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THE EFFECT OF FUNGAL PROTOCATECHUATE 3,4-DIOXYGENASE ON SODIUM LIGNOSULPHONATE FRACTIONS

MARIA WOJTAŚ-WASILEWSKA and JOLANTA LUTEREK

Department of Biochemistry, Maria Curie-Sklodowska University, 20-031 Lublin, Pl. M. Curie-Sklodowska 3, Poland

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Key Word Index—*Pleurotus ostreatus;* fungus; protocatechuate 3,4-dioxygenase; lignosulphonate; lignin transformation.

Abstract—Protocatechuate 3,4-dioxygenase (protocatechuate: oxygen 3,4-oxidoreductase, EC 1.13.11.3) from the white-rot fungus *Pleurotus ostreatus* was incubated with two fractions of sodium lignosulphonate (NaLS) obtained from sulphite waste liquor. By means of gel-permeation chromatography on Sephadex G-50 and UV sepctral analysis, it was shown that there was a decrease in the aromatic structures of the NaLS fractions. Oxygen uptake studies confirmed that the observed transformations of the NaLS frasctions was an enzyme catalysed process. The same method revealed differences in the affinity of the dioxygenase for the NaLS fractions.

INTRODUCTION

Despite many years of research, the pathways for the fungal degradation of lignin are known only as far as their overall details. In particular, little is known about the enzymes which participate in this important process [1–6].

It is suggested that the attack of white-rot fungi on lignin is carried out in part via low-M_r aromatic compounds [7–10]. It is also suggested that in the course of the process in question the partial cleavage of aromatic nuclei, still bound in the lignin polymer, takes place [11, 12]. The same was also postulated by Haider and Grabbe [13] who based their hypothesis on the results of their research carried out several years ago.

In the course of the last 10 years, Kirk and his co-workers have published a series of papers which describe the probable chemical reactions and changes to which lignin is subjected during the decay of spruce and birch wood by the white-rot fungus *Phanerochaete chrysosporium* [10, 11, 14–16]. On the basis of the detailed chemical analysis of heavily degraded lignins the oxidative character of the process was confirmed. Further they discovered extracellular lignin-degrading enzymes in *P. chrysosporium* which included non-specific peroxidases and oxygenases [5, 17].

One approach to the problem of fungal degradation of lignin is to isolate specific enzymes from lignolytic fungi and to investigate their effects on lignin, lignin-derived polymers or lignin substructure model compounds. This method has been used to establish the function of laccase and peroxidase in lignin degradation [2, 18–23].

The purpose of this study was to investigate the effects of the fungal enzyme protocatechuate 3,4-dioxygenase on sodium lignosulphonates (NaLS). A purified preparation of dioxygenase obtained in our laboratory from the lignolytic fungus *Pleurotus ostreatus* was employed [24]. As a model of lignin, two fractions of NaLS were used because unlike natural lignin they are readily soluble in water. The fractions of NaLS were separated from cellulose industry wastes and then purified [25].

RESULTS AND DISCUSSION

It has been shown that *P. ostreatus* grows well in a medium containing yeast waste liquor* and that it utilizes major elements (lignosulphonates) in the waste as a carbon source [26]. A subsequent study in which *P. ostreatus* was incubated with pure NaLS of various M_rs obtained from cellulose industry wastes, confirmed that *P. ostreatus* is able to metabolize lignosulphonates [25]. Evidence was also obtained for the stimulating effect of NaLS on the activities of the phenolic oxidases, laccase and peroxidase as well as protocatechuate 3,4-dioxygenase [27]. This finding suggested that there is direct participation of these enzymes in NaLS transformations. The investigations carried out so far show that the major effect of peroxidase, separated from the fungus *Trametes versicolor*, of which belongs to the same group of lignin degraders as *P. ostreatus*, is the polymerization of lignosulphonates [22]. Research on fungal laccase indicates that the effect of this enzyme on lignin and lignin substructure model compounds is similar [23, 28, 29].

