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Biochemical and Biophysical Research Communications 313 (2004) 17–21
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First synthesis of a polysaccharide-supported lignin model compound and study of its oxidation promoted by lignin peroxidase

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Received 13 November 2003

Abstract

Veratrylchitosan, a polysaccharide-supported lignin model compound, has been synthesised by covalently attaching 3-(3,4-dimethoxybenzyloxy)propionic acid to the polysaccharide chitosan through an amide linkage. When this polymer was used as a substrate in the oxidation promoted by lignin peroxidase (LiP), significant decomposition of the lignin model resulted in the formation of veratraldehyde. The oxidation mechanism involves an initial transfer of one electron from chitosan to the active species of LiP (LiP I) followed by C_α–H deprotonation of an aromatic cation radical. A benzylic radical is then formed which is further oxidised to a benzyl cation. Reaction with water and hydrolysis of the hemiacetal then lead to veratraldehyde formation. An increase in the yields of the oxidation product is observed in the presence of the mediator 2-chloro-1,4-dimethoxybenzene, thus indicating that a more efficient degradation results from the transfer of an electron from the polymer to the radical cation of the mediator.

Keywords: Chitosan; Electron transfer; Lignin model compound; Lignin peroxidase; Mediation

Lignin is a highly irregular three-dimensional biopolymer composed of oxygenated phenylpropane units linked together by different bonds [1]. It is synthesised by plants mainly to provide strength, rigidity, and protection from oxidative processes and from attacks by microorganisms [1,2]. The oxidative degradation of lignin is a process of fundamental importance not only because it can convert lignin into low molecular weight aromatic compounds useful for the industrial preparation of a number of chemicals [3], but also because the selective degradation of lignin and its removal from the carbohydrate component of wood is a key process in the pulp and paper industry [4]. In view of the complexity of the structure of lignin and of the difficulty of studying its degradation products, lignin model compounds (LMCs) are frequently used to mimic the structure and reactions of native lignin. Recently, trimeric, tetrameric, and oligomeric LMCs have been synthesised in order to get more complex and heavy structures [5,6]. However, these models cannot mimic some of the properties of lignin associated to its polymeric character. Thus, in the past years several polymer-supported LMCs have been synthesised by attaching phenolic LMCs to polystyrene via benzyl ether type linkages [7,8] or via a trityl ether linkage [9], or by end-capping polyethylene glycol through an ether linkage [10]. LMCs containing aldehydic groups have been also attached to chloromethylated polystyrene by the use of the Wittig reaction [11]. However, to our knowledge, no polymers have been synthesised with the lignin fragments grafted onto a polysaccharide backbone, a structure which better mimics the interlinkages of the wood components. Along this line, we have synthesised a water soluble polymeric structure, veratrylchitosan, where a very simple lignin model, the carboxylic ether derivative of veratryl alcohol **1**, has been grafted to chitosan through an amide linkage (Scheme 1) and briefly studied its reactivity towards lignin peroxidase.

Lignin peroxidase (LiP, EC 1.11.1.7) is a ferric hemoprotein isolated from the ligninolytic cultures of the white-rot basidiomycetous *Phanerochaete chrysosporium* [12,13] which has been shown to catalyse the oxidative depolymerisation of lignin [14–16]. The active species of

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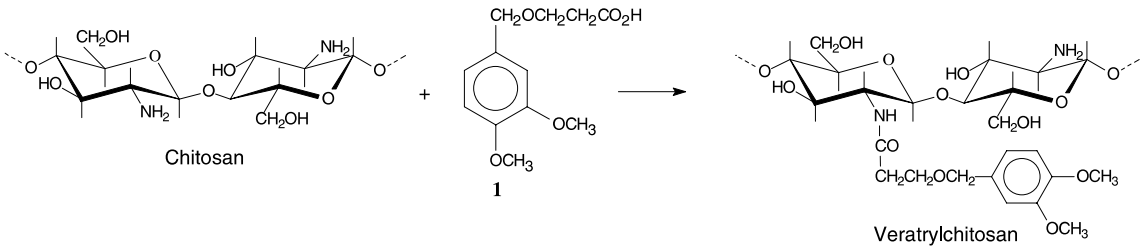
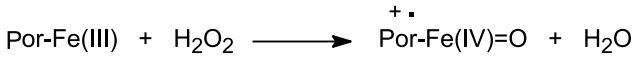
doi:10.1016/j.bbrc.2003.11.083

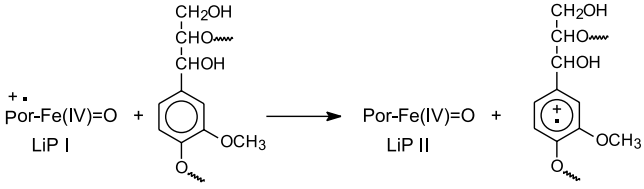


Scheme 1. Synthesis of veratrylchitosan.

the enzyme, LiP compound I (LiP I), is formed by oxidation of the native enzyme by H_2O_2 (Eq. (1)). It is believed that LiP I is able to oxidise the lignin polymer via the transfer of one electron from an aromatic ring with the formation of an aromatic radical cation and the reduced form of LiP compound II (LiP II) (Eq. (2)) [14,15,17].



