Enzymatic polymerization of amphiphilic alkyl tyrosine derivatives from emulsions

Rupmoni Sarma a, K. Shridhara Alva b, Kenneth A. Marx a, Sukant K. Tripathy a, Joseph A. Akkara c, David L. Kaplan c

a Department of Chemistry, Centers for Advanced Materials and Intelligent Biomaterials, University of Massachusetts, Lowell, MA 01854, USA
b Department of Physics, Centers for Advanced Materials and Intelligent Biomaterials, University of Massachusetts, Lowell, MA 01854, USA
c Biotechnology Division, US Army Natick Research, Development and Engineering Center, Natick, MA 01760, USA

Abstract

Horseradish peroxidase catalyzed polymerizations of amphiphilic derivatives of d- and l-isomers of tyrosine have been carried out in micellar solutions. The rate of polymerization has been found to be maximum at a pH of 6.1–6.2 and showed a second-order dependence on the monomer concentration. This enzyme shows stereospecificity towards the d-isomer compared to the l-isomer, which is consistent with the aqueous reaction with underivatized isomers of tyrosine. The resulting polymers, having a molecular weight of 2700, have been characterized by UV-Vis spectroscopy and 1H NMR. The polymer shows surface activity at the air-water interface unlike the corresponding monomer, which aggregates under identical conditions.

Keywords: Micelles; Polyphenol; Horseradish peroxidase; Enzymatic polymerization

1. Introduction

Phenolic based polymers have been the focus of interest for industrial applications. Horseradish peroxidase (HRP) catalyzed polymerization of phenols has been studied in monophasic aqueous or organic solvents as well as in reverse micelles [1–5]. These methods result in an insoluble cross-linked polymer. Cross-linked polymers, due to their insoluble nature, are difficult to process for industrial applications. Polymers of alkyl derivatives of phenols are easily processable, but they pose problems of common solvent for the enzyme catalyzed reactions. Though the reverse micellar technique is useful in the polymerization of alkyl derivatives of the monomers, in the case of long alkyl chain derivatives, the monomer becomes soluble in the bulk organic solvent instead of staying at the oil–water interface. Polymerization of these long alkyl chain derivatives could be accomplished by forming micelles in aqueous medium. In micelles, the polar phenolic moieties are exposed towards the bulk aqueous phase, in which the enzyme is dissolved, while the hydrophobic chain forms the micellar core.

Amphiphilic molecules, bearing distinct hydrophobic and hydrophilic segments aggregate in aqueous media, forming various molecular organizations such as micelles, vesicles and membranes. Amphiphiles having one long aliphatic chain form micelles when their concentration exceeds a critical value known as the critical micellar concentration [6]. Micelles are in a state of dynamic equilibrium with non-aggregated amphiphiles in solution [6–8]. Therefore, one of the incentives to study polymerization of monomers in micelles was to chemically fix these loose monomeric micellar structures.

This paper presents a novel method for the polymerization of long chain derivatives of phenols. Compounds studied here are the decyl ester d-(DEDT) and l-(DELT) isomers of tyrosine whose structures are shown in Fig. 1. The HRP catalyzed polymerization of l- and d-isomers of tyrosine is known to exhibit limited stereospecificity, with the reaction of the d-isomer about twice as fast as the l-isomer [9]. Here, we also report on the stereospecificity of the enzyme catalyzed polymerization reaction towards the isomers of the long chain derivatives of tyrosine.

2. Experimental procedures

Horseradish peroxidase, (EC 1.11.1.7) was purchased from the Sigma Chemical Company, St Louis, MO. 30%
Hydrogen peroxide was purchased from Aldrich Chemical Company, Milwaukee, WI. The tyrosine derivatives were prepared and supplied by Prof. Braja Mandal at the Illinois Institute of Technology, Chicago, IL. All other chemicals used here were at least of analytical grades and were utilized as received.