The participation of dioxygenase catalysed reactions in the biodegradation of lignin has been considered by several groups of researchers [11–15, 30, 31], who incubated native lignin, its artificial substructure compounds (dehydropolymer of coniferyl alcohol) or lignocellulose material with selected fungi. The results of these studies proved that dioxygenation reactions of the aromatic rings in lignin took place. This conclusion was based either on the release of $^{14}CO_2$ from [ring-^{14}C] coniferyl alcohol dehydropolymer (^{14}C-DHP) or on the results of chemical and physicochemical analysis of lignins

* Waste product of cellulose production by sulphite pulping method containing 77.6% of chemically modified lignin.

isolated from wood previously exposed to fungal degradation.

We have investigated whether the protocatechuate 3,4-dioxygenase from the lignolytic fungus *P. ostreatus* [24] exhibits aromatic ring cleavage activity towards the NaLS fractions used as lignin models.

Sodium lignosulphonates were isolated from cellulose industry wastes and separated into five fractions by means of gel filtration chromatography on Sephadex G-50. Each NaLS fraction had a UV absorption maximum at 280 nm due to the aromatic structures of the various ring substituents. The purification procedure and full physicochemical characteristics of NaLS fractions can be found in previous reports [22, 25]. The M_rs of five NaLS fractions were determined by means of gel permeation chromatography on a Sephadex G-50 column: Fr.I($M_r \times 10^3$, 83), II(25), III(8.5), IV(5), V(2.4).

The fractions of approximate M_rs 8500 and 83 000 were chosen for further study. On gel filtration, both NaLS fractions after incubation with dioxygenase showed no change in M_r(Fig. 1). The only changes were a decrease of absorption at 280 nm (compared to the controls) indicating probable aromatic cleavages by dioxygenase (Fig. 1). The above findings were supported by the UV absorption spectra (in the region of 200–350 nm) recorded before and after incubation of the NaLS fractions with dioxygenase (Fig. 2). Although the shape of the curves after incubation was almost unchanged the decrease of absorption intensity, especially in the case of the high-M_r NaLS, was observed for the whole spectrum. As judged from the oxygen uptake when protocatechuate 3,4-dioxygenase was incubated with the NaLS fractions it was obvious that the transformation of these substrates was an enzyme catalysed process. Thus oxygen consumption for the high and low M_r fractions was 34 and 27 per cent, respectively, of the value recorded for the proper substrate of dioxygenase i.e. protocatechuic acid. The same method (i.e. the measurement of oxygen uptake) was also used to determine the Michaelis–Menten constant (K_m) of dioxygenase for both NaLS fractions. The values for the high and low M_r fractions were respectively 48 and 673 nM. It is of particular interest that the lower K_m value for the enzyme was obtained for the high M_r NaLS fraction. This suggests a higher affinity of protocatechuate 3,4-dioxygenase towards this substrate.

The results of this study have confirmed the opinion of several authors [11–15] that lignin is degraded by white-rot fungi, partly by the cleavage of the aromatic rings bound in the polymer. The products of these dioxygenase-catalysed reactions are then decomposed by other lignolytic enzyme systems into carbon dioxide and water.

