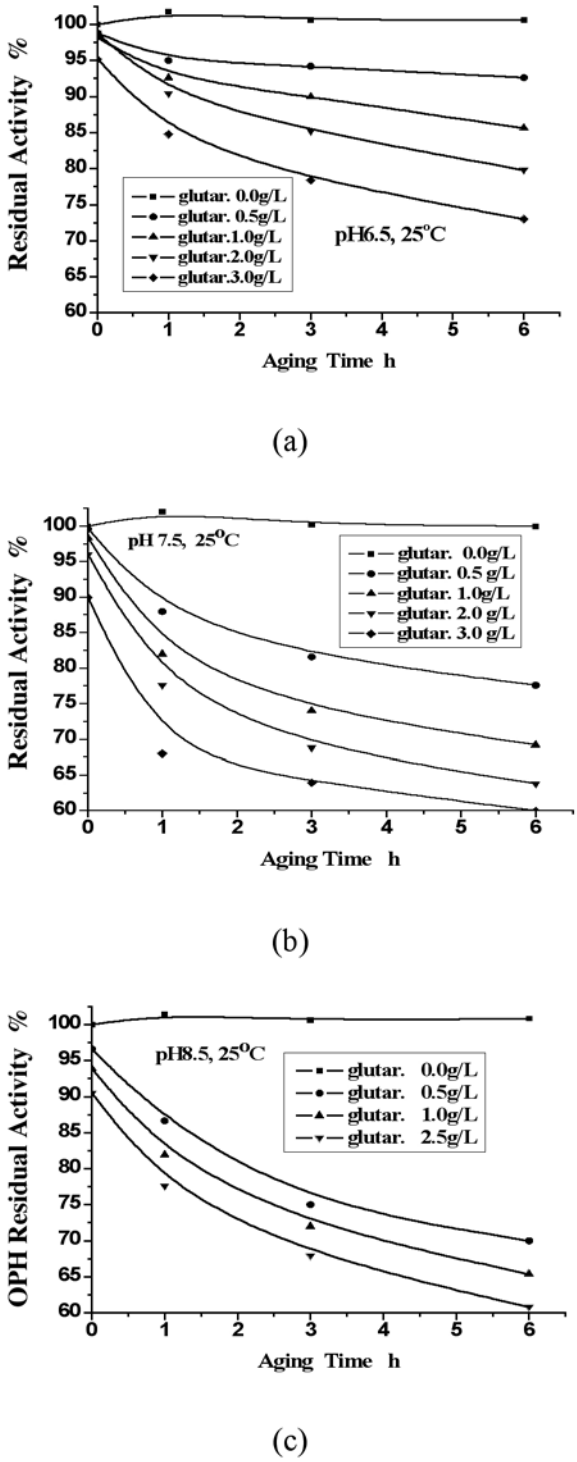

Figure 2. Tolerance of OPH to glutaraldehyde at various pHs (a): pH 6.5;(b): pH7.5; (c):pH8.5.

We see that the enzyme activity decreases with increasing aging time in the presence of glutaraldehyde at these three pHs. Note that enzyme activity shows almost no effective within 6 hours without glutaraldehyde. But enzyme activity decreases with increasing glutaraldehyde concentration and pH. The activity decrease is greatest at pH 8.5, less at pH 7.5 and least at pH 6.5. The reaction of glutaraldehyde with protein is strongly dependent on pH and the glutaraldehyde concentration;[19] hence, more inter- and intra-molecular crosslinks form at high pH and high glutaraldehyde concentration. This may lead to modification of the enzyme molecular configuration, resulting in enzyme activity reduction.

We also see that the residual OPH activity after 6 hours remains at 80% in 2 g/L glutaraldehyde solution and pH 6.5, but it is only about 65% for pH 7.5 and 8.5 at the same glutaraldehyde concentration (2 g/L). While it is quicker and easier to couple the enzyme with support using both high glutaraldehyde concentration and pH, enzyme activity suffers significantly. Consequently, the glutaraldehyde concentration should be lower than 2g/L and the pH less than 7.5 during OPH immobilization.

Factors Affecting Immobilization of OPH on Hide Powder

OPH Immobilized on Different Hide Powders

10mg of crude enzyme was immobilized on the 500mg portions of different hide powders under otherwise identical conditions. Activities of immobilized OPH on different hide powders are shown in Table I.