The monomer (d- and l-isomers of tyrosine decyl esters) was taken up in water. A few drops of HCl were then added to lower the pH to about 3, where the amino group of the tyrosine derivatives are completely protonated, enabling the solubility of the monomer in aqueous medium through micellar formation. The pH conditions were controlled so that there is no ester hydrolysis. The pH was then adjusted to the desired value, by adding drops of 1 M solution of dibasic phosphate. The enzyme HRP was then added, followed by a 3% solution of H$_2$O$_2$ to initiate the polymerization. All kinetic parameters were measured by UV-Vis spectroscopy. The reaction was followed by measuring the increase in the absorbance at 290 nm, during the course of the reaction, using a Perkin-Elmer Lambda-9 UV-Vis near IR spectrophotometer. The initial slope of the absorbance verses time curve was taken as the measure of the rate of the polymerization reaction.

The polymer was isolated by evaporating the water in a roto-vapor. Chloroform was then added to extract the polymer. Gel permeation chromatographic (GPC) analysis was done on a GBR mixed bed linear column, Jordi Associates, Inc., Bellingham, MA. A UV detector at 270 nm was used to follow the elution. The eluent was DMF containing 1% lithium bromide. The flow rate was kept at 1 ml min$^{-1}$. $^1$H NMR experiments were done using a Bruker 250 MHz NMR spectrometer.

Surface properties of the monomer and the polymer were studied using a Lauda film balance with a trough (obtained from GMBH and Co., KG, Lauda-Königshofen, Germany). The monomer and the polymer were dissolved in chloroform (approximately 1 mg in 1 ml of the solvent) and then spread onto the aqueous subphase at 20 °C. The surfactant was compressed at a constant rate and the pressure–area isotherm was recorded.

3. Results and discussion

The enzymatic polymerization of amphiphilic tyrosine derivatives DEDT and DELT were followed spectroscopically. Fig. 2 presents the absorption spectra recorded during the enzymatic polymerization of DEDT at a pH of 6.0. The spectra were recorded at 2 min time intervals. [HRP] = 10 units, [DEDT] = 0.31 mM, [H$_2$O$_2$] = 10 mM.

Fig. 3. The absorption spectra of (a) DEDT, (b) DEDT treated with hydrogen peroxide, (c) DEDT treated with horseradish peroxidase and (d) DEDT treated with horseradish peroxidase and hydrogen peroxide at pH 6.0. [HRP] = 10 units, [Monomer] = 0.31 mM, [H$_2$O$_2$] = 10 mM.

In order to establish that the change in absorbance is due to enzymatic polymerization, a series of control experiments were done. The micellar solution of DEDT was exposed to various experimental conditions and then extracted to chloroform. Fig. 3 depicts the absorption spectra of (a) DEDT, (b) DEDT treated with hydrogen peroxide, (c) DEDT treated with horseradish peroxidase and (d) DEDT treated with horseradish peroxidase and hydrogen peroxide. It is evident from the figure that only curve (d) shows the extended absorption into the visible region, characteristic of the extended backbone chain conjugation resulting from the enzymatic polymerization of DEDT.

Both micellar formation and the enzyme kinetics are influenced by the solution pH. The solution pH was varied between 5.5 and 6.5 and the rate of change of absorbance was
measured for both DEDT and DELT. Fig. 4 depicts the rate of change of absorbance as a function of solution pH for DEDT and DELT respectively. It can be observed from the figure that both systems show an optimum pH of 6.1 to 6.2 for the reaction. Increased turbidity limited the spectroscopic investigation at pH values over 6.5.