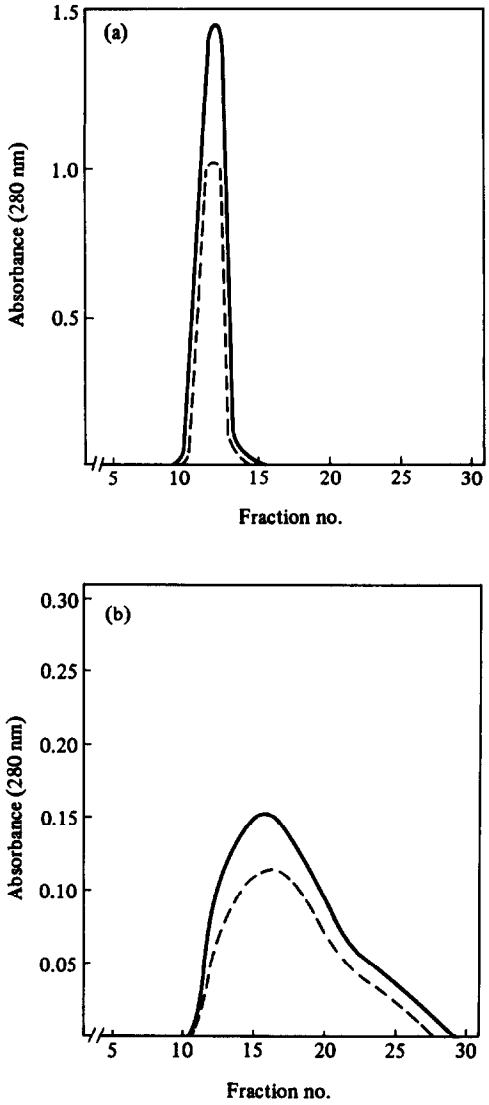

Fig. 1. Analytical gel filtration chromatography on Sephadex G-50 of NaLS fractions. a, fraction I (M_r 83 000); b, fraction III (M_r 8500)-----, Samples treated with active enzyme;——, samples treated with boiled enzyme (30 min). Incubation mixture (5 ml, see Experimental) was introduced into the column (1.5 × 90 cm) and eluted with water. Fractions of 3 ml were collected at a flow rate of 12 ml/hr.

EXPERIMENTAL

Organism and culture conditions. P. ostreatus (Jacqu.) Fr. No. 53 obtained from The Department of Plant Anatomy and Physiology of the J. E. Purkyne University (Brno, Czechoslovakia) was grown in Roux flasks at 26° in a liquid medium according to ref. [24]. *p*-Hydroxybenzoic acid at a final concentration of 0.02% was added to the 4-week-old culture. After 12 hr incubation with the inducer the mycelium was harvested by filtration through a Büchner funnel, washed several times with distilled water and used directly or stored as a wet packed cell at −20°.

Purification of the enzyme. Protocatechuate 3,4-dioxygenase was purified from the extract of *p*-hydroxybenzoate induced cells of *P. ostreatus* according to the procedure described in ref. [24]. The purification involved $(NH_4)_2SO_4$ fraction (0.55 saturation, pH 8.0 attained from NH_3 soln), DEAE-cellulose and Sephadex G-200 chromatography. All steps were carried out at 0–4°. The buffer applied (15 mM Tris–HCl, pH 8.0) contained 0.5 mM 2-mercaptoethanol and 0.1 mM EDTA. To minimize proteolysis *p*-methylsulphonyl fluoride (1 mM) was added to the homogenization buffer.

Enzyme activity was determined spectrophotometrically by measuring the decrease in absorbance at 290 nm or by measuring oxygen uptake with a Clark electrode. Both assays were described previously in refs [24, 32].

Protein determination. Protein was determined spectrophotometrically by measuring the difference in absorption at 228.5 and

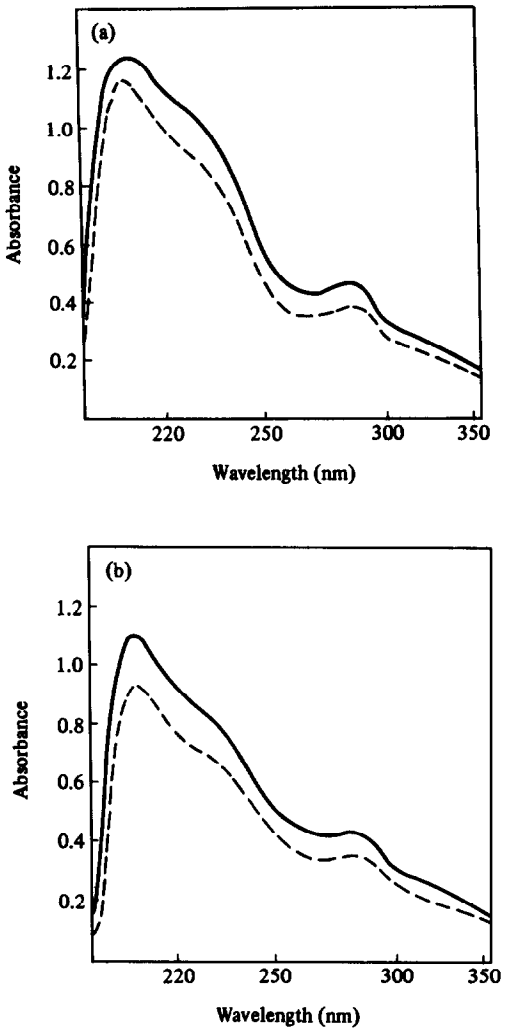

Fig. 2. UV absorption spectra of NaLS fractions in 15 mM Tris–HCl buffer, pH 8.0. a, Fraction I (M_r 83 000); b, fraction III (M_r 8500).-----, Samples treated with active enzyme;——, samples teated with boiled enzyme (30 min).

234.5 nm according to ref. [33]. Bovine albumin was used as a standard.

Isolation and purification of Na-*lignosulphonates.* The methods employed were carried out according to ref. [25]. Sulphite waste liquor, after the removal of fermentable sugar by alcoholic fermentation, obtained from Cellulose Plant at Niedomice (Poland) was used for *n*-octadecylamine extraction of lignosulphonic acids. The crude preparation of NaLS was separated into five fractions by gel filtration chromatography on a Sephadex G-50 column (1.5 × 185 cm) using H_2O as an eluent. Samples of 3 ml were collected at a flow rate of 12 ml/hr. A of the effluent was measured at 280 nm. The samples corresponding to the peaks in the elution profile were freeze-dried.

M_r *determination.* The average M_rs of NaLS fractions were determined by gel filtration on Sephadex G-50 with 0.1 M NaCl as an eluent. For calibration of the Sephadex G-50 column (0.9 × 58 cm), hemoglobin (M_r 68 000), trypsin (23 000), cytochrome *c* (11 800) and insulin (5700) were used (3 mg in a vol. of 1 ml). V_0 of the column was determined using Dextran Blue 2000. Fractions of 0.5 ml were collected at two min intervals.

Incubation of Na-*lignosulphonate fractions with protocatechuate 3,4-dioxygenase.* Two NaLS fractions of M_r 8500 and 83 000, respectively, were used in the experiment. The incubation mixture contained 2 mg NaLS fraction I (M_r 83 000) or III (M_r 8500) and 5 ml enzyme (0.04 mg/ml, spec. act. 266.07). After 3 hr incubation at 37° with shaking, the reaction was stopped by adding 0.5 ml 4 M HCl. To the reference sample (B) containing buffer instead of substrate and the same amount of enzyme, HCl was added at zero time. Then the mixture was kept for 10 min on a boiling waterbath before incubation. The control sample (C) containing substrate untreated by the active enzyme was prepared in the same manner. After acidifying, the ppt. from the samples (A, B and C) was removed by centrifugation. The supernatants were neutralized with 10% NaOH. The samples were diluted × 5 (fr. I) and × 3 (fr. III) with the buffer before spectrophotometric analysis.

Spectrophotometric analysis. NaLS oxidation by the dioxygenase was examined spectrophotometrically. The UV absorption spectrum of sample A was recorded against the reference sample B. The spectrum of sample C was recorded using the buffer as a reference.

Sephadex G-50 chromatography was performed before and after incubation of the NaLS fractions with dioxygenase. The neutralized samples A and C (5 ml) were introduced separately into the column (1.5 × 90 cm) and eluted with H_2O. Fractions of 3 ml were collected at a flow rate 12 ml/hr and were assayed spectrophotometrically at 280 nm using H_2O as a reference.

Oxygen uptake in the presence of NaLS fractions I or III (M_r 83 000 and 8500) was measured with a Clark O_2-electrode. The reaction mixture contained in a final vol. of 3 ml: 29.25 μmol Tris–HCl buffer, pH 8.0, the enzyme (30.25 μg, spec. act. 164.1) and various concentrations of the substrate (0.006–0.2 μmol NaLS fr. I or 0.3–3.0 μmol NaLS fr. III).

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