(1)



(2)

Native lignin in wood is inaccessible for large enzymes such as LiP, therefore, the hypothesis has been made that small molecules (mediators) may diffuse in the access channel and form an electron shuttle allowing lignin oxidation [15]. In this study in order to compare the results in the absence and in the presence of mediators the LiP promoted oxidation of veratrylchitosan was also carried out in the presence of 2-chloro-1,4-dimethoxybenzene.

Materials and methods

Materials. All the reagents and solvents were of the highest purity available and used without further purification. The concentration of H_2O_2 was determined by titration with permanganate [18]. LiP was prepared and purified as described in the literature [19]. The concentration of the enzyme solution was determined spectrophotometrically ($\varepsilon_{409\,nm} = 169\,mM^{-1}\,cm^{-1}$) [20]. VA methyl ether was synthesised according to the literature [21].

Commercial sample of chitosan, low molecular weight $M_r \sim 150{,}000$, ca. 10% acetylated, was supplied by Fluka and used as received. The veratryl ether derivative **1**: 3-(3,4-dimethoxybenzyloxy)propionic acid, was synthesised by reaction of 3,4-dimethoxybenzylalcohol with β-propiolactone at 150 °C for 24 h [22]. The compound was identified by ^{1}H NMR analysis ($CDCl_3$): δ 6.89–6.80 (m, 3H, ArH); δ 4.49 (s, 2H, $ArCH_2O$); δ 3.88 (s, 3H, OCH_3); δ 3.87 (s, 3H, OCH_3); δ 3.74 (t, 2H, $OCH_2CH_2CO_2H$); and δ 2.65 (t, 2H, $OCH_2CH_2CO_2H$).

Veratrylchitosan was prepared by coupling of the veratryl ether derivative [23] by dissolving 100 mg of chitosan (low molecular weight M_r 150,000, Fluka, 10% acetylated), 80 mg of **1**, and 44 mg of the condensing agent 1-hydroxybenzotriazole (HBT) in 20 ml of morpholinoethanesulfonate buffer 0.05 M, pH 5.5, for 90 min under magnetic stirring. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was then added. After 24 h at room temperature the mixture was dialysed against distilled water until the conductivity of the external bath was <3 μS/cm. The polymer solution was then lyophilised and thorough removal of any unreacted **1** was achieved by repeatedly washing the polymer with MeOH:H_2O 7:3 until no significant amount of **1** was detected by HPLC in the washing solution. After air-drying product characterisation was performed by ^{1}H NMR in D_2O in the presence of 0.1% deuterated trifluoroacetic acid, using a Bruker 200 MHz NMR: δ 6.99 (ArH); δ 4.48 ($ArCH_2O$); δ 4.0–3.4 (OCH_3, OCH_2CH_2CO, CH chitosan); δ 3.13 (NH_2); δ 2.57 (OCH_2CH_2CO); and δ 2.03 (CH_3CO). The polymer showed 40% of functionalisation of the total amino groups with the veratryl ether derivative.

Methods. ^{1}H NMR spectra were recorded on a Bruker AC200P spectrometer. GC-MS analysis was performed on a HP5890 GC (OV1 capillary column, 12 m × 0.2 mm) coupled with a HP5970 MSD. GC analysis was performed on a Varian 3400GC (OV1 capillary column, 25 m × 0.2 mm).

Product analysis study. As much as 3.7 mg of veratrylchitosan (corresponding to 5.8 μmol of **1**) was first dissolved in 4 mL of 50 mM Ar-saturated sodium tartrate buffer (pH 3.5) containing 5% CH_3CN at 25 °C by magnetic stirring and then LiP (1 U) was added followed by H_2O_2 (12 μmol) which was added gradually in 1 h using an infusion pump. The reaction mixture was extracted with dichloromethane, the organic phase was separated by centrifugation for 5 min and analysed by GC. The only product observed was veratraldehyde (0.23 μmol, 4% referred to the amount of **1** in veratrylchitosan). Negligible amounts of veratraldehyde were observed in the absence of either LiP or H_2O_2. The aqueous phase was concentrated and analysed by ^{1}H NMR. No significant changes in the spectrum of the polymer were observed which might indicate its decomposition under the reaction conditions.

