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0273-2289/98/73-2/3—0012$11.00

Chemical Modification of Horseradish Peroxidase with Several Methoxypolyethylene Glycols

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Received October 19, 1997; Accepted February 23, 1998

ABSTRACT

The chemical modification of ϵ-NH_2 lysine residues of horseradish peroxidase (E.C. 1.11.1.7) with several mPEG was carried out. The modified enzymes were studied through chromatographic and electrophoretic methods; the extent of mPEG linking was determined using ^{1}H-NMR. Peroxidase, modified with mPEG ranging from 350 to 5000, activated with 4-nitrophenylchloroformate (mPEGpn), showed a better thermal stability than the native, but there was no correlation between the length of the polymer adduct and the improvement. The enzyme was modified with mPEG (5000) activated by cyanuric chloride (mPEGcc). The number of modified lysine increased, but the thermal behavior of mPEGcc peroxidase was similar to those of mPEGpn enzymes. In all cases, the modification did not markedly change the stability in organic solvents.

Index Entries: Covalent modification; peroxidase; chromatographic separation; thermoinactivation; organic solvents.

Nomenclature: PEG, polyethyleneglycol; mPEG, monomethoxypolyethyleneglycol; mPEGpn, monomethoxypolyethyleneglycol activated with p-nitrophenylchloroformate; mPEGcc, monomethoxypolyethyleneglycol activated with cyanuric chloride; mPEG350, mPEG of MW350 (and so on); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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INTRODUCTION

Modification of proteins have provided important knowledge on protein properties such as stability, solubility in organic solvents, or extreme conditions. Chemical modification of proteins was extensively studied using various polymers *(1–4)*, and many works were focused on the use of PEG *(5,6)*, which reduces immunogenicity and increases blood clearance time for enzymotherapeutic applications *(7)*. PEG-modified proteins are soluble in selected organic solvents because of the amphipatic nature of the polymer *(8)*.

Covalent attachment of PEG to various enzymes was reported (trypsin *(6)*, ribonuclease *(9)*, superoxide dismutase *(9)*, lipase *(10)*, peroxidase *(11)*. Their use in catalyzing reactions in organic solvents or in biotechnological processes presents a great potential *(4,5)*. In the present investigation, horseradish peroxidase (HRP, EC 1.11.1.7) was chosen with a view to developing an organic phase enzyme electrode (OPEE) for the detection of phenols and breakdown products of pesticides.

This work focuses on the modification and improvement of properties (thermal inactivation and solvent stability) of HRP through attachment to mPEGs. The polymer chosen has mol wt of 350, 2000, and 5000, and two types of activation: *p*-nitrophenylformate and cyanuric chloride. Some properties of the modified HRP are described: the rate of modified amino acids using ^{1}H-NMR spectroscopy, the change of surface properties of the modified enzyme, and the stabilization of enzymatic activity according to the temperature and the addition of solvents.

MATERIAL AND METHODS

Horseradish peroxidase (Type XII) (EC 1.11.1.7), mPEGs MW = 5000, 2000, and 350, mPEG cyanuric chloride, *p*-nitrophenylchloroformate, 2,2′-azino-*bis*(3-ethylbenz-thiazoline-6-sulfonic acid), and 3 (trimethylsilyl) propane sulfonic acid sodium salt were supplied by Sigma (St. Louis, MO). Protein BCA reagent was supplied from Pierce (France). Anhydrous solvents obtained from Aldrich (Milwaukee, WI) contained less than 0.005% water and were packaged under nitrogen. All other reagents were of analytical grade.

Activation of Monomethoxypolyethyleneglycols

The mPEGpn (MW 5000 and 2000) were synthesized following the reaction of monomethoxypolyethyleneglycols with *p*-nitrophenylchloroformate in dichloromethane, in the presence of triethylamine, as previously described by Sartore et al. *(12)*. The products were purified after

precipitation in diethylether. The method was slightly modified for mPEG (MW 350). After the first step (reaction with *p*-nitrophenylchloroformate), the dichloromethane was evaporated under vaccum. The yellow oil obtained was then dissolved in ethylacetate and washed with several solutions: water, 1 *M* $NaHCO_3$, 0.5 *N* HCl, and water. After drying on sodium sulfate, ethylacetate was evaporated under vaccum.

