Aromatic ring cleavage of veratryl alcohol by *Phanerochaete chrysosporium*

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Received 17 July 1985

Two oxidation products of veratryl alcohol were isolated from ligninolytic cultures of *Phanerochaete chrysosporium*. After purification the compounds were characterized by ^{1}H-NMR, mass spectrometry and infrared spectrometry. The structural information suggests that the compounds are two isomers of a ring cleavage product from veratryl alcohol. Both compounds were also found as by-products when veratryl alcohol was oxidized to veratraldehyde with the crude extracellular ligninase preparation.

Aromatic ring cleavage *Veratryl alcohol* *Ligninase white-rot fungi*

1. INTRODUCTION

A lignin degrading basidiomycete, *Phanerochaete chrysosporium*, accumulates veratryl alcohol in culture medium during secondary ligninolytic metabolism when it grows in glucose medium [1]. Lundquist and Kirk [2] showed that this compound comprises about 60% of the chloroform extractable radiolabelled material after 6 days of cultivation when the organism was grown on [^{14}C]glucose. Veratryl alcohol has been shown to activate the ligninolytic system as well as to increase the level of ligninolytic enzymes in *P. chrysosporium* [3,4]. The ligninase oxidizes veratryl alcohol to veratraldehyde in the presence of H_2O_2. However, since the aldehyde is readily reduced back to alcohol the alcohol accumulates in the cultures.

Extracellular aromatic ring cleavage by *P. chrysosporium* has been suggested by Chen et al. [5] for the β-*O*-4 substructure in lignin and was actually shown to occur by Umