Covalent Modification of Mushroom Tyrosinase with Different Amphiphic Polymers for Pharmaceutical and Biocatalysis Applications

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ABSTRACT

Two different poly(ethylene glycol) derivatives (linear, mol wt 5000 and a branched form, mol wt 10000) and a new polymer (poly-[acryloylmorfoline], mol wt 5500) were covalently bound to the enzyme tyrosinase. The polymer–protein conjugates were studied with a view to their potential pharmaceutical application and to their use for the bioconversion of phenolic substrates in organic solvents. V_{max} and K_m for the dopa-dopaquinone conversion, thermostability, stability toward inactivation by dopa oxidation products, half-life in blood circulation, and behavior in organic solvents for the different adducts were investigated. Arrhenius plots for the dopa-dopaquinone conversion were also obtained in order to study the effects of temperature on the different enzyme forms. Covalent attachment of the polymers increased enzyme stability in aqueous solution and the solubility in organic solvents. However, organic solvent solubilization brought about loss of enzyme conformation as assessed by CD measurements, which is accompanied by a nonreversible loss of catalytic activity.

Index Entries: PAcM; PEG; branched-PEG; protein-surface modification; poly(ethylene glycol); poly(acryloylmorfoline); tyrosinase; enzyme therapy; bioconversion.

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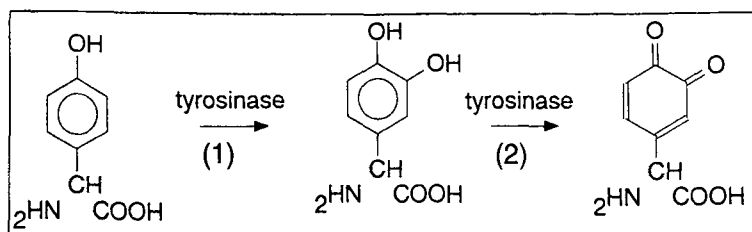
Scheme 1. Monophenol monooxigenase (1) and orthodiphenol:O_2 oxido-reductase (2) reactions catalyzed by tyrosinase on L-tyrosine and L-dopa.

INTRODUCTION

Tyrosinase is an enzyme showing two catalytic activities, namely the monophenol monooxigenase (EC 1.14.18.1) and the orthodiphenol:O_2 oxidoreductase (EC 1.10.3.1) activities (Scheme 1). Its main substrates are tyrosine (4 hydroxyphenylalanine) and dopa (3,4 dihydroxyphenylalanine), but it can also catalyze the regioselective oxidation of other aromatic compounds (1).

The ability of the enzyme to catalyze the tyrosine–dopa conversion is of interest for its therapeutic application, since many diseases are known to be related to abnormal catecholamine levels in different areas of the body. Parkinson's disease, Huntington's chorea, pheochromocytoma, and different types of hypertyrosinemia are among the pathologies that could take advantage of therapy with tyrosinase (3,4). Miranda et al. have recently investigated the possibility of trapping this enzyme in multi-lamellar liposomes to reduce some of the disadvantages related to its therapeutic application (4). These disadvantages include toxicity of the oxidation products, high risk of immunogenicity, and short blood circulation time. In addition to the potential therapeutic applications, the selective tyrosine–dopa conversion is of interest in biocatalysis. Many workers are investigating the use of enzymes in bioreactors for industrial production of L-dopa (5). Moreover, the enzyme can regioselectively oxidize different monophenols even when suspended in organic solvent (6).