The oxidation in the presence of mediator was carried out under the same experimental conditions described above by adding a solution of 0.32 μmol of 2-chloro-1,4-dimethoxybenzene in CH_3CN (100 μl) to the buffered solution of the substrate. The only product observed was veratraldehyde (0.52 μmol, 9% referred to the amount of **1** in veratrylchitosan). When the reaction was carried out with a lower concentration of veratrylchitosan (1.7 mg corresponding to 2.65 μmol of **1**) and of 2-chloro-1,4-dimethoxybenzene (0.16 μmol), the yield of veratraldehyde increased significantly (0.35 μmol, 13% referred to the amount of **1** in veratrylchitosan).

The oxidation of VA methyl ether was carried out by dissolving the substrate (1 mg, 5.5 μmol) and LiP (1 U) in 4 mL of 50 mM Ar-saturated sodium tartrate buffer (pH 3.5) containing 5% CH_3CN at 25 °C.

H_2O_2 (12 μmol) was then added gradually in 1 h using an infusion pump. The reaction mixture was extracted with dichloromethane and the organic phase was analysed by GC. The only product observed was veratraldehyde (1.2 μmol, 22% referred to the substrate).

Results and discussion

The new polymer-supported lignin model compound veratrylchitosan was prepared by coupling of 3-(3,4-dimethoxybenzyloxy)propionic acid (**1**) with chitosan in a morpholinoethanesulfonate buffer 0.05 M, pH 5.5, in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) as condensing agent and 1-hydroxybenzotriazole (HBT) as additive to prevent the formation of ester linkages [23]. Product characterisation, performed by ^{1}H NMR in D_2O in the presence of 0.1% deuterated trifluoroacetic acid, showed 40% of functionalisation of the total amino groups with the veratryl ether derivative (Fig. 1).

To determine if a direct oxidative degradation of lignin by lignin peroxidase can occur we used veratrylchitosan as substrate for the LiP promoted oxidation. The direct oxidation of lignin by LiP has been questioned since an interaction between the lignin polymer and the heme prosthetic group of the enzyme is generally considered unlikely in view of the restricted access channel from the LiP surface to the heme group [24]. Therefore, the hypothesis has been made that small molecules (mediators), like veratryl alcohol (VA), may diffuse in the access channel and form an electron shuttle allowing lignin oxidation [15,25,26]. The results obtained so far using polymeric substances as substrates for LiP are not clear. For example, the discoloration of the polymeric anthraquinone dye Poly R-478 by LiP is greatly stimulated by the presence of a mediator like VA, 1,4-dimethoxybenzene or 2-chloro-1,4-dimethoxybenzene [27], whereas extensive oxidation of a water soluble PEG-linked model was observed in the absence of mediators [10]. In our study in order to compare the results in the absence and in the presence of mediators the LiP promoted oxidation of veratrylchitosan was also carried out in the presence of 2-chloro-1,4-dimethoxybenzene [28].

The LiP promoted oxidation of veratrylchitosan was carried out, under argon at 25 °C, by gradual addition of 12 μmol of H_2O_2 to a buffered solution (pH 3.5) of veratrylchitosan (3.7 mg) and LiP (1 U). The reaction mixture was then extracted with dichloromethane. The only product observed by GC analysis of the organic phase was veratraldehyde (0.23 μmol, 4% referred to the amount of **1** in veratrylchitosan, Table 1, entry 1). Negligible amounts of veratraldehyde were observed in the absence of either LiP or H_2O_2. The aqueous phase was concentrated and analysed by ^{1}H NMR. No significant changes in the spectrum of the polymer backbone were observed [29].

When the oxidation was carried out, under the same experimental conditions, in the presence of 2-chloro-1,4-dimethoxybenzene (0.32 μmol) as the mediator the yields of veratraldehyde increased significantly (0.52 μmol, 9% referred to the amount of **1** in veratrylchitosan, Table 1, entry 2).