TABLE I
Immobilized OPH Activities on Different Hide Powders (U/g).

	Untanned	Glutaraldehyde tanned			Cr tanned
		5% Glutar.	10% Glutar.	15% Glutar.	0.57% Cr_2O_3
Activity (U/g)	7.05	6.82	5.81	2.84	2.45
Residual activity (%) after ten use cycles	83.06	76.68	55.00	42.47	30.24

Note: Immobilizing conditions: pH 7.5; total 20% glutaraldehyde dosage; 20°C

Hide powder is a fibrous material, which is generally insoluble in water. The tanned hide powder has less water-absorption, less swelling and higher porosity; so, it may have larger absorbability and affinity to enzyme proteins, as compared to untanned hide powder. But, Table 1 shows that the tanned hide power exhibits low enzyme activity yield compared to untanned hide powder, and the immobilized enzyme activity decreases with increasing tanning extent. Glutaraldehyde is a bifunctional reagent mainly capable of reacting with the surface amine groups of enzyme and support, through the formation of Schiff bases and Michael adducts. Hence, the enzyme loading amount on collagen fibers is related to the number of free amino groups in the collagen molecules. The free amino group content in native collagen (white hide powder) is 0.325 mmol/g.[20] With the rise in tanning extent (glutaraldehyde offer), the collagen's free amino groups decrease, and consequently, the number of available enzyme coupling points is reduced. Also, glutaraldehyde binding to the amino groups leads to a remarkable reduction of the cationic charge of collagen molecules and to a great decrease in the isoelectric point. These changes influence the attraction of collagen fibers to enzyme molecules, which is the first necessary stage of combination between them. Hence, the amount of enzyme bound to hide powder decreases in glutaraldehyde tanned hide powders. When the glutaraldehyde dosage during tanning is greater than 10%, the decrease in enzyme activity yield is remarkable. And reusability of the immobilized enzyme also dramatically decreases.

Enzyme activity yield in the chromium-tanned hide powder is the lowest, overall. More likely, available chromium may complex with the OPH enzyme differently than with glutaraldehyde, i.e. too close to active site decreasing its activity even more.

Glutaraldehyde-tanned and chromium-tanned hide powders are not suitable supports for immobilizing OPH. Hence, white hide powder was used as the support in the following experiments.

Effect of pH

10 mg of crude OPH was immobilized on 500 mg of white hide powder at varying pH. The tolerance of OPH to glutaraldehyde (Figure 2) shows that enzyme activity quickly decreases in glutaraldehyde solution at pH 8.5. So, enzyme immobilization was performed from pH 6.5 to 8.0. The pH effect in the coupling reaction is presented in Figure 3. Maximum activity is observed at pH 7.5 and immobilized OPH activity dramatically decreases up to pH 8.0. The glutaraldehyde reaction with the amino groups of the protein support is dependent on pH, so the reaction becomes easier with increasing pH.[20]Hence, the amount of bound enzyme should increase with increasing pH. It is easy to accept the increasing activity of immobilized enzyme with the pH increase in the range of 6.5 to 7.5. The loss of the immobilized

enzyme activity beyond pH 7.5 may be attributed to the formation of more intra- and inter-molecular crosslinks of enzyme proteins and/or compromise of the enzyme's active site. Therefore, pH 7.5 was chosen as the optimal pH for OPH immobilization.

Effect of Glutaraldehyde Dosage

OPH immobilization on white hide powder was conducted at pH 7.5 with varying glutaraldehyde dosages, from 10 to 25 percent based on hide powder weight. In order to avoid the detrimental effect of high glutaraldehyde concentration on enzyme activity, glutaraldehyde was added in two to five separate additions. A 5% offer was added each time and the interval was 60 min. Hide powder has a large affinity to glutaraldehyde, with a hide taking up to 21% glutaraldehyde.[19] Hence, the glutaraldehyde concentration in the immobilization solution was less than 3g/L, even at a 20% dosage. The effect of glutaraldehyde dosage on immobilized enzyme activity is shown in Figure 4. The immobilized enzyme activity increases with increasing glutaraldehyde dosages from 5% to 20%, peaking at 20%. Up to 25% dosage there is a negative effect on immobilized enzyme activity. Therefore, 20% dosage is optimal for the OPH immobilization on hide powder.

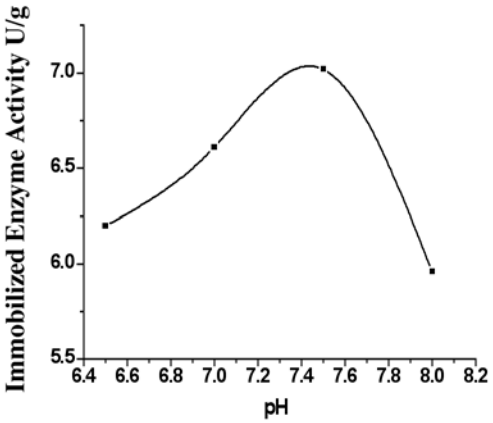

Figure 3. Influence of pH on enzyme immobilization
Immobilizing conditions: 20% glutaraldehyde dosage; 20°C.

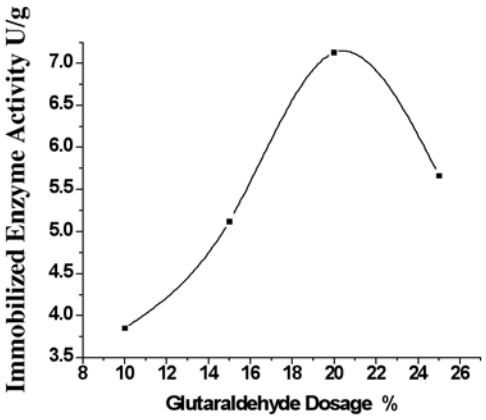

Figure 4. Influence of Glutaraldehyde dosage on enzyme immobilization
Immobilizing conditions: pH 7.5; 20°C.

The metered addition method for glutaraldehyde was also studied. A total offer of 25% glutarldehyde was accomplished in three ways. Method A: OPH solution was added to the hide powder suspension with stirring for 60min, then a 25% offer of glutaraldehyde was added in five separate additions over a time interval of 60min. Method B: the hide powder was initially tanned with 5% glutaraldehyde for 60min, then OPH solution was added with stirring for another 60min, finally, the remaining glutaraldehyde (20%) was added in four times over a time interval of 60min. Method C: this method was similar to Method B, but only 10% glutaraldehyde was used to tan hide powder, and then the remaining 15% glutaraldehyde was added in three increments. The effect of adding methods of glutaraldehyde is presented in Table II. Obviously, the pretanning of glutaraldehyde leads to reduced activity, i.e. in methods B and C. The reason is the same as that of low enzyme activity yield in tanned hide powder, i.e. the pretanning of glutaraldehyde leads to the reduction of amino groups on collagen molecules.

Actually, most of the added glutaraldehyde combines with hide powder and mainly plays a tanning agent role. Hence, hide powder hydrothermal stability is improved after enzyme immobilization. Table III shows the thermal denaturation temperatures (T_d) of hide powers with immobilized OPH with different dosages of glutaraldehyde. With increasing glutaraldehyde dosage T_d increases. So the hide powder with immobilized enzyme can be used at a rather high temperature.

TABLE II
Influence of Addition Methods of Glutaraldehyde on Enzyme Immobilization.

Addition method	A	B	C
Activity (U/g)	5.66	4.23	4.12

Immobilizing conditions: pH 7.5; 25% glutaraldehyde dosage; 20°C

TABLE III
T_d of Hide Powder Immobilized OPH with Different Glutaraldehyde Dosages.

Glutar. Dosage %	0	5	10	15	20
T_d *°C	65.5	70.6	76.0	77.5	79.9

*: T_d was determined in a differential scanning calorimeter (NETASCH-Geratebau GmbH Thermal Analysis)

Effect of Time and Temperature

It has been mentioned that glutaraldehyde was added in timed multiple additions. We found that the residual enzyme activity in solution quickly reached equilibrium in 60 min after each glutaraldehyde addition, so, the 60min interval was accepted as standard practice. After the last addition of glutaraldehyde, the suspension was stirred for another 4 hours. Longer stirring time did not remarkably increase immobilized enzyme activity, and the activity would slightly decrease if treating time was more than 6 hours.