Enzyme Modification

Several mPEGpn (350, 2000, 5000) were added in excess (10 molar excess over HRP NH_2lysine) to peroxidase (3.5 mg/mL dissolved in 0.1 *M* borate buffer, pH 9.1). The resulting mixture was stirred at 25°C for 3 h. The method is identical with mPEGcc, but with only 1 h coupling reaction. The excess of mPEG was removed by ultrafiltration using YM 10 or YM 30 membranes (Amicon, Danvers, MA; cut-off, respectively, MW 10.000 and 30.000).

^{1}H-NMR Spectroscopy

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were observed at 20°C, using a JEOL (Tokyo, Japan) EX-400 NMR spectrophotometer operating at 400 MHz. Solutions (from 50–200 μg) of mPEG were prepared in D_2O, with an internal 3-(trimethylsilyl)propane sulfonic acid sodium salt (DSS). Preparations of HRP and mPEG-HRP were dissolved in D_2O, and the ^{1}H-NMR chemical shift of mPEG linked to the protein was determined using DSS as internal standard.

Activity Measurement

The peroxidase activity was determined at 37°C, by monitoring the oxidation of 1 m*M* 2,2′-azino*bis*(3-ethylbenzothiazoline-6-sulfonic acid) in the presence of 0.05 m*M* hydrogen peroxide in 1 mL of phosphate buffer, 25m*M*, pH 7.0. The increase in the reaction product was recorded at 412 nm (using a Hewlett-Packard, Evry, France spectrophotometer equipped with a thermostated cell), by adding 250 μL of enzyme solution.

Protein Measurement

Samples reacted with the Pierce reagent (2:1; v/v) for 1 at 60°C. Protein concentration was determined by measuring the absorbance at 562 nm with a Hewlett-Packard spectrophotometer, using bovine serum albumin (BSA) as standard.

Chromatographic Methods

The solutions containing both the peroxidase and the mPEGs-peroxidase were eluted through a gel-filtration column (TSK-gel G3000 SW Tosohaas, flow rate 0.5 mL/min) with a 0.05 *M* phosphate buffer, pH 8.0. Hydrophobic interactions chromatography was used to separate, and to determine the changes in, surface hydrophobicity of each peroxidase (TSK-gel phenyl 5 PW-Tosohaas column, flow rate 0.5 mL/min) by using a linear gradient of ammonium sulfate (from 1.7 to 0.0 *M*). The eluent was collected in 0.5-mL fractions. The enzymatic activities were determined in each fraction.

Electrophoresis

SDS-PAGE was performed on 8% acrylamide gels in a Trisglycine buffer, pH 8.3. Samples were incubated for 5 min at 100°C in a loading buffer containing β-mercaptoethanol (5%) and SDS (2.3%). The gels were stained with 0.1% (w/v) Coomassie blue in a mixture of 10% acetic acid, 10% ethanol, and 0.5% glycerol. The broad mol wt calibration kit (20.1–94.0 kDa) consisted of the following proteins with the corresponding mol wt values: phosphorylase b (94.0), albumin (67.0), ovalbumin (43.0), carbonic anhydrase (30.0), and trypsin inhibitor (20.1).

Thermal Stability

The residual activity of the peroxidases was assayed after various incubation periods at 60°C in bi-distilled water. The residual activity was also measured after an incubation period of 15 min, in the range of 30–80°C. Thermal denaturation was stopped by keeping aliquots in ice. The results were expressed as percentages of residual activity as a function of both the incubation time and the temperatures.

Stability in Organic Media

Tubes containing 50 μL HRP solution (4 μg), prepared in 25 m*M* phosphate buffer, pH 7.0, were lyophilized. 200 μL of organic solvent were added to these tubes under continuous shaking for 30 min. The enzyme was then extracted with 1 mL phosphate buffer, and the enzymatic assay was performed, using either 200 μL of the aqueous phase (in the case of water-nonmiscible solvents) or 240 μL of the mixture in the case of water-miscible solvents. Dichloromethane, benzene, toluene as water-nonmiscible solvents, and methanol and acetonitrile as water-miscible solvents were used. For each solvent, experiments were carried out in triplicate. The results are expressed as percents of the remaining activity; 100% corresponds to the incubation in the buffer.