Previous researchers have reported that HRP shows stereospecificity with the oxidative coupling reaction rate of d-tyrosine being twice as fast as that of L-tyrosine [9]. Fig. 5 summarizes a similar observation for the enzymatic polymerization of the two isomers of tyrosine in the micellar state. It can be observed that the enzyme shows specificity towards the d-isomer over the L-isomer. It is interesting to note that the reaction rate does not follow the normal Michaelis-Menten behavior with varying substrate concentration. The curves follow a second-order substrate concentration dependence with regression coefficients of 0.998 and 0.994 for d-tyrosine and L-tyrosine derivatives respectively. This suggests that the oxidative free radical coupling reaction involves a second-order rate determining step following the enzymatic generation of free radicals. Moreover, the size and shape of the micelles are affected by the monomer concentration, which in turn may also contribute to the observed second-order relation [10–12]. However, we do not presently have any experimental evidence to support this assertion.

Though the enzyme reaction was very rapid from a kinetic point of view, for complete conversion of the substrate, the reaction was allowed to continue for an extended period of time. The reaction product was then isolated and used for further characterization. Fig. 6 plots the absorption spectra of DEDT polymer, where the monomer concentration was varied for fixed concentration of the enzyme and hydrogen peroxide. It will be noted from the figure that the absorption band becomes broader with increasing monomer concentration. This indicates that the conjugation length of the resulting polymer depends on the monomer concentration. In a separate set of experiments, it was observed that the conjugation length also depends on the reaction temperature. The polymers obtained at 30 °C show an extended conjugation as compared to that obtained at 20 °C or 40 °C.

GPC analysis indicated that the polymer resulting from DEDT has an average molecular weight of the order of 2700 daltons. The polydispersity of 1.5 confirms that the polymerization reaction involved free radical initiation [13]. The free radical initiation of the polymerization reaction can lead to the complexity of the polymer structure. Further, GPC analysis indicated that the monomer conversion was about 90%. From 1H NMR studies of the monomer and the polymer of DEDT (data not shown), we observe that the line widths of the polymer are larger as compared to that of the monomer. The aromatic proton signals of the polymer show that the concentrations of both ortho and meta protons have been decreased equally during the reaction. This indicates that both the ortho and meta positions of the phenolic moiety are involved in the polymerization.

For industrial applications, one needs to obtain a thin film of the polymer with controlled thickness. In a preliminary study, we have attempted to form monolayers of the polymer and the corresponding monomer at the air–water interface. Fig. 7 summarizes the isotherms of both the monomer and the polymer of DEDT. It can be observed that the monomer does not show any appreciable surface activity. The molecules aggregate at the air–water interface, which on continued compression shows increased surface pressure. The observed molecular area of 4 Å² does not correspond to the monomer molecular dimensions, where one expects a molecular area of at least 20 Å² due to the alkyl chain. This may be due to the fact that the monomer has an alkyl chain of 10 carbon
atoms, which does not provide a balance of hydrophobic and hydrophilic groups in the amphiphilic molecule, a feature that is essential to form a monolayer. On the other hand, the polymer shows a well defined isotherm, with a molecular area for the repeat unit of 30 \( \text{Å}^2 \). The hydrophobic groups of polymers have a great influence on their monolayer properties. However, previous studies have indicated that polymers with quite short tail groups can form stable monolayers [14]. The effective contribution of the hydrophilicity of the tyrosine moiety is lowered due to polymerization, which in turn provides a balance of hydrophobic and hydrophilic properties to the polymer.

4. Conclusions

Horseradish peroxidase can catalyze the oxidative polymerization of amphiphilic derivatives of both the \( d \)- and \( l \)-isomers of tyrosine in micellar solution. The rate of the reaction is maximum at a pH 6.1 to 6.2 for both the isomers. The enzyme demonstrates stereospecificity, catalyzing the reaction of the \( d \)-isomer faster than the \( l \)-isomer. \(^1\)H NMR indicates that the polymers are formed by substitution equally at both ortho and meta positions of the benzene ring. The polymer of the \( d \)-derivative has a molecular weight of 2700. The polymer, further, shows surface activity at the air–water interface of a Langmuir trough unlike the corresponding monomer, which aggregates under identical conditions.

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