The effect of coupling temperature on enzyme immobilization is shown in Table IV. The highest immobilized enzyme activity was obtained at 20°C. Low temperature does not benefit enzyme coupling to hide powder, and high temperature leads to loss of enzyme activity. Hence, 20°C was chosen as the optimal temperature for OPH immobilization.

TABLE IV
Effect of Temperature on Enzyme Immobilization.

Reacting Temp. °C	4	15	20	25
Activity (U/g)	5.75	5.83	6.62	6.15
Immobilizing conditions: pH 7.5; 20% glutaraldehyde dosage				

Effect of Added Enzyme Amount

5 to 15 mg of crude OPH was immobilized on 500 mg of white hide powder. The enzyme activities of hide powders immobilized with different amounts of OPH, as well as enzyme activity yields are shown in Figure 5. Immobilized enzyme activity increases with increasing added enzyme amount, and immobilized enzyme activity seems to reach the highest point when the added amount of enzyme reaches 10 mg/500 mg-hide powder. Since the coupling positions for enzyme molecules in collagen are limited, when all of the coupling points are occupied the immobilized enzyme activity will not increase further. But the relative activity yield of immobilized enzyme decreases with increasing added enzyme amount. It is only 35% at the 10 mg-enzyme/500mg-matrix dosage. Actually, collagen is a protein that is rich in attachment points for enzyme proteins. Enzyme proteins can also bind with collagen through ionic interactions, hydrogen bonds and van der Waals interactions. Thus, collagen fibers should have a rather large loading capacity for enzyme proteins. But, in our experiments, the OPH preparation was a crude enzyme mixture with a relatively low enzyme activity. It certainly contained a rather large proportion of non-enzyme proteins, which competed for binding positions on collagen. During enzyme immobilization, some amount of enzyme activity was probably lost activity to some extent. Therefore, the yield of immobilized enzyme activity was not very high. If a high purity OPH was used, the yield of immobilized enzyme would likely be higher.

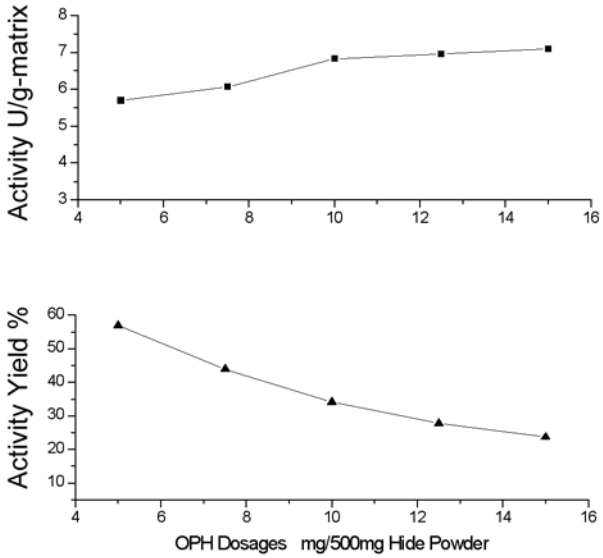

Figure 5. Effect of amount of added enzyme on OPH immobilization Immobilizing conditions: pH 7.5; 20% glutaraldehyde dosage; 20°C.

Overall, according to the results obtained in the above study, the optimal conditions for each step in the immobilization procedure are as follows; white hide powder carrier, 10 mg-enzyme/500mg-hide powder enzyme dosage, 20% glutaraldehyde offer, and immobilization at pH 7.5 and 20°C.