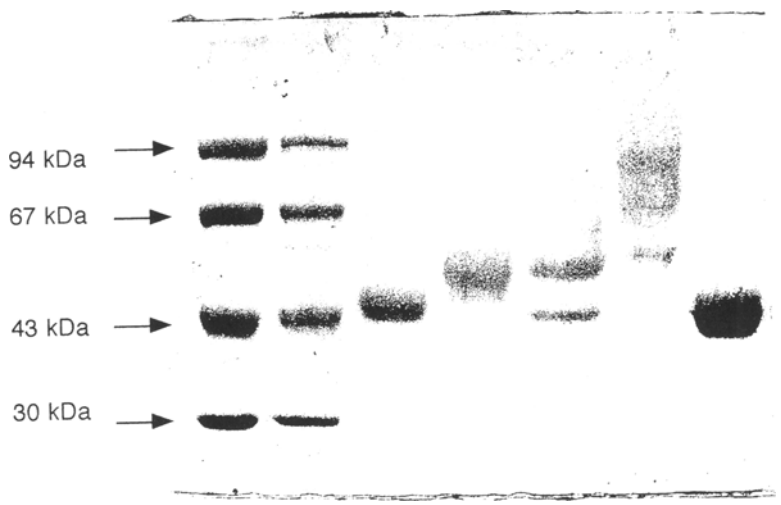


Fig. 1. Electrophoretic separation of the peroxidase and the modified peroxidases. Lanes 1 and 2 represent a standard protein kit; lane 3, modification with mPEGpn350; lane 4, modification with mPEGpn2000; lane 5, modification with mPEGpn5000 (coupling reaction during 1 h); lane 6, modification with mPEGpn5000 (coupling reaction during 3 h); lane 7, the native peroxidase.

RESULTS AND DISCUSSION

Characterization of mPEGs-Modified Peroxidase with mPEG of Various Mol Wt (350, 2000, and 5000) Activated by *p*-nitrophenylchloroformate

The authors first investigated the effect of several mPEGpns length. HRP did not lose activity during the modification step, and the excess mPEG was eliminated by ultrafiltration. Aliquots of each sample were studied using chromatographic and electrophoretic methods. All these methods showed no remaining native HRP after 3 h of coupling reaction (Figs. 1 and 2).

First, electrophoretic behavior of native and modified forms of HRP was studied (Fig. 1). Lane 7 shows the native HRP, and lanes 3, 4, and 6 the modified HRP, with, respectively, mPEGpn 350, 2000, and 5000. No more native HRP is detected after modification, and the electrophoretic mobility decreases as the mPEG adduct increases. It is known that grafting PEG polymer leads to an anomalously low mobility *(13)*. The polymer changes the electrophoretic mobility of proteins, and SDS-PAGE gels can give only a rough estimation of the modification degree. The mol wt change at lane 3 was below 5 kDa, and so was nonsignificant. The mol wt calculated from lanes 4 and 6 are 52 and 57 kDa, indicating maximal values close to 4 and 3 mPEG molecules linked to each HRP molecules.