The aim of this work was to determine the effects of covalent modification of the tyrosinase surface with three different amphiphic polymers. Covalent attachment of PEG chains on various enzymes' surfaces has proven to be very useful for such applications as drugs or in biocatalysis (8–10). Polymer-modified enzymes are often less immunogenic and antigenic, and show a longer half-life in blood than the unmodified forms (11,12). Such polymer conjugates can therefore be better therapeutic agents than the native enzymes. In addition to this therapeutic aspect, some PEG-modified enzymes show increased solubility in organic solvents, without loss of activity (7–10). Therefore, the covalent modification of enzymes with amphiphic polymers is currently a key technique for optimization of biocatalysis in organic solvent.
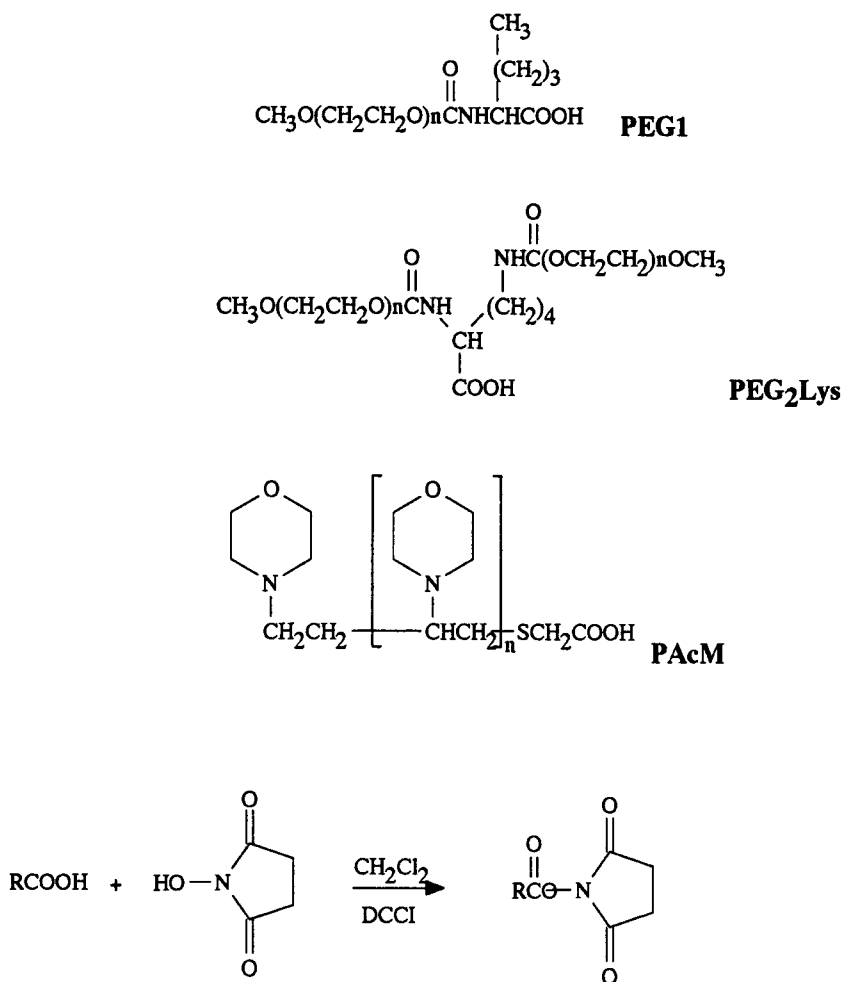
CH3
|
O   (CH2)3
|| |
CH3O(CH2CH2O)nCNHCHCHCOOH   **PEG1**

O
||
O   NHC(OCH2CH2)nOCH3
|| |
CH3O(CH2CH2O)nCNH   (CH2)4
        CH
        |
        COOH   **PEG2Lys**

CH2CH2—[—CHCH2—]n—SCH2COOH   **PAcM**

RCOOH + HO—N   →(CH2Cl2 / DCCI)→   RCO—N

Fig. 1. Linear monomethoxypoly(ethyleneglycol) with nor-leucine as a spacer arm (PEG1); α,ε-di-monomethoxypoly(ethyleneglycol)lysine (PEG2); poly(acriloil-morfoline) (PAcM), and the carboxylic group activation reaction for protein amino group coupling.

In this work, linear monofunctional poly(acryloylmorfoline) (PAcM) of mol wt 5500 (*15*), linear monomethoxypoly(ethylene glycol) (PEG1) of mol wt 5000 (*13*), and a branched monofunctional derivative of the above mPEG (PEG2Lys) (*14*) (Fig. 1) were used to modify mushroom tyrosinase surface. For each conjugate, V_{max}, K_m, thermostability, stability toward inactivation by the reaction products, and half-life in blood circulation were investigated. Arrhenius plots showing the temperature dependence of the dopa oxidation rate and the ΔH for reaction activation were also obtained. Moreover, the effects of the various polymer coatings on solubility and activity in organic solvents were investigated.