Characterization of Immobilized OPH

pH and Temperature-activity Profiles

The enzyme activities of free and immobilized OPH on hide powder, at different pH and temperatures, are shown in Figure 6 and Figure 7, respectively. Immobilized enzyme has almost the same optimal pH and temperature as free enzyme. The optimal pH range for both free and immobilized enzyme is from 8.5 to 10.5. The optimal temperature range is from 40 to 50°C.

pH and Thermal Stability of Immobilized Enzyme

Free enzyme solution and immobilized enzyme suspension with varying pH were kept in a 32°C water bath for 14 hours. Their residual activities are shown in Figure 8. Free enzyme solution and immobilized enzyme suspension at pH 9 were also incubated for one hour at selected temperatures. Their residual activities are shown in Figure 9. From Figure 8, pH stability of immobilized enzyme is found to be much better than that of free enzyme. The immobilized enzyme activity has no loss when incubated for 14 hours at pHs lower than 9, while the activity of free enzyme decreases 10% at pH 6.0 and 15% at pH 9.0. When the pH is greater than 9.0, the activity decreases dramatically with increasing pH for both immobilized and free enzymes, but especially for free enzyme. The activity loss of free enzyme is 65% at pH 11, while immobilized enzyme only loses 15% activity.

Figure 9 shows the difference in thermal stability between immobilized enzyme and free enzyme. It is apparent that the immobilized enzyme has enhanced thermal stability compared to free enzyme. With increasing incubation temperature, the free enzyme activity decreases almost linearly; it loses 50% activity after incubation at 50°C for one hour. But the immobilized enzyme has no activity loss for the same incubation conditions. When the temperature is greater than 50°C, the immobilized enzyme stability also decreases dramatically.

Kinetics Parameters

Immobilized enzyme and free enzyme were reacted with different concentration paraoxon solutions at 30° and pH 9.0. The Lineweaver-Burk plots of free enzyme and immobilized enzyme are shown in Figure 10 and the values of Michaelis constant (K_m) and maximum reaction rate (V_{max}) were obtained. The free enzyme showed a K_m value of 0.215 mM and a V_{max} value of 7.633μM/min. The K_m and V_{max} for the immobilized enzyme were 0.388 mM and 6.25μM/min, respectively. The kinetic constants for immobilized enzymes are highly dependent on the extent of external and internal mass transfer resistances. Changes in apparent K_m values upon immobilization are generally considered as an indication for diffusional limitation. In contrast to free OPH, immobilized OPH showed a slightly higher K_m value. This indicates the likelihood of transport impedance for the immobilized enzyme reaction. Consequently, the immobilized OPH gave a lower V_{max} value compared to the free OPH. Unlike other enzymes immobilized on collagen-membrane, the increase in K_m value for immobilized OPH is small. This can be attributed to the highly porous structure of tanned collagen fibers. Hence, mass transport is relatively unrestricted in hide powder compare to a collagen membrane.

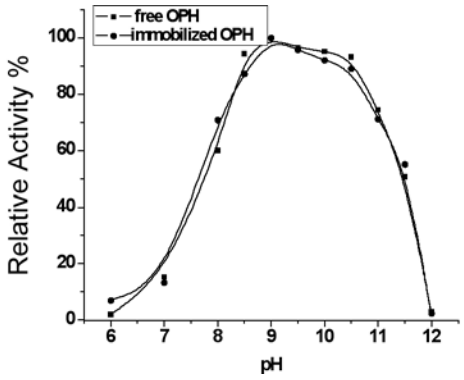

Figure 6. pH-activity profile.

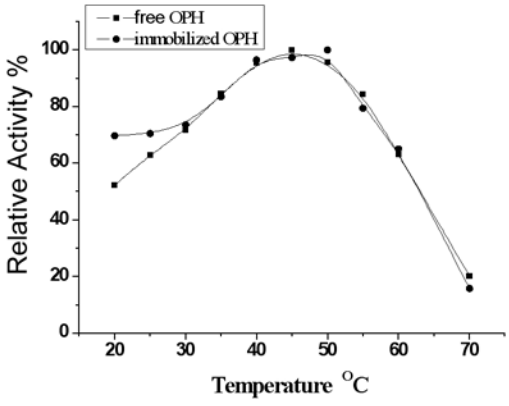

Figure 7. Temperature-activity profile.