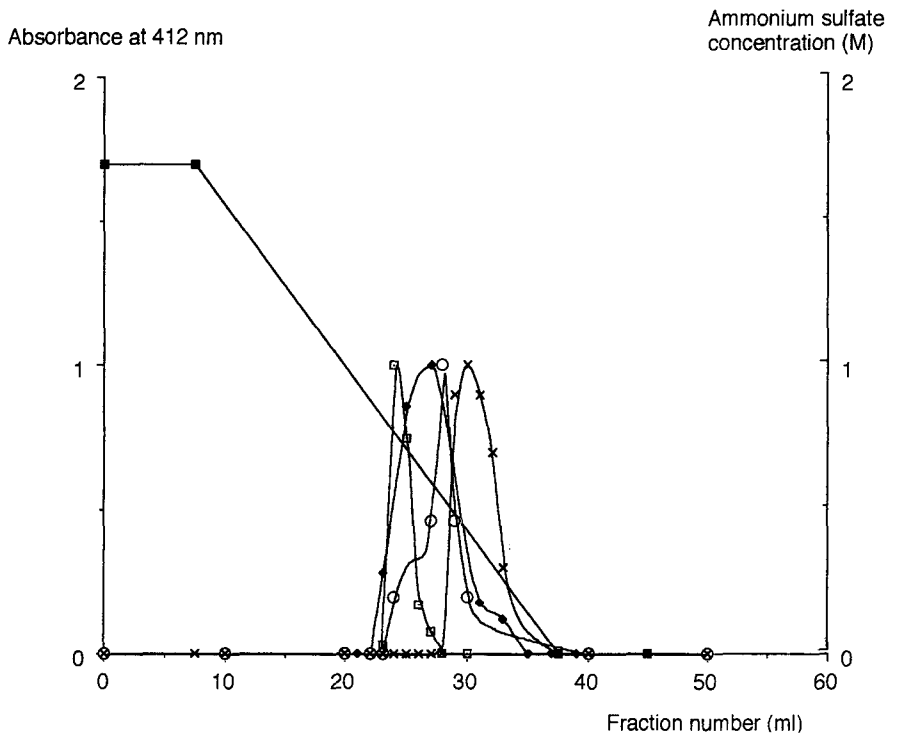


Fig. 2. Elution patterns in hydrophobic interactions chromatography of the (□) native peroxidase and the mPEGpn-peroxidases: (◆) mPEG350-peroxidase, (○) mPEG2000-peroxidase, (×) mPEG5000-peroxidase. Gradient (■).

Each sample was then eluted through a size-exclusion chromatography (Table 1). When the size of the mPEG adduct increases, the K_{av} decreases, but the differences between mol wts cannot be used to estimate the number of mPEG molecules linked to the protein. This result is consistent with the assumption that polyethyleneglycol chains are linked onto the surface of the proteins, and increase its Stocke's radius much more than the corresponding mol wt *(13)*. Under these conditions, gel filtration cannot be used to estimate the modification degree of a mPEG-protein.

Moreover, the surface properties of the modified mPEGs-peroxidase change: The polymer increases the surface hydrophobicity of the proteins. This property can thus be used to separate the native from the modified form, using hydrophobic interactions chromatography (Fig. 2). The hydrophobicity increases with the size of the mPEG, and the modified proteins are eluted at lower ammonium sulfate concentrations. The elution profile also shows shoulders, probably indicating an heterogenous mixture of modified proteins.

Table 1
Partition Coefficient in Gel Filtration of Peroxidase and Modified Peroxidases

	Peroxidase			
		Modified		
Sample	Native	mPEG350	mPEG2000	mPEG5000
K_{av}[a]	0.50	0.45	0.39	0.33

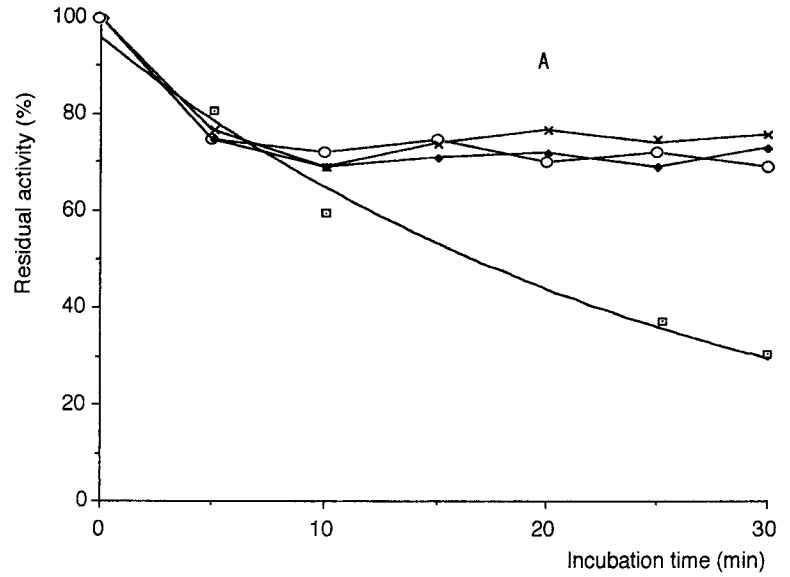
[a] $K_{av} = (V_e - V_0)/(V_t - V_0)$
V_0 and V_t represent, respectively, the dead volume and the total volume of the column; V_e, the elution volume of the sample.

