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Chemical Modification of Horseradish Peroxidase with Ethanal–Methoxypolyethylene Glycol: Solubility in Organic Solvents, Activity, and Properties

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The oxidation of polyethylene glycol monomethyl ethers (MW 350, 1900, and 5000) by the Moffatt–Swern method to the corresponding aldehyde is described. These aldehydes are used to modify horseradish peroxidase (HRP) by a reductive amination. The modification of two to three ε-NH_2 groups of the enzyme was observed. The isoelectric point of the native HRP (p*I* 8.8) was shifted to p*I* 5.5 on modification. The modified enzymes have an activity close to that of the native enzyme. Only the enzyme modified with the aldehyde MW 5000 (HRP 5000) was soluble and active in organic solvents like toluene, dioxane, and methylene chloride. In toluene, HRP 5000 was more sensitive to hydrogen peroxide inhibition than in buffer. At room temperature, it is more stable in toluene than in buffer.

INTRODUCTION

Chemical modifications of proteins with polyethylene glycol derivatives have been made initially with the purpose to increase the half-life and to reduce the immunoreactivity of enzymes in the treatment of various diseases (*1–7*). These modifications were performed in aqueous solutions where the modified enzymes remained soluble so that the effect of the modification on their activity and their physical properties could be determined. Later it was found that these modified enzymes were soluble and active in organic solvents. The modified enzymes were hydrolases—lipase (*8–10*), α-chymotrypsin (*11–15*), trypsin (*15–17*), thermolysin (*18*), and papain (*19–21*)—and oxidoreductases—catalase (*22*), peroxidase (*21, 23–25*), and cholesterol oxidase (*26*). It was shown that they could be used as a catalyst of chemical reactions for biotechnological applications (*27*).

The PEG[1] derivatives used most are mono- and dichlorotriazine polyethylene glycol ether (*16, 28*) but a number of other methods have been developed for the

[1] Abbreviations used: PEG, polyethylene glycol; aldehyde MW 350, MW 1900, and MW 5000, ethanal ω-methoxypolyethylene glycol of average molecular weights 350, 1900, and 5000; TNBS, 2,4,6-trinitrobenzenesulfonic acid; HRP, horseradish peroxidase; HRP 5000, peroxidase modified with aldehyde MW 5000; SDS, sodium dodecyl sulfate.

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$$CH_3\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_{n-1}\text{-}CH_2\text{-}CH_2OH$$

1

$$CH_3\text{-}O\text{-}(CH_2\text{-}CH_2\text{-}O)_{n-1}\text{-}CH_2\text{-}CHO$$

2

coupling of PEG to proteins (*3, 14, 20, 21, 25, 26, 29, 30*). The commercial availability of polyethylene glycol monomethyl ethers **1** and the selective method for the oxidation of hydroxyl groups to carbonyl groups (the Moffatt–Swern method) (*31, 32*) led us to prepare the corresponding aldehydes, ethanal ω-methoxypolyethylene glycols **2,** and to link them to accessible amino groups of proteins by a reductive amination. We describe here the preparation of these aldehydes **2,** their linkage to horseradish peroxidase, and the activities of the modified enzymes (*33*).

MATERIALS AND METHODS

Horseradish peroxidase [EC 1.11.1.7] of grade I and bovine serum albumin (fraction V) were obtained from Boehringer Mannheim (FRG). The RZ value (Reinheitszahl, i.e., purity number, absorbance (402 nm)/absorbance (280 nm)) which is a measure of the hemin content, was about 3.0 (*34*). Organic solvents of highest commercially available purity were used without further purification. The polyethylene glycol monomethyl ethers of indicated average molecular weight were from EGA Chemie (Steinheim, FRG). Trinitrobenzenesulfonic acid, *o*-phenylenediamine, and 4-aminoantipyrine were supplied by Fluka (Buchs, Switzerland). Hydrogen peroxide (30% solution) was purchased from Prolabo (Paris).