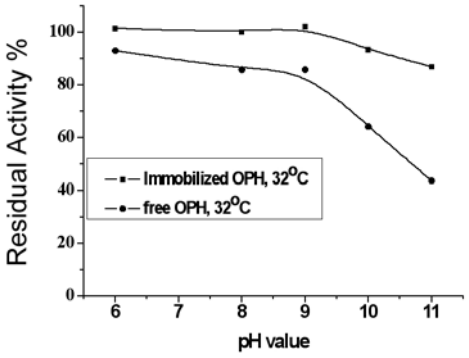

Figure 8. pH stability of enzymes.
(incubated for 14h at 32°C)

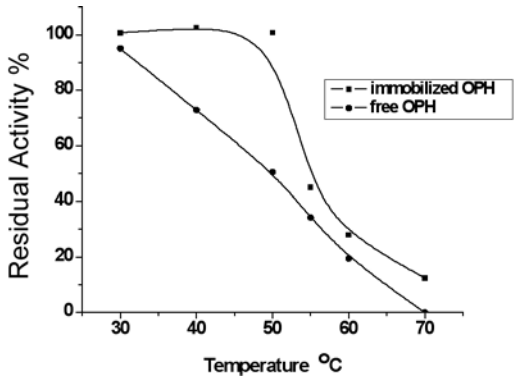

Figure 9. Temperature stability of enzymes.
(incubated for 1h at pH 9)

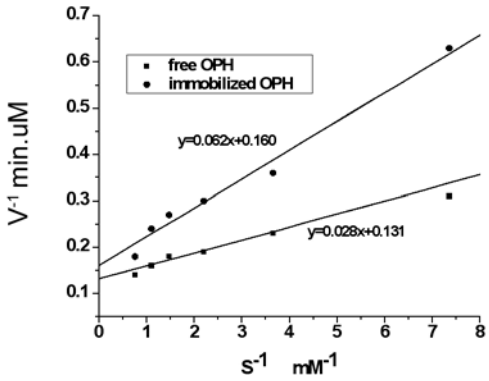

Figure 10. Lineweaver-Burk plots for free and immobilized enzymes.

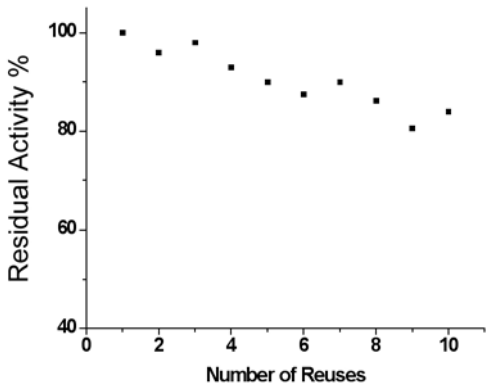

Figure 11. Operational stability of immobilized OPH on the collagen fibers.

Operational and Storage Stability

Increased operational stability of immobilized enzymes is essential in order to achieve the overall cost benefits. The ability of the immobilized enzyme to retain activity is important for operational stability. Reusability of the immobilized OPH was assessed by measuring the activity repeatedly over a period of 10 days at 24 hours intervals. After each activity test, the hide powder immobilized OPH was washed and air dried. As shown in Figure 11, immobilized OPH retained about 85% of its initial activity after 10 reuse cycles.

Storage stability of immobilized OPH was also investigated. The hide powder immobilized OPH was kept at 20°C for 8 months. Activity testing results showed that the residual activity of the immobilized OPH remained at 98%.

Conclusions

Insoluble collagen fiber (white hide powder) is more easily obtained and prepared than soluble collagen, and it is more suitable for use as the filler of a column reactor than collagen membrane. A crude organophosphorous hydrolase preparation with 1030U/g activity was made from *Flavobacterium sp*. The crude OPH was immobilized onto hide powder through glutaraldehyde crosslinking. Compared to glutaraldehyde-tanned and chromium-tanned hide powders, OPH immobilized on white hide powder (untanned) showed higher enzyme activity. The optimal conditions for OPH immobilization onto hide powder were found to be pH 7.5, 20°C, 20% glutaraldehyde offer provided in four separate additions, and 10 mg-OPH/500 mg-hide powder enzyme loading amount. The hide powder immobilized OPH had an enzyme activity of about 7U/g and the relative enzyme activity yield was about 35%. The immobilized enzyme showed the same temperature and pH profiles as the free enzyme, and it displayed superior pH and thermal stability, as compared to the free enzyme. The K_m value of the immobilized enzyme was a little higher (0.388mM) than that of the free enzyme (0.215mM). The reusability test showed that about 85% activity was retained after being used 10 times. The storage stability test showed that the residual activity was 98% after storage for 8 months at 20°C.

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