Native HRP contains six lysine residues and a blocked-N terminus *(14)*. The ^{1}H-NMR spectra indicate a modification of 1.7 lysine residues; the modified peroxidase seems to be a mixture of one mPEG- and two mPEG-enzymes.

The stability of the PEGpn-modified peroxidase was investigated by increasing the temperature. All the mPEGs-peroxidase shows an increased thermostability, compared to the native enzyme (Fig. 3A). After 30 min at 60°C, the modified peroxidase were stable: The polymer adduct, whatever its length, has the same protecting effect. No correlation was found between the mol wt of mPEG and the enhanced stability.

Ranging temperature from 40–80°C, mPEG greatly stabilizes HRP (Fig. 3B). The native enzyme is inactivated, but the modified peroxidases remain catalytically active. The protecting effect is not clearly correlated to the length of the polymer adduct; however, mPEG (5000) seems to have the most protective effect at high temperature.

The stability of the peroxidase in organic solvents was also carried out. Figure 4 shows that the peroxidase remains active with all the solvents used. The lowest activity was observed in the water-miscible solvents, suggesting that the enzyme-bound water controls the activity of the peroxidase. This well-known phenomenon, called water stripping, has been described by Gorman and Dordick *(15)*. According to these authors, water desorption is complete within the first minutes of contact with the solvents (explaining the choice of 30 min of incubation). It is generally observed that water-soluble organic solvents act as denaturing agents of enzymes *(16–18)*. The reasons of enzyme stability changes have been previously discussed in the literature *(19,20)*. Briefly, water-miscible solvents cause an unfolding of native protein structure by solvation of interior nonpolar residues, in addition to competing with water for structure-forming hydrogen bonds, which are necessary in order to maintain the native struc-



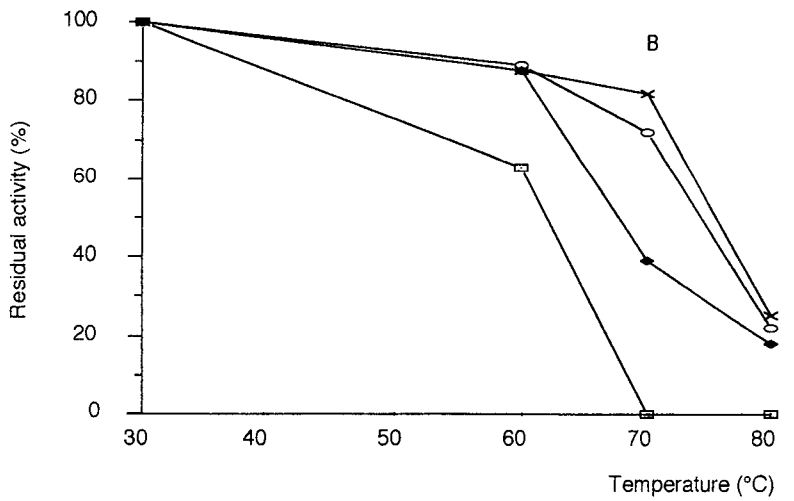


Fig. 3. **(A)** Residual activity of the peroxidase and the mPEGpn-peroxidases (1.7 μg/mL) at 60°C in bi-distilled water; and **(B)** residual activity of the peroxidase and the mPEGpn-peroxidases after 15 min incubation at various temperatures. (□) native peroxidase, (◆) mPEG350-peroxidase, (○) mPEG2000-peroxidase, (×) mPEG5000-peroxidase.