EXPERIMENTAL

Reagents

Linear monomethoxypoly(ethylene glycol) (mol wt 5000 Dalton) with norleucine as a spacer arm (*13*) and branched PEG_2Lys (*14*) were prepared as described previously or purchased from Shearwater polymers (Huntsville, AL). Poly(acryloylmorfoline) was prepared according to Ranucci et al. (*15*) and the 5500-Dalton fraction obtained by fractional precipitation was used. Also, this polymer will soon be available from Shearwater polymers. Mushroom tyrosinase, L-dopa, and *p*-cresol were obtained from Sigma (St. Louis, MO), and *N*-hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide were purchased from Fluka Chemie AG. Trinitrobenzenesulfonic acid (TNBS) was obtained from ICN (Cleveland, OH), and *N*-succinimidyl-[2,3-³H]propionate was obtained from Radiochemical Center (Amersham Bucks, UK). UV-Vis determinations were performed on Perkin Elmer λ2 and λ5 spectrophotometers, Circular dichroism spectra were recorded with a Jasco J-500 spectropolarimeter. All the organic solvents used for synthesis and biocatalysis experiments were of "analytical" grade, except for THF, which was HPLC-grade.

Preparation of the Activated Polymers

The three polymers have a single terminal carboxylic group (Fig. 1), and are activated as the succinimidyl esters using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in dry organic solvent (*13*). The degree of polymer activation was measured after ester hydrolysis and spectrophotometric evaluation of the released *N*-hydroxysuccinimide ($\epsilon_{260 \text{ nm}} = 8700$). The degree of activation was usually more than 90%.

Preparation of Protein-Polymer Adducts

For conjugate preparation, the polymers were slowly added to enzyme (44 lys, mol wt 128,000 Dalton [*1,2*]) solution (10 mg/2 mL of 0.2*M* borate buffer, pH 8.0) under vigorous mixing. A threefold molar excess of polymer over the protein lysine amino groups was used. After a 1-h reaction, the modified proteins were separated from *N*-hydroxysuccinimide and excess polymer by ultrafiltration (Amicon PM 10) using 0.1*M* phosphate buffer, pH 7.0, and then lyophilized. The degree of protein modification was evaluated by colorimetric trinitrobenzenesulfonate assay (TNBS) (*17*), or by determination of norleucine or lysine content after hydrolysis and amino acid analysis. Enzyme concentration was measured either spectrophotometrically (ϵ_{280} 1% = 24.9) (*1*) or by biuret method (*16*).

Ortodiphenol:O₂ Oxidoreductase Activity Evaluation

The ability of the enzyme to catalyze dopa oxidation was measured according to the method of Fling et al. (*18*). In the standard assay, total

volume was 1 mL, and the enzyme was 5×10^{-3} mg/mL in $0.1M$, pH 7.0, phosphate buffer. Dopa was $5 \times 10^{-3}M$ and the reaction was followed spectrophotometrically by measuring dopachrome formation at 475 nm. The reaction rate was calculated considering that 1 mol of dopachrome is formed for every 2 mol of dopa oxidized and that $\Delta\epsilon_{475 \text{ nm}}$ for the dopa–dopachrome conversion is 3600 (*1*). For V_{max} and K_m determination, the same amount of enzyme as in the standard assay was used, whereas the substrate concentration was between $1.25 \times 10^{-3}M$ and $1.25 \times 10^{-4}M$. The assays were carried out at different temperatures in a thermostated spectrophotometer in order to evaluate the effect of the temperature on the K_m value.

Thermostability

Solutions of the native enzyme or the different polymer adducts (0.2 mg/mL in $0.1M$, pH 7.0 phosphate buffer) were sealed in glass vials, and the dopa oxidase activity was assayed after 15 min of incubation at the desired temperature. The difference in thermal stability among the different enzyme forms could also be evaluated by incubating the enzyme (0.2 mg/mL in $0.1M$, pH 7.0, phosphate buffer) at 55°C and plotting the decrease in activity vs time.

Enzyme Inactivation by Dopa Oxidation Products

Four hundred microliters of the enzyme solution (0.2 mg/mL in phosphate buffer) were added to an equal volume of dopa solution ($1.25 \times 10^{-2}M$ in the same buffer), and 50-μL aliquots of this mixture were assayed for dopa oxidase activity at scheduled times.

Arrhenius Plots and Evaluation of the Reaction Activation ΔH

The initial dopa oxidation rate was measured at selected temperatures (between 18.5 and 45°C) in thermostated cuvets of a UV-Vis spectrophotometer. The ln of the reaction rate was plotted vs 1/T, and ΔH was calculated using the Arrhenius equation and suggestions of Dixon and Webb (*19*).