Synthesis of Ethanal ω-Methoxypolyethylene Glycol

The methoxypolyethylene glycol of molecular weight 350, 1900, and 5000 were dried under vacuum for 12 h. A solution of dimethyl sulfoxide (48 mmol) in methylene chloride (5 ml) was added dropwise to a cold (−78°C) solution of oxalyl chloride (22 mmol) in methylene chloride (25 ml). The rate of addition was controlled so that the temperature did not increase above −55°C. After 10 min, a solution of polyethylene glycol monomethyl ether (10 mmol) in methylene chloride (10 ml for ether MW 350 and MW 1900; 20 ml for ether MW 5000) was added. The temperature was kept at −20°C for 1 h. After addition of 1,4-diazabicyclo[2.2.2]octane (50 mmol), the reaction medium was warmed to room temperature. Water (50 ml) was then added, and the reaction mixture was extracted with

methylene chloride. The organic layer was separated, dried over magnesium sulfate, and evaporated. In the case of aldehyde MW 350 the residue was chromatographed on a silica gel column (Merck, 70–230 mesh) with 7 : 1 methylene chloride–methanol as the eluant. The fractions were analyzed by thin-layer chromatography (Merck Kielselgel $60F_{254}$). The fractions giving a positive response with the 2,4-dinitrophenylhydrazine test were pooled and evaporated. The aldehydes MW 1900 and MW 5000 were precipitated from their solution in acetone with ether. The precipitate was filtered and dried under vacuum for 24 h.

The aldehyde content was determined with the oxime formation test. The hydroxylamine solution was prepared by adding ethanol (80 ml) and a 0.5 M potassium hydroxide solution in methanol (90 ml) to hydroxylamine hydrochloride (4 g) dissolved in water (8 ml). A solution of the aldehyde (200 to 300 mg) dissolved in benzene (20 ml) was added to the hydroxylamine solution (10 ml). After 1 h at 70°C, the solution was cooled to 20°C, and methanol (30 ml) was added. Excess of base was titrated with a 0.2 M hydrochloric acid solution. The blank value was determined in the absence of aldehyde.

Modification of Peroxidase with the Aldehydes

To a solution of peroxidase (0.72 mM, 5 ml) in 0.1 M borate buffer, pH 8, were added aldehyde MW 350 (0.26 M) and sodium borohydride or sodium cyanoborohydride (0.26 M). After 2 or 5 h at 20°C, the modified enzyme solution was dialyzed with 10 changes (500 : 1 dilution factor) against 1 mM citrate buffer, pH 5, over 3 days, and then lyophilized.

For the modification of HRP with the aldehydes MW 1900 and MW 5000, a similar procedure was applied. Peroxidase (50 μM) was modified in a 0.1 M borate buffer, pH 8, with aldehyde MW 1900 or MW 5000 (20 mM) in the presence of sodium cyanoborohydride (20 mM). After 3 h at 20°C, the modified enzyme solution was dialyzed as above, and then lyophilized.

Determination of the Concentration of Protein

The concentrations of native and modified peroxidase were determined with the Bradford method (*35*) using bovine serum albumin as the standard. For the peroxidase dissolved in organic solvent, a known volume of the solution was evaporated to dryness under vacuum. The protein was then dissolved in buffer and the concentration was determined with the Bradford method. The absorption at 404 nm (ε = 91,000 M^{-1} cm^{-1}) was also used to determine the peroxidase concentration in buffer and in organic solvents (*23*). The concentrations determined with both methods agreed.

Activity of Native and Modified Peroxidases in Buffer

The enzymatic activity was determined at 20°C in a 0.1 M citrate buffer, pH 5, by following the absorbance increase at 446 nm (ε = 6000 M^{-1} cm^{-1}) in the presence of 75 mM *o*-phenylenediamine and 15 mM hydrogen peroxide (*36*). The activity was expressed in mmol oxidized *o*-phenylenediamine min^{-1} (mg pro-

tein)$^{-1}$. For the determination of the Michaelis constant of hydrogen peroxide, the concentration of hydrogen peroxide was varied from 0.25 to 15 mM.

The pH dependence of the activity at 20°C was studied in 0.1 M citrate buffer, pH 3, 3.5, 4, 4.5, 5, 5.5, and 6.