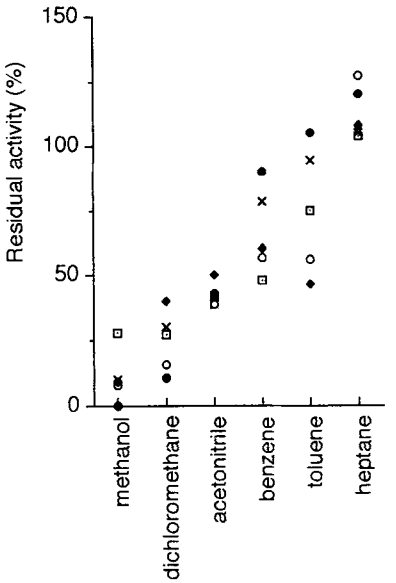


Fig. 4. Study of the stability: Activity of native peroxidase (□) and modified peroxidase: (◆) mPEGpn350, (○) mPEGpn2000, (×) mPEGpn5000, and (●) mPEGcc5000 after 30 min incubation in different organic solvents. Measurement conditions: 25 m*M* phosphate buffer, pH 7.0, 37°C, 1m*M* ABTS, 0.05 m*M* hydrogen peroxide.

ture *(16)*. In accordance with this, the authors found that methanol and acetonitrile rapidly destabilized HRP. The modification by mPEG does not markedly improve the stability of the enzyme after contact with these solvents. Moreover, no correlation was found between the mol wt of the mPEG adducts and the residual activity.

A new approach in biotechnological processes is to use enzymes modified with PEG. The modified enzymes are soluble in organic solvents, such as benzene, toluence, and chlorinated hydrocarbons, and exhibit high enzymic activities in these solvents. Inada et al. *(21)* showed that modified peroxidase efficiently catalyzes its respective reactions in these solvents. The authors have checked, in this paper, that the modified HRP is as stable as the native enzyme in benzene, toluene, dichloromethane, and heptane. The dichloromethane rapidly inactivated both native and modified peroxidase when the other water-nonmiscible solvents kept the enzymes stable. It is possible to correlate the enzyme stability with a physicochemical parameter of the solvents: log P, defined as the logarithm of the partition coefficient in a standard octanol–water two-phase system. As predicted by Laane et al. *(22)*, HRP was found to be unstable when log P < 2 (dichloromethane), variable when $2 \leq \log P < 4$ (benzene, tolunene), and stable when log P ≥ 4 (heptane). Moreover, mPEG is not soluble in heptane: The stability of the native and of the modified peroxidase are

quite similar in this case (the activation noted with heptane cannot be explained). But, when mPEG is soluble (e.g., in benzene and toluene), the remaining activity of the native and of the modified peroxidase are quite different, perhaps suggesting different partition coefficients for modified and nonmodified HRP.

Characterization of mPEGs-Modified Peroxidase with mPEG Mol Wt 5000 Activated with *P*-Nitrophenylchloroformate and Cyanuric Chloride

The authors then investigated the changes caused by another mPEG activation. mPEGcc is less specific than the mPEGpn. The dichlorotriazine reacts strongly with the primary amines, but also with other functional groups, such as hydroxyl and sulfhyrdyl; the phenylchloroformates react cleanly with the primary amines (*23*). As with mPEGpn, no loss of activity was observed during the coupling reaction. After 1 h coupling reaction, the reaction with mPEGcc is complete, but it is not complete with mPEGpn, as shown in Fig. 1, lane 5, and in Fig. 5A. The chromatographic methods were also applied to this modified enzyme. The gel filtration profile (Fig. 5B) shows an increasing weight for the peroxidase modified by mPEGcc, compared to the one modified with mPEGpn. The hydrophobic interactions chromatography (Fig. 5A) exhibits a more hydrophobic behavior, and the gel electrophoresis a lower electrophoretic mobility of mPEGcc-peroxidase, compared to the mPEGpn-enzyme (data not shown). All the differences noted between the two modified enzymes are consistant with a higher degree of modification with the cyanuric chloride activation, which means a higher mol wt for the mPEGcc HRP than for the mPEGpn HRP. The result is confirmed by the titration of mPEG content of the modified HRP with ^{1}H-NMR spectroscopy. This method indicates a modification of 2.7 lysine residues.

The thermal stability (data not shown), as well as the inactivation by organic solvents (Fig. 4), of the mPEGcc-HRP are similar to those obtained with mPEGpn.