Solubility in Organic Solvent

The desired organic solvent (1 mL of chloroform or tetrahydrofuran) was added to aliquots of the lyophilized enzyme powder corresponding to 3 mg of protein (either native or polymer adducts), and the mixture was vigorously mixed for 10 min. The undissolved fraction was removed by centrifugation, and an exact volume of the clear supernatant was dried. The protein content of the residue was then evaluated by biuret assay after being redissolved in aqueous medium.

Monophenol-Monooxigenase Activity in Organic Solvent

Aliquots of the lyophilized powder corresponding to 1 mg of enzyme were added to 1 mL of a *p*-cresol solution $2.5 \times 10^{-2}M$ in $CHCl_3$ or THF.

The reaction was monitored spectrophotometrically by following the appearance of the 4-methyl-*o*-quinone at 495 nm.

Circular Dichroism Spectra

The circular dichroism spectra of native tyrosinase in aqueous medium (0.1 mg/mL in 0.01M phosphate, 0.15M NaCl, pH 7.2) and of the PEG1 and PAcM adducts in CHCl$_3$ were recorded from 190 to 320 nm. The enzyme solutions in organic solvent were obtained, as in the solubility experiments, by suspending the lyophilized enzyme powder in the solvent and removing the undissolved fraction by centrifugation.

Pharmacokinetics Experiments

Native tyrosinase (2 mg) or the polymer conjugate corresponding to 2 mg of native enzyme were treated with 10 μL of ^{3}H-NSP in borate buffer, 0.2M, pH 8.0. The excess ^{3}H-NSP was removed by ultrafiltration, and 1000 μL of the samples concentrated up to 1 mg/mL were injected into the tail vein of Wistar rats (2 mo old, weight about 250 g). The amount of radiolabeled enzyme in the blood strain was evaluated from blood samples withdrawn from the animal heart under ether anesthesia. Radioactivity was measured on the serum obtained after blood ultracentrifugation (12,000 rpm, 2 min). t/2α and t/2β for the absorption and elimination phases were obtained from biexponential curves. Best fittings were obtained using "SIGMA plot" software.

RESULTS AND DISCUSSION

Figure 1 reports the structure of the three polymers used for tyrosinase conjugation: monomethoxypoly(ethylene glycol), single chain with norleucine as spacer arm (PEG1), "tween PEG" with lysine as spacer arm (PEG2), and poly(acryloylmorfoline),—COOH terminating single chain (PAcM). The chemistry of coupling, which is the same for the three polymers, is also reported.

Effects of Polymer Conjugation on Enzymatic Properties

1. Table 1 shows the degree of lysine modification, V_{max}, K_m, and V_{max}/K_m values for dopa oxidation performed at 30°C. Although different polymer structures were involved, a similar extent of modification in the three different polymer conjugates was obtained using the same molar ratio of activated polymer to enzyme amino groups (3:1). The kinetic parameters among the different conjugates are also comparable.

   Data of Table 1 indicate that polymer modification with linear PEG1 and PAcM slightly increases enzyme activity, mainly through a V_{max} change since the K_m value is only slightly

Table 1

Degree of Lysine Modification, V_{max}, K_m, and V_{max}/K_m Values Evaluated at 30°C for Native Tyrosinase and the Three Different Polymer Conjugates

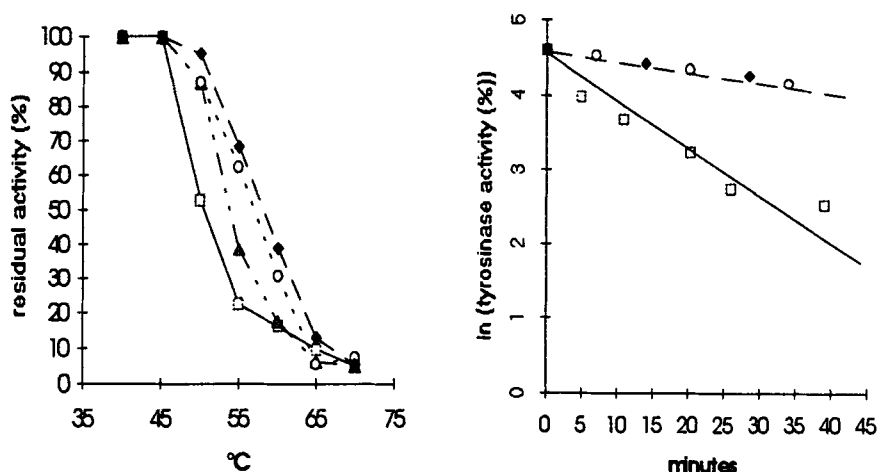| Compound	Degree of lysine modification	K_m, M^{-1}	V_{max}, min^{-1}	V_{max}/K_m
Native tyrosinase		5.72×10^{-4}	1211	212
PEG1-tyrosinase	67.6%	5.30×10^{-4}	1480	279
PEG2-tyrosinase	66.8%	5.68×10^{-4}	1144	201
PAcM-tyrosinase	66.3%	6.40×10^{-4}	1798	281

