0273-2289/02/98-100/0365/$11.75

Production of Chelating Agents Through the Enzymatic Oxidation of Acetosolv Sugarcane Bagasse Lignin

ADILSON R. GONÇALVES* AND MAURO A. SOTO-OVIEDO

Departamento de Biotecnologia — FAENQUIL, Cx. Postal 116, 12600-000 Lorena-SP, Brazil, E-mail: adilson@debiq.faenquil.br

Abstract

Oxidation of lignin obtained from Acetosolv pulping of sugarcane bagasse was performed by polyphenoloxidase (PPO) using glycerol or polyethyleneglycol to increase the number of carbonyl and hydroxyl groups in lignin, and to improve its chelating capacity. Increase in the absorption in UV-spectrum related to α-carbonylphenolic and α,β-unsaturated structures was observed in all the experiments. The chelating properties of the original and oxidized lignins were compared by monitoring the amount of Cu^{2+} bound to lignin by gel permeation chromatography. The chelating capacity of original Acetosolv lignin was 354 mg Cu^{2+}/g lignin. On the other hand, lignin oxidized with PPO/O_2 showed an increase of 73% in chelating capacity in relation to the original lignin. The chelating capacity of lignin oxidized with PPO/O_2/glycerol was 110% higher than that of the original lignin. Glycerol stabilizes PPO, increasing its half-life. Average molecular weight ($\overline{MW}$), measured by size-exclusion chromatography, was smaller for the oxidized lignins than for the original Acetosolv lignin. This result suggests that quinones can eventually be formed through the action of PPO, but are not polymerized. The chelating capacity of oxidized lignins increases with the incorporation of vicinal hydroxyl groups.

Index Entries: Oxidation of lignin; polyphenoloxidase; sugar cane bagasse; Acetosolv; chelating agents.

Introduction

Chelating agents have been extensively studied with a view to removing heavy metals from aqueous solutions *(1,2)*. Substances with high chelating capacity contain functional groups with high electronic density, such as carbonyls, amines, thiols, hydroxyls and aromatic rings *(3–5)*.

*Author to whom all correspondence and reprint requests should be addressed.

Lignin, a very adequate substance for use as a chelating material, contains various of these groups.

Sugarcane bagasse is a promising lignin source. In Brazil, there are large sugarcane plantations and the principal products are sugar for alimentary purposes and ethanol used as fuel and fuel additive for automobiles. The production of sugarcane bagasse is 5×10^6 to 10×10^6 ton/yr *(6,7)*; the largest part is burned for energy production and 10% of the sugarcane bagasse remains as excess, which corresponds to 1.0×10^6 ton/yr *(8)*. Lignin can be easily obtained from the bagasse through separation techniques (pulping and steam explosion, for example).

Enzymatic systems are promising in the oxidation of lignins. Polyphenoloxidases (PPO) catalyze the aerobic oxidation of monophenols incorporating vicinal hydroxyls. Further oxidation of vicinal dihydroxyderivatives leads to *o*-quinones *(9,10)*. PPO can oxidize lignin producing cresols or quinone structures, increasing the number of chelating groups in the lignin macromolecule. This oxidized lignin can be used in the removal of metals present in industrial effluents *(11)*. In this work lignin obtained from Acetosolv pulping of sugarcane bagasse was oxidized with comercial PPO to improve its chelating properties.

Materials and Methods

Enzymatic Oxidation of Bagasse Acetosolv Lignin

Acetosolv pulping of depithed sugarcane bagasse was carried out as described by Benar *(12)*. Lignin was isolated from the pulping liquor by precipitation in water. The Acetosolv sugarcane bagasse lignin was oxidized by polyphenoloxidase (PPO) [Sigma Phenolase EC 1.10.3.1 (0.88 μg/g lignin)] in homogeneous phase, using O_2 bubbled at 30 mL min^{-1}, 30 mg of lignin, and, in some experiments, 0.1% (m/v in respect to solvent volume) of glycerol or polyethyleneglycol in 3 mL of phosphate buffer:dioxane 3:1 (v/v) at pH 6.6, 30°C for 4 h, without agitation. Oxidized lignin was recovered after precipitation with HCl and analyzed by UV in the range of 200–400 nm in a Hitachi U-2001 spectrometer.

Chelating Capacity Determination

The chelated complexes were quantified using a glass column filled with a suspension of Sephadex G-10 in the mobile phase *(13)*. The mobile phase was composed of 0.58 g NaCl, 1.21 g Tris-buffer (hydroxymethylaminomethane), and 0.128 g of $CuCl_2 \cdot 2H_2O$ in 800 mL and the pH was adjusted to 8.0. Samples were prepared with 17 mg of original or oxidized lignins in 10 mL of a 0.04 mol L^{-1} NaOH aqueous solution. The solution or filtrate (500 μL) was introduced into the top of the column. Samples were collected during the elution of 1 mL every 2 min, added to a diethyldithiocarbamate solution and the absorbance of the resulting complex was measured at 440 nm in an Hitachi U-2001 spectrometer. For the calibration curve six standard Cu^{2+} solutions were used.

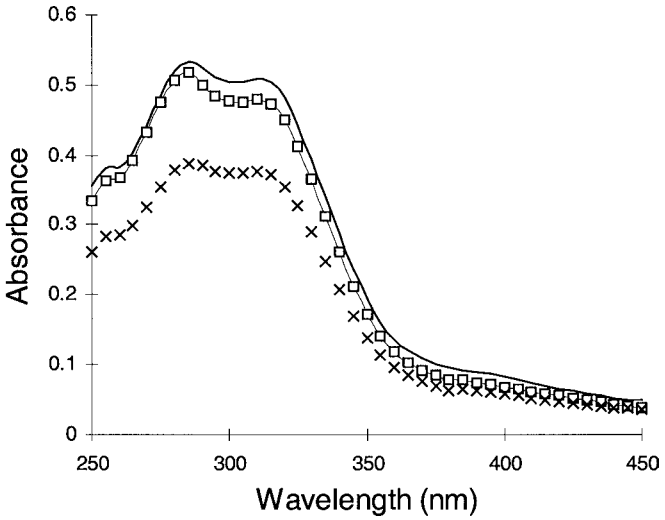

Fig. 1. UV-spectra of Acetosolv lignin (—) and oxidized lignins from Expt 2 (×××) and from Expt 3 (-□-□-□-).

Molecular Weight Distribution

The determination of molecular weight distribution of the lignins was performed by size-exclusion chromatography using a glass column filled with a suspension of Sephadex G-50. The mobile phase was NaOH 0.5 mol L^{-1} at 0.5 mL min^{-1}. The samples (500 µL of 1 g L^{-1} lignin in NaOH 0.04 mol L^{-1}) was introduced at the top of the column. Samples were colected during the elution of 4.5 mL and the absorbance measured at 280 nm (λ_{max} for lignin) in a CINTRA 20 spectrometer. For the calibration a set of proteins was used: albumin 66 kDa; carbonic anhydrase 29 kDa; cytochrome C 12.4 kDa, and aprotinin 6.5 kDa. Total exclusion volume (V_t) was determined with blue dextran (1000 kDa) and total permeation volume (V_0) with acetone. Elution volumes (V_e) were converted to Kd using the relation $Kd = (V_e - V_0)/(V_t - V_0)$.

Results and Discussion

The enzymatic system was effective for the oxidation of the Acetosolv lignin, since in all the experiments an increase in the relative absorption at 280 nm (related to α-carbonylphenolic structures) was observed. This absorption increase was measured in relation to the band at 310 nm, corresponding to α,β-unsaturatedstructures (Fig. 1). The yield of oxidized lignin was higher than 80%, and only a small fraction of the lignin was lost as low-molecular-weight compounds.

Similarly to the action on monophenols *(9,10)*, the action of PPO should increase the number of hydroxyl and carbonyl groups (as quinones) in lignin, and improves its chelating properties (Table 1). Lignin oxidized

Table 1
Parameters and Results of the Enzymatic Oxidation of Acetosolv Lignin with 0.88 µg PPO/mg Lignin

Experiment Number	Oxygen (mL min^{-1})	Glycerol (% m v^{-1})	Polyethyleneglycol (% m v^{-1})	Chelating capacity (mg Cu^{2+}/g lignin)
	Original Acetosolv lignin			354
1	—	0.0	0.0	414
2	30	0.0	0.0	615
3	30	0.1	0.0	748
4	30	0.0	0.1	413

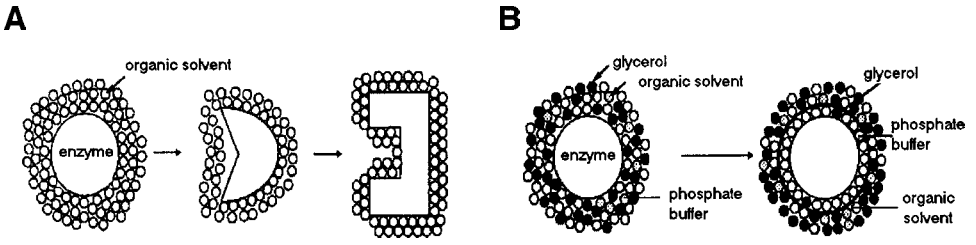

Fig. 2. **(A)** Denaturation of the enzyme in an organic solvent. **(B)** Schematic presentation of the effect of the polyol on the stabilization of the enzyme.

with PPO/O_2 showed a 73% increase in chelating capacity in relation to the original lignin (Expt 2).