CONCLUSION

The authors modified HRP with mPEG, varying its activation. We used several methods to compare the rate of modification, each being consistent with the others: The number of mPEG adducts is more important with mPEGcc than with mPEGpn. Moreover, the modification led to an improvement of the thermal stability with the mPEG activation methods and length (350, 2000, and 5000). The first experiments carried out in organic media were not sufficient to draw a general conclusion. The stability in some organic solvents can be improved by the enzyme modification.

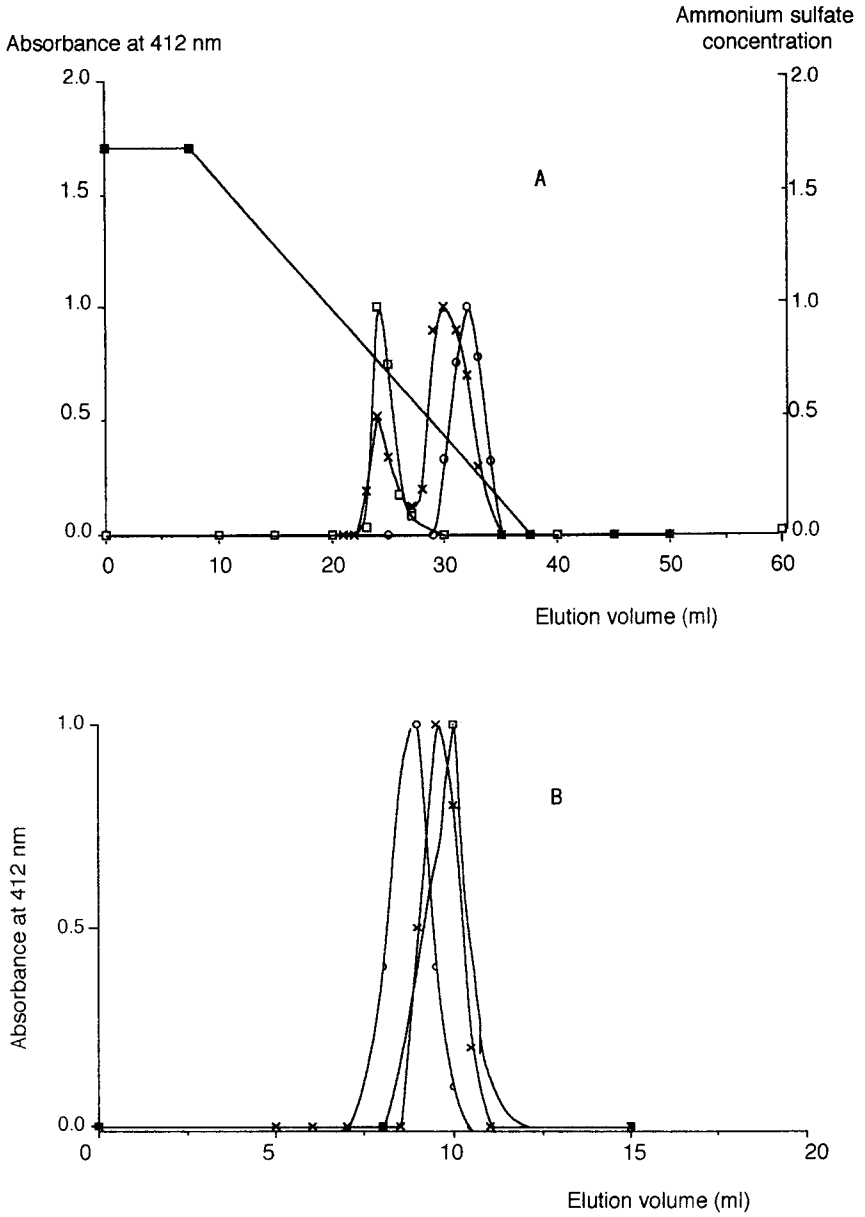


Fig. 5. Elution patterns in hydrophobic interactions chromatography **(A)** and gel filtration **(B)** of the (□) native peroxidase and the mPEG5000-peroxidases: (×) mPEGpn-peroxidase, (○) mPEGcc-peroxidase. Gradient (■).

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