Titration of Amino Groups

The number of amino groups of native and modified peroxidases was determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (*37*). To a 10 μM solution of native or modified peroxidase in water (200 μl) were added TNBS (2 mg) and sodium bicarbonate (4 mg). After 30 min, 1.5 h, 2.5 h, 3.5 h, and 4.5 h at 40°C in the dark, the medium was chromatographed on a Sephadex G-25 (Pharmacia) column (1.5 × 12 cm) with a 50 mM sodium chloride solution as the eluant, to eliminate excess of TNBS and sulfite ions. The fractions (2.5 ml) containing the enzyme, detected by its absorption at 404 nm, were pooled. The reaction extent was determined by measuring the absorbance at 340 nm (ε = 14,000 M^{-1} cm^{-1}) after addition of a 0.15 M HCl and 2% SDS solution (600 μl) to an aliquot of the protein solution (200 μl). The concentration of the TNBS-modified protein was determined with the chromogene, heme pyridine method (*38*). The contribution of heme absorption at 340 nm was determined using an absorption coefficient of 27,700 M^{-1} cm^{-1}.

Isoelectric Point Determination

The determinations of the isoelectric point of native and modified (MW 5000) peroxidases were accomplished with the PhastSystem from Pharmacia, using the methodology recommended by the supplier.

Peroxidase Activity in Toluene

The activity of the modified peroxidase in water-saturated toluene was determined by following the absorbance increase at 488 nm in the presence of 75 mM *o*-phenylenediamine and 0.15 mM hydrogen peroxide. The concentration of HRP 5000 was 4 × 10^{-8} M. Hydrogen peroxide in toluene was prepared by vigorously mixing the aqueous solution of hydrogen peroxide and toluene. The aqueous layer was removed after settling. The concentration of hydrogen peroxide in the toluene layer was determined iodometrically (*39*).

RESULTS

Synthesis of the PEG Aldehydes

ω-Methoxypolyethylene glycols having average molecular weights 350, 1900, and 5000 were oxidized by the Moffat–Swern method to the corresponding aldehydes. Aldehyde MW 350 was an oil, whereas aldehydes MW 1900 and MW 5000 were solids. The content in aldehyde group was determined by the oxime forma-

tion and found to be 51, 57, and 67% for the aldehydes MW 350, MW 1900 and MW 5000, respectively. The presence of aldehyde group was confirmed by ^1H NMR (signal at 9.8 ppm in CD_2Cl_2), by IR spectroscopy (band at 1740 cm^{-1} in carbon tetrachloride) and by the formation of phenylhydrazone. The aldehydes were used without further purification.

Peroxidase Modification

The reductive alkylation of peroxidase with the different synthesized aldehydes (10 molar excess of aldehyde MW 350 and 60 molar excess for aldehydes MW 1900 and MW 5000 over the amino group content of peroxidase) was performed in the presence of sodium borohydride (*43*) or sodium cyanoborohydride (*44*). This latter is a more selective reducing agent since, at pH 8, it reduced Schiff bases without affecting the aldehyde groups (*44*). At the end of the reaction, the RZ dropped from the initial value of 3.0 to 2.7. This was the only change in the spectrum of modified HRP when compared to native HRP.

The degree of modification of HRP during the reductive alkylation was determined by the TNBS method (*37, 38*). A 4.5-h reaction time was necessary for a complete modification of the amino groups of native HRP with TNBS: 6 amino groups were titrated. For HRP 350, 1900, and 5000, 3, 3.5, and 3.8 amino groups were showed to be modified with TNBS, respectively. It appears that 3, 2.5, and 2.2 amino groups out of 6 were alkylated by aldehydes MW 350, MW 1900, and MW 5000. Amino acid analyses on native and modified HRP confirmed these results.

Activity and Physical Properties of Modified Peroxidases in Buffer

The reductive alkylation of HRP with PEG aldehydes did not change the enzymatic activity when the reaction was stopped after 2 h (Table 1). Longer reaction

TABLE 1

Activity of Native and Modified Horseradish Peroxidases

Chemical treatment	Activity
None	40
MW 350/$NaBH_4$/2 h	40
MW 350/$NaBH_3CN$/2 h	35
MW 350/$NaBH_3CN$/5 h	30
MW 1900/$NaBH_3CN$/3 h	45
MW 5000/$NaBH_3CN$/3 h	35

Note. Activity was determined with 15 mM H_2O_2 and 75 mM *o*-phenylenediamine in a 0.1 M citrate buffer pH 5 at 20°C. It is expressed in mmol oxidized *o*-phenylenediamine min^{-1} (mg protein).$^{-1}$.