Fig. 2. Thermostability of native and polymer-conjugate tyrosinases. □ Native tyrosinase, Δ PEG1-tyrosinase, ○ PEG2-tyrosinase, ◆ PAcM-tyrosinase, (A) residual activity after 15 min of incubation at different temperatures; (B) inactivation rate at 55°C incubation.

affected. This phenomenon, which has been reported by other authors with other enzymes following surface modification *(20,21)* or addition of small amounts of organic solvents *(22,23)*, is apparently related to formation of a more flexible conformation of the conjugate. The overall conformation of the protein after polymer linking remained unchanged, as assessed by CD evaluation in the far UV.

2. The polymer–tyrosinase conjugates present increased thermal stability as shown in Fig. 2, where the residual activity after 15 min of incubation is plotted vs the incubation temperature. The temperature at which 50% inactivation is achieved was calculated from the curves, and is 50.5°C for native tyrosinase and 53.5, 57.5, and 60°C for the PEG1, PEG2, and PAcM adducts,
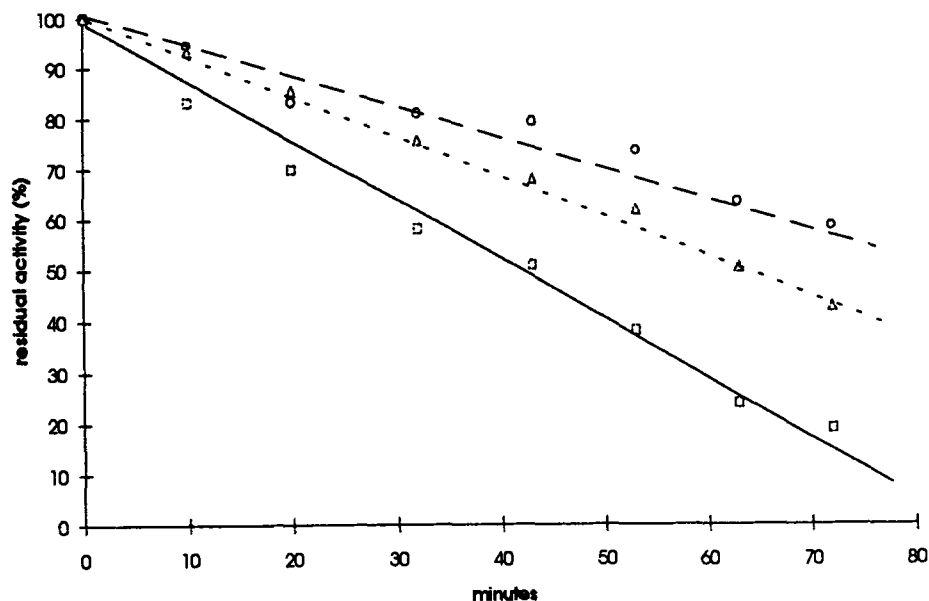
Fig. 3. Enzyme inactivation by orthodiphenol oxidation products. □ Native tyrosinase, △ PEG1-tyrosinase, ○ PEG2-tyrosinase.

respectively. The increase in thermal stability is greatest for the PEG2 and PAcM adducts, as is also evident from Fig. 2B, where the rate of inactivation at 55°C for the native enzyme and that of the last two conjugates are compared. Inactivation follows first-order kinetics with approximate inactivation half-lives of 10.5 min for native and PEG1-modified enzymes and of 63 min for the PEG2 and PAcM adducts.