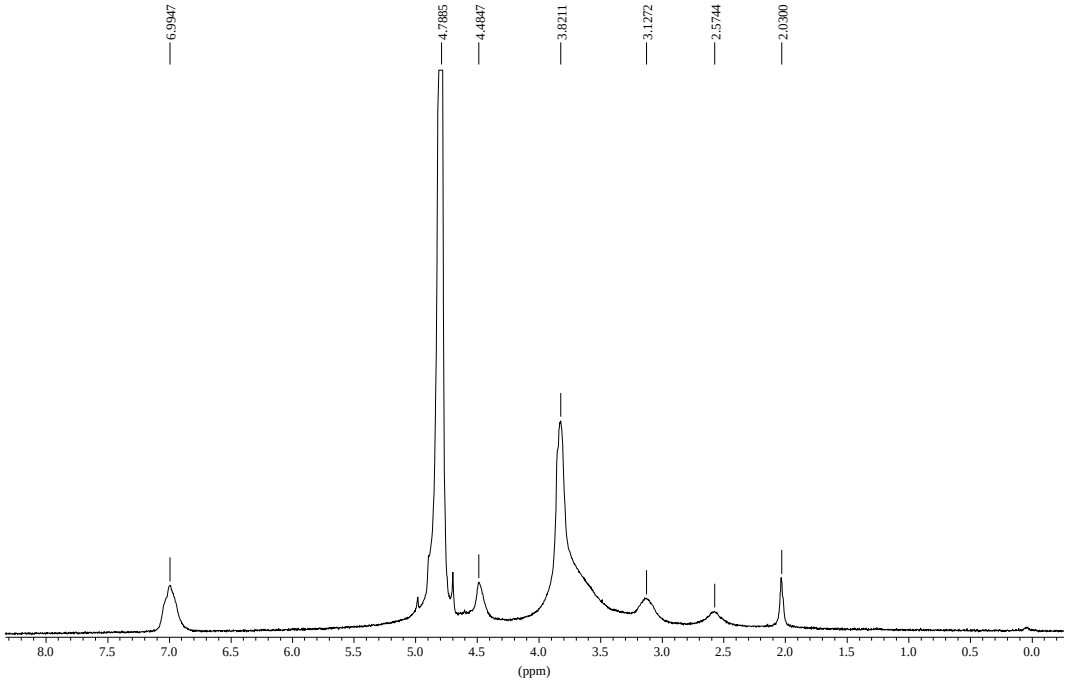


Fig. 1. ^{1}H NMR spectrum (200 MHz) of veratrylchitosan in D_2O in the presence of 0.1% deuterated trifluoroacetic acid.

Table 1
Yields of veratraldehyde in the LiP-catalysed oxidation of veratrylchitosan and VAMe[a]

Entry	Substrate	Mediator (μmol)[b]	Yields[c]
1	Veratrylchitosan	—	4
2	Veratrylchitosan	0.32	9
3[d]	Veratrylchitosan	0.16	13
4	VAMe	—	22

[a] Veratrylchitosan (3.7 mg corresponding to 5.8 μmol of **1**) or VAMe (5.5 μmol) in 4 mL of 50 mM Ar-saturated sodium tartrate buffer (pH 3.5) containing 5% CH_3CN at 25 °C, LiP (1 U), and H_2O_2 (12 μmol).

[b] 2-Chloro-1,4-dimethoxybenzene.

[c] Yields are referred to the amount of **1** in veratrylchitosan or to the amount of substrate in the oxidation of VAMe.