TABLE 2

Solubility of HRP 5000 in Various Organic Solvents

Solvent	Solubility (mg/ml)
Toluene	0.15
Methylene chloride	0.15
Dimethylformamide	0.15
Dioxane	0.30
Dimethylsulfoxide	0.50
Cyclohexane	0.0
Tetrahydrofuran	0.0
Ethyl acetate	0.0

times (5 h) tended to reduce the activity by about 20%. At a constant concentration of *o*-phenylenediamine (75 mM), a 2.2 mM Michaelis constant of hydrogen peroxide was determined for native and modified enzyme. No inhibition was found with concentration of hydrogen peroxide up to 15 mM. The pH of maximum activity was shifted from pH 5.0 for native HRP to pH 3.5 for HRP 5000 (results not shown).

Native HRP has an isoelectric point of 8.8 in agreement with the value for HRP isoenzyme C (*45–47*) whereas HRP 5000 had one of 5.5. The peroxidase is a thermostable enzyme (*48*). Native and modified HRP had the same thermostability in buffer up to 80°C (results not shown).

Solubility and Activity of Modified Peroxidases in Organic Solvents

The native and modified (HRP MW 350 and MW 1900) peroxidase did not show any detectable solubility in toluene and were not further studied in other solvents; on the other hand, HRP MW 5000 was soluble in several organic solvents. Water seemed to be necessary to render the modified enzyme soluble and active in organic solvents. Reproducible results were obtained by using water saturated solvents (Table 2).

The incubation of HRP 5000 in the presence of hydrogen peroxide and *o*-phenylenediamine led to the formation of a compound having a λ_{max} shifted from 446 nm in buffer to 488 nm in toluene. The enzyme concentration had to be increased to 10 nM to detect any activity with 75 mM *o*-phenylenediamine. The modified enzyme was rapidly inhibited at concentrations of hydrogen peroxide above 0.2 mM; hence, the enzymatic activity tests were carried out at 0.15 mM of H_2O_2. Under these conditions, the rate was linearly related to the concentration of HRP 5000 and found to be 20 μmol min^{-1} mg^{-1}. As in water, 2-methoxyphenol and the couple 4-aminoantipyrine/phenol were also oxidized by the modified peroxidase in toluene.

The action of HRP 5000 on water-insoluble potential substrates was studied: anisole, 1,2-dimethoxybenzene, and benzyl, 4-methoxybenzyl, 3,4-dimethoxy-

benzyl, and 4-hydroxy-3-methoxybenzyl alcohols. No spectral change was detected with these compounds and it was concluded that they were not substrates of the modified peroxidase in toluene. The recovery of unreacted starting materials were confirmed by HPLC.

At 20°C, the modified peroxidase is quite stable in toluene where its half-life was found to be 500 min. In 0.1 M citrate buffer pH 5, the half-lives were only 250 and 220 min for the modified and the native enzymes, respectively.

In dimethylformamide, the modified peroxidase did not show any activity. However, in dioxane and methylene chloride, an activity close to that in toluene was found with *o*-phenylenediamine. In dimethyl sulfoxide, a comparatively low activity was detected.

DISCUSSION

The chemical oxidation of *O*-methoxypolyethylene glycol could be successfully carried out according to the Moffatt reaction modified by Swern (*31, 32*). This reaction involved only volatile reagents and by-products so that the PEG chains could be easily freed from undesired compounds. The unreacted methoxypolyethylene glycol chains should not influence the reductive alkylation of proteins; hence, the modification of HRP was performed with the mixture of unreacted and oxidized PEG chains by taking into account the amount of aldehyde groups. PEG aldehydes have a greater advantage over the frequently used triazine PEG derivatives in that they do not absorb light in the region where proteins do.

The amino-terminal group of native HRP is blocked so that only the ε-NH_2 of the Lys residues (six according to the sequence) can be alkylated with the PEG aldehydes (*49*). The titration of free amino groups with TNBS showed that two to three polyethylene glycol chains had bound to the enzyme. In these conditions the enzymatic activity remained virtually unchanged with respect to native HRP. The modification of HRP performed with 2,4-bis(methoxypolyethylene glycol) 6-chloro-*s*-triazine (MW 5000), resulting in the alkylation of 60% of their amino groups (three to four PEG chains per HRP molecule), showed a 30% activity decrease in buffer (*23*). When the ε-NH_2 groups were modified with dithioester functionalized PEG chains (*21*) or when the PEG chains were coupled on the glycolic part (*25*), the enzymatic activity, in buffer, remained also close to that of native HRP.