3. The ortodiphenol oxidation products are known to inactivate tyrosinase irreversibly (24). Polymer modification increases the stability toward such inactivation as shown in Fig. 3. The half-life for inactivation, calculated from the curves in Fig. 3, was 43 min for the native enzyme, and 63 and 85 min for the PEG1 and PAcM adducts, respectively. The inactivation seems to be, as suggested by Wood and Ingraham (24), owing to a covalent reaction between the oxidation products and the enzyme, and probably involves a basic residue on the enzyme active site. These authors also suggest that the newly formed quinone may, before leaving the active center, undergo Michael reaction with a free amino group on the enzyme, namely a lysine. If this is the case, the data in Fig. 3 indicate that the lysine involved in this inactivation is not reached by the polymer during modification, because total inactivation is achieved also for the modified enzymes. The reduction in the inactivation rate seems to indicate that the polymer might act by sterically

Table 2
ΔH and "Break Point" Temperature (bp) for the Dopa Oxidation Activation
Process Calculated for Native Tyrosinase and the PEG1 and PAcM Conjugates

Compound	bp, °C	ΔH below bp, cal	ΔH over bp, cal
Native tyrosinase	27.8	15,100	8560
PEG1-tyrosinase	31	12,600	8120
PAcM-tyrosinase	31.5	13,150	7330

"hiding" such a lysine from the quinone. This is expected, since the hydrated polymer cloud is known to hinder the approach of small substrates, proteases, and antibodies to PEG-modified enzymes (25). Another explanation is that the reaction turnover for the modified enzymes is higher than for the native one as assessed by the V_{max} increase. The quinone would therefore remain for a shorter time near the active center, and the inactivation reaction would be slowed.

4. The temperature dependence of the reaction rate for native tyrosinase was studied, and Arrhenius plots derived from the same data can be approximated by two straight lines having different slopes and intersecting at a "break point." Each part of the broken line corresponds to a different ΔH for the reaction activation process. This phenomenon is common for enzyme-catalyzed reactions, and different explanations have been suggested by several authors (19). The activity/temperature dependence was also obtained for the PEG1 and PAcM conjugates and, in both cases, a break point in the Arrhenius plot was found. The temperature at which the break point occurs is 27.5°C for the native enzyme and 31.0 and 31.5°C for the PEG1 and PAcM adducts, respectively. This study could be conducted because the K_m value is not affected by the temperature, as previously verified following Dixon and Webb's suggestions (19). The ΔH for the activation process before and after the break point was calculated for each compound, (Table 2). There seems to be a correlation between the "break" temperature and the thermal stability of the different compounds, even though the enzyme is stable at the temperature at which such "break" occurs. The ΔH values shown in Table 2 show that the enthalpy value below the break point is nearly twice that of the one over this temperature. This phenomenon occurs both for the native protein and for the modified forms. On the other hand, polymer modification does not substantially affect the ΔH values, and we can therefore conclude that the polymer molecules do not interfere with the activation process.

Table 3
Pharmacokinetic Parameters for Native Tyrosinase
and the Polymer Conjugates After iv Injection in Wistar Rats

Compound	$t/2\alpha$, Distribution, min	$t/2\beta$, Elimination, min
Native tyrosinase	28	722
PEG1-tyrosinase	6.5	513
PAcM-tyrosinase	8.5	475
PEG2-tyrosinase	36	1576

Pharmacokinetic Behavior

The pharmacokinetic profiles for native tyrosinase and the three polymer conjugates after iv injection in Wistar rats were analyzed using "SIGMA plot" curve fittings. $t/_2\alpha$ and $t/_2\beta$ calculated from biexponential curves assuming a biocompartimental system are shown in Table 3. Polymer modification was found to affect the enzyme's pharmacokinetic parameters, even though modification is not dramatic. Of interest is the fact that no significant difference is observed between the PEG1 and the PAcM conjugates either in the distribution or in the elimination phase. On the other hand, the pharmacokinetic profiles are different from those observed for native tyrosinase and the PEG2 conjugate. Distribution occurs faster for the PEG1 and PAcM conjugates when compared to the native enzyme, $t/_2\alpha$ being 6.5 and 8.5 min for the two polymer conjugates and 28 min for the native form. On the contrary, PEG2 slows down the distribution, $t/2\alpha$ for such polymer adduct being 36 min. Similar behavior is observed with the elimination parameters: $t/2\beta$ for PEG1 and PAcM conjugates being lower when compared to the native enzyme, whereas the PEG2 modification increases the body residence. Therefore, only the modification with PEG2 increases tyrosinase's residence in the bloodstream, both by reducing its distribution and slowing its elimination. This could be of interest in view of therapeutic applications. Considering the high molecular weight of native tyrosinase itself (ca. 128,000), and the renal ultrafiltration cutoff of approx 66 kDa, we can presume that elimination is probably owing to another mechanism. This would also explain why the increase in the molecular weight resulting from polymer conjugation is not increasing the body residence for the PEG1 and PAcM conjugates. Proteolytic digestion or specific receptors may be involved. Further studies on the enzyme stability toward proteolytic digestion (data not reported) have been carried out, and demonstrated high resistance for both the native form and the polymer-modified ones excluding a direct digestion mechanism in the elimination. The positive result obtained with the PEG2 conjugate may probably be related to an increased masking of some enzyme critical sites that are apparently important for recognition by the elimination machinery. Further investigations should be carried out to understand better the mechanism involved in the clearance.

Table 4
Solubility of Native Tyrosinase
and Different Polymer Adducts in Organic Solvents

Compound	Solubility in CHCl$_3$, mg/mL	Solubility in THF, mg/mL
Native tyrosinase	4.6×10^{-3}	Undetectable
PEG1-tyrosinase	1.015 ± 0.05	4.93×10^{-2}
PAcM-tyrosinase	0.623 ± 0.1	—
PEG2-tyrosinase	0.603 ± 0.1	3.73×10^{-2}

Structural and Enzymatic Studies in Organic Solvents

Since tyrosinase is able to catalyze the regioselective hydroxylation of phenols other than its natural substrate, tyrosine, its monophenol-hydroxylase activity could be a very useful tool for the organic chemist. A general limitation in the use of enzymes in organic chemistry is their lack of solubility in organic solvents. However, the lack of solubility can be circumvented by the use of cosolvents (DMSO or other water-miscible solvents), by carrying out the reaction in a two-phase environment, by solubilizing the enzyme in surfactant aggregates (26,27), or by adsorbing the undissolved enzyme on different polymeric matrices (6,28). Another possibility is to attach amphiphic polymers covalently to the enzyme surface to increase its solubility in the organic solvent without losing the catalytic activity. PEG has often been successfully used for this purpose (8–10,29), but also other polymers might be interesting: for example, PAcM has been recently used in our laboratory to solubilize the enzyme lipase for biocatalysis purposes (30). The solubility in CHCl$_3$ of tyrosinase modified with our polymers increased dramatically as shown in Table 4. Linear PEG was slightly more effective in this respect, but no significant difference was observed among the three different polymer adducts we studied. Similar data were obtained in our laboratory with the enzyme superoxide dismutase modified with the same polymers (unpublished results).