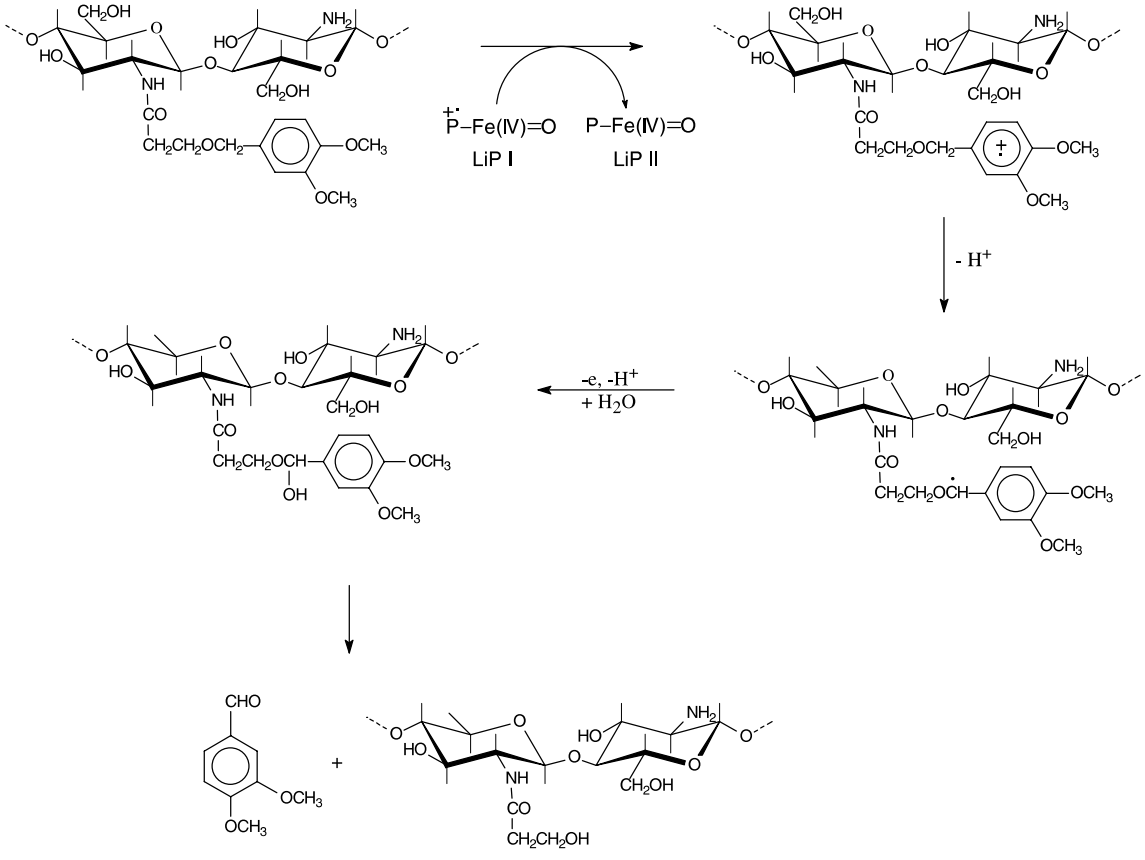
[d] Veratrylchitosan (1.7 mg corresponding to 2.6 μmol of **1**).

When the reaction was carried out with a lower concentration of veratrylchitosan (1.7 mg, corresponding to 2.6 μmol of **1**) and of 2-chloro-1,4-dimethoxybenzene (0.16 μmol), better results and higher yields of veratryl aldehyde were obtained (0.35 μmol, corresponding to 13% referred to the amount of **1** in veratrylchitosan, Table 1, entry 3). This is likely due to the limited solubility of veratrylchitosan in the buffer solution.

As a comparison, in the LiP/H_2O_2 promoted oxidation of the methyl ether of veratryl alcohol (VAMe), carried out in the same experimental conditions, the only product observed was veratraldehyde (1.2 μmol, 22% referred to the substrate, Table 1, entry 4).

The results reported in Table 1 clearly indicate that LiP is able to catalyse the direct oxidation of a bulky substrate like veratrylchitosan. The recent hypothesis of an interaction of lignin with the LiP surface and a "non-heme edge" long range electron transfer site involving a redox-active tryptophan residue Trp 171 [30] is thus supported by our results. Formation of veratraldehyde can be rationalised according to the pathway described in Scheme 2. The oxidation of veratrylchitosan involves the transfer of one electron to LiP I leading to the formation of an aromatic cation radical and the reduced form of the oxocomplex LiP II. Subsequently, the radical cation undergoes C_α–H deprotonation, a typical reaction of alkylaromatic radical cations [31], to form a carbon centred radical which is further oxidised to a benzyl cation. The latter is eventually converted to veratrylaldehyde after reaction with water and hydrolysis of the hemiacetal which leaves the amino groups of chitosan derivatised with a β-hydroxyamide function.

The efficiency of oxidation of the polymer-bound lignin model compound is much lower than that of its free form (compare entries 1 and 4), a result that could be explained by the access to different active sites by the



Scheme 2.

two forms of the LMC (the restricted access channel from the LiP surface to the heme group for VAMe and the LiP surface for veratrylchitosan), or by the higher affinity of the enzyme for the smaller molecule. The significantly higher yields of veratraldehyde in the oxidation of veratrylchitosan in the presence of the mediator 2-chloro-1,4-dimethoxybenzene (compare entries 1 and 2) indicate that a more efficient degradation results from the transfer of an electron from the polymer to the radical cation of the mediator, being it free or bound to the enzyme. In this context, we feel that veratrylchitosan could represent a good polymeric model to test the efficiency of different mediators in the oxidation of lignin promoted by enzymes such as LiP or laccase.

Work in this area is continuing with the attachment of more complex lignin models, like dimeric or trimeric ones, to chitosan.

Acknowledgments

We thank the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR), and the University "La Sapienza" for financial support. We also thank Professor V. Crescenzi for his help with the synthesis of veratrylchitosan.

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