Enzyme denaturation, which results from small changes in the temperature, pH, ionic strengh, pressure, and solvent nature of the medium, is still a limiting factor for its application. Nevertheless, enzymatic stability can be achieved, for example, by adding polyols to the medium as demonstrated by Matsumoto et al. *(14)*, and, as a consequence, enzymes have been utilized in the analysis and production of chemicals. Polyols are the best aqueous solvent for preventing denaturation and maintaining the native conformation of the enzymes. Since the direct action of a polyol cannot be detected, its effect was evaluated by the chelation power of the oxidized lignin (Table 1).

Lignin oxidized with PPO/O_2/glycerol had a chelating capacity 110% higher than that of the original lignin (Expt 3), showing that glycerol stabilized PPO and increased its half-life. Glycerol interacts more effectively with the enzyme, which is maintained in its quaternary form (Fig. 2). The high viscosity of the polyethyleneglycol is a barrier for the dissolution of low-polarity compounds *(15)* and so there was no significant difference between Expt 1 and 4 concerning the chelating capacity.

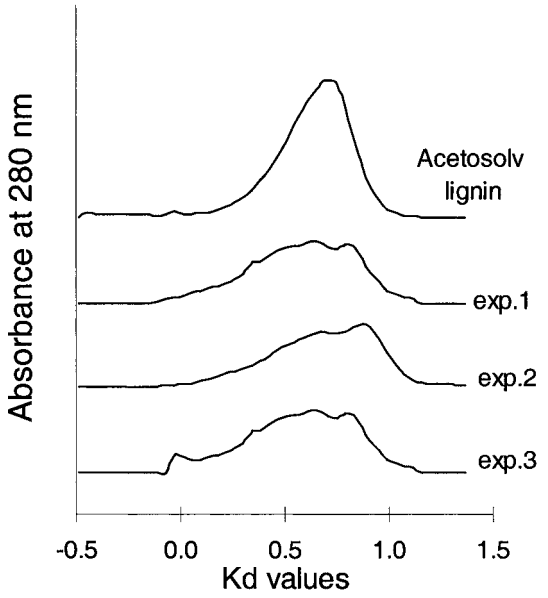

Fig. 3. Molecular weight distribution for the original Acetosolv and oxidized lignins.

The chelating capacity of the oxidized lignin (Expt 1) in flask open in the air was only 17% higher than that of the original lignin, showing the influence of shaking and O_2 concentration.

The distribution of molecular weight of original and oxidized lignins is shown in Fig. 3. The Acetosolv lignin had a monomodal distribution and the oxidized lignins showed bimodal profiles. Table 2 presents the weight-average ($\overline{MW}$) and number-average ($\overline{MN}$) molecular weight and polydispersity (D) values for the lignins.

The $\overline{MW}$ of oxidized lignins was smaller than the $\overline{MW}$ of the original lignin, showing an inhibition of polymerization reactions. Lignin oxidized by PPO/air (Expt 1) presented the smallest $\overline{MW}$ value, but the number of polar groups incorporated in the lignin was not high, since its chelating capacity was only 17% higher than that of the original Acetosolv lignin. An opposite behavior was observed for lignin oxidized with PPO/O_2/glycerol (Expt 3), with high values of $\overline{MW}$ and chelating capacity.

The increase in $\overline{MW}$ values was also accompanied by an increase in polydispersity and a closer evaluation of this analysis was performed with the distribution of $\overline{MW}$ values fractioned in selected molecular weight ranges (Table 3). Oxidized lignins showed a high fraction with $\overline{MW}$ <1000 Da, which was not present in the original Acetosolv lignin. The original lignin was probably hydrolyzed through clivages of β-O-4 ether bonds. More than 85% of the original Acetosolv lignin had $\overline{MW}$ between 2.0×10^3 and 10.0×10^3 Da. For the oxidized lignins this fraction corresponded to only 8–26%. The increase in the fraction with $\overline{MW}$ higher than 2000 Da in the oxidized lignin was correlated with the increase in chelating capacity of these lignins.