The physical properties of HRP 5000 are quite different from that of the native enzyme: it did not bind to CM-Trisacryl-Sephadex, even at low ionic strength, whereas the native enzyme did (*47*). Similar behavior has been reported in the literature (*2, 17*). The observed lower isoelectric point could essentially be explained by a decrease of the pK_a of the alkylated ε-NH_2 groups of the modified enzyme. Indeed ethylamine has a pK_a value of 10.47 (*50*) while that of methoxyethylamine was 9.45 (*51*). So the introduction of one PEG chain should lead to a decrease of the pK_a of the modified Lys residue of about one unit. Nevertheless the large shift of three units remains unexplained. As for other PEG-bound HRP (*21, 25*), a lowering of the optimal pH was shown for HRP 5000 but the relation

with the observed shift of the isoelectric point is only speculative. The introduction of the PEG chains via the glycolic part of HRP showed a decrease of the optimal pH of 1.1, while the ε-NH_2 groups were not modified (*25*).

Regarding to the solubilities of the polymer-bound enzymes the results clearly demonstrate the importance of the polymer chain length: modified MW 350 and MW 1900 peroxidases were not soluble in toluene as shown by the absence of absorption at 404 nm as that of the native HRP, even in the presence of aldehyde MW 5000; on the other hand, HRP 5000 was soluble in several organic solvents. Other PEG-bound HRP, soluble in organic solvents, have also been described (*21, 23, 25*) and the results are compiled in Table 3. Water, chloroform, and dichloromethane appear as the solvents in which the modified enzymes were soluble and active. In dimethylformamide, the enzymes were always soluble but inactive; in hydrocarbons, they were insoluble and inactive.

HRP 5000 is active in toluene. The enzymatic activity was determined using hydrogen peroxide and *o*-phenylenediamine as the hydrogen donor. The oxidation product of *o*-phenylenediamine had a λ_{max} shifted from 446 nm in buffer to 488 nm in toluene. Likely, it was not due to a different reaction product, but to a medium effect on their spectral properties (*23*). At an hydrogen peroxide concentration of 0.15 mM the initial rate in toluene (20 μmol min^{-1} mg^{-1}) corresponded to 0.05% of the maximal rate in buffer. Assuming an ε of 6000 M^{-1} cm^{-1} for the oxidized *o*-phenylenediamine, the maximal rate of 2,4-bis(methoxypolyethylene glycol) 6-chloro-*s*-triazine-modified peroxidase, determined with other substrates concentration, was 94 μmol min^{-1} mg^{-1} (*23*). This discrepancy could be due to the different assay conditions. It is known that the activity of PEG-bound enzymes in organic solvents, like benzene or toluene, depends on the concentration of water in the reaction medium (*12, 17, 24*); hence, the difference between the initial rates could be due to the amount of water present in the medium. Concentrations of hydrogen peroxide above 0.2 mM rapidly inhibited HRP 5000 in toluene, while in buffer, no inhibition was detected during the assay with concentrations of hydrogen peroxide up to 15 mM. It is possible that in toluene, the hydrogen peroxide

TABLE 3

Solubility and Activity of Various PEG-Bound HRP in Organic Solvents[a]

	Hydrocarbons[b]	Toluene	Ethyl acetate	Dioxane	$CHCl_3$ or CH_2Cl_2	DMF	H_2O
Native HRP	–	–	–	–	–	–	++
HRP 5000	–	++	–	++	++	+	++
PEG-HRP[c]	–	+	n.d.	+	++	+	++
PEG-HRP[d]	–	++	–	–	++	+	++

[a] -, insoluble; +, soluble; ++, soluble and active; n.d.: not determined.

[b] Hexane, heptane, or cyclohexane.

[c] Described in (*21*).

[d] Described in (*25*).

replaces the water molecule as the sixth ligand to iron ion, leading to the inhibition of HRP 5000. The enzyme was more stable in toluene than in water. This observation was found for other enzymes and was attributed to a more structural rigidity of the enzymes in organic solvents preventing the native conformation from unfolding (*52*). In view of the activity and the stability of HRP 5000 in organic solvents, the modification with aldehyde MW 5000 should be extended to other enzymes to render them soluble and active in organic solvents.

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