Unfortunately, our tyrosinase conjugates showed no activity for the phenolic substrates we tested, neither in CHCl$_3$ nor in the more hydrophilic THF. The lack of activity after organic solvent solubilization is probably owing to irreversible denaturation of the enzyme. In fact, activity could not be recovered even after the organic solvent was removed and the protein was redissolved in buffer. On the other hand, inactivation did not occur when the native enzyme was suspended in the organic media since substrate conversion did occur, although at a very slow rate. The data obtained suggest that an irreversible conformational change takes place in the protein structure after solubilization. This was also confirmed by the circular dichroism spectra at low wavelength of the polymer conjugates performed in CHCl$_3$ and shown in Fig. 4. A dramatic change in protein secondary structure is evident when these spectra are compared
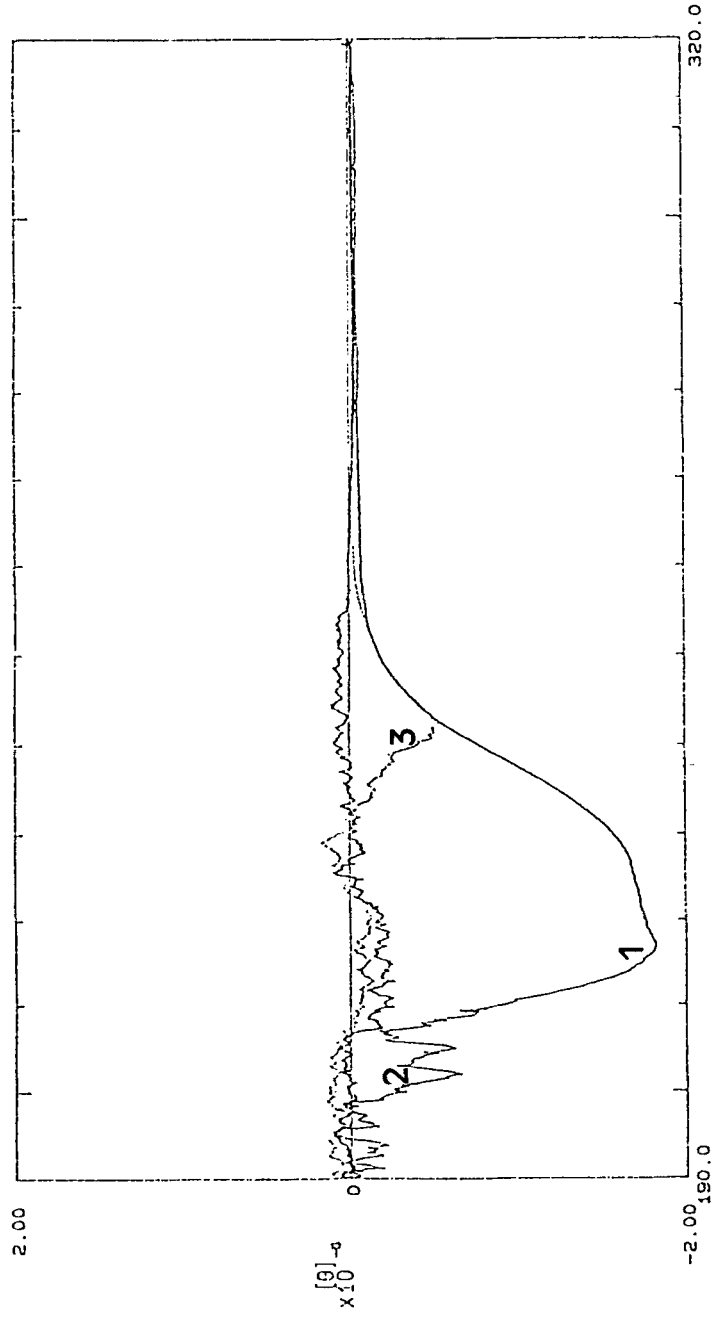
Fig. 4. Circular dichroism spectra of native tyrosinase (1) in aqueous solution (0.2 mg/mL in 0.1M rosfate buffer, pH 7), (2) and of the PEG1 (2) and PAcM (3) cojugates in CHCl$_3$.

to the one of the native enzyme (Fig. 4) or to those of the polymer conjugates in aqueous buffer. These results demonstrate that the use of amphiphic polymers to improve enzyme application in organic solvent biocatalysis cannot be considered a general procedure.

CONCLUSIONS

In this work, three different polymers (PEG1, PEG2, and PAcM) were covalently bound to the enzyme tyrosinase in order to evaluate potential use of the different conjugates either for improvement of pharmacological applications or in biocatalysis. Enzyme activity in aqueous medium was not substantially affected by the polymer coating, whereas enzyme stability was increased as demonstrated by the higher resistance of the polymer conjugates to heat and to inactivation by dopa oxidation products.

Polymer modification affects the pharmacokinetic profile of tyrosinase, each polymer influencing the distribution and elimination parameters in a different way. PEG1 and PAcM slightly decrease the enzyme's body residence, whereas PEG2 acts in the opposite direction by reducing both the distribution and the elimination. In this way, PEG2-tyrosinase remains in the bloodstream for a longer time, and this, together with the already well-known advantages of polymer modification (low immunogenicity and antigenicity and higher stability), is of interest for its application as a drug.

The solubility of the polymer-modified enzymes in organic solvents increased dramatically. Unfortunately, this solubilization brought about total enzyme inactivation that is related to loss of protein conformation. This demonstrates that the known strategy of coating an enzyme with amphiphic polymers to improve its use in biocatalysis in organic solvents cannot be extended to tyrosinase. On the other hand, the increased conformational stability shown by the polymer conjugates in aqueous media could be useful in biocatalysis of water-soluble substrates.

ACKNOWLEDGMENTS

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