Table 2
Average Molecular Weights and Polydispersity of Original and Oxidized Lignins

Lignin	$\overline{MW}$ ($\times 10^3$ Da)	$\overline{MN}$ ($\times 10^3$ Da)	*D*
Original Acetosolv	5.2	3.7	1.4
Expt 1	1.1	0.2	5.5
Expt 2	1.9	0.2	9.5
Expt 3	2.4	0.3	8.0
Expt 4	3.4	0.4	8.5

Table 3
Fractionation of Lignins in Selected Molecular Weight Ranges

Molecular weight range (Da)	Original (%)	Expt 1 (%)	Expt 2 (%)	Expt 3 (%)
<1000	0.0	77.9	65.6	53.5
1000–2000	5.8	10.9	14.7	11.9
2000–4000	40.8	5.6	7.1	14.7
4000–10,000	44.4	2.6	8.6	11.0
>10,000	9.1	2.9	4.0	6.1

Actually, the property measured by size exclusion chromatography is the hydrodynamic volume. Lignin has a globular structure, stabilized by hydrogen bonds *(16)*. This structure is distorted when hydroxyl groups are introduced into the lignin, whose hydrodynamic volume increased as a consequence. The enzyme was stabilized by glycerol, and its activity was maintained for a longer time. More vicinal groups were incorporated and so quinones could be also formed. The $\overline{MW}$ should have increased with the quinone polymeryzation whereas the chelating capacity should have decreased.

Conclusions

Glycerol improved the oxidation of sugarcane bagasse Acetosolv lignin with PPO. The half-life of the enzyme increased and oxidized lignin with 110% more chelating capacity was obtained. Lignin was also hydrolyzed by decreasing the fractions with high molecular weight. The higher the $\overline{MW}$ values were the higher was the chelating capacity. Effectiveness of the enzyme resulted in increased polar groups in the lignin, causing an increase in the hydrodynamic volume and, as a consequence, an increase in the measured $\overline{MW}$.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional do Desenvolvimento Científico e Tecnológico. The authors thank M.E.M. Coelho for technical assistance.

References

1. Levine, N. M. (1986), in *Polyelectrolytes for Water and Wastewater Treatment*, Removal of Heavy Metals from Water, 2nd ed., Schwoyer, W. L. K., ed., CRC Press, NY, pp. 47–59.
2. Lo, K. S. L. and Chen, Y. H. (1990), *Sci. Total Environ.* **90,** 99–116.
3. Geckeler, K., Lange, G., Eberhardt, H., and Bayer, E.(1980), *Pure Appl. Chem.* **52,** 1883–1905.
4. Geckeler, K., Bayer, E., Shkinev, V. M., and Spivakov, B. Y. (1988), *Naturwissenschaften* **75(4),** 198–199.
5. Geckeler, K. E. and Volchek, K. (1996), *Environ. Sci. Technol.* **30,** 725–734.
6. Burgi, R. (1988), *A Granja* **44(484),** 16–26.
7. Molina, J. W. F., Ripoli, T. C., Geraldi, R. N., and Amaral, J. R. (1995), *STAB-Açúcar, Álcool e Subprodutos* **13(5),** 28–31.
8. Armas, C. M. and Bianchi, E. (1990), *STAB, Açúcar, Álcool e Subprodutos* **8(5/6),** 41–45.
9. Burton, S. G. (1994), *Catalysis Today* **22,** 459–487.
10. Estrada, P., Baroto, W., Castillon, M. P., Acebal, C. and Arche R. (1993), *J. Chem. Technol. Biotechnol.* **56,** 59–65.
11. Gonçalves, A. R., Nascimento, L. R. P., Cotrim, A. R., and Silva, F. T. (1996), *V Seminário de Hidrólise Enzimática de Biomassas*, Universidade Estadual de Maringá, Brazil.
12. Benar, P. (1992), *Tese de Mestrado,* Instituto de Química-UNICAMP, Campinas, Brazil.
13. Deacon, M. and Smyth, M.R. (1993), *J. Chromatogr. A.* **657,** 69–76.
14. Matsumoto, M.; Kida, K., and Kondo, K. (1997), *J. Chem. Technol. Biotechnol.* **70,** 188–192.
15. Illanes, A. and Barberis, S. (1994), *Serie de Biología Biotecnologia de Enzimas*, Catálisis Enzimática en Fase Orgánica, Ed. Univ. Valparaiso, Chile.
16. Jurasek, L. (1997), *Molecular Modeling of Fibre Walls*, International Symposium on Wood and Pulping Chemistry, 9, Montréal Proceedings, Canada, pp. E5-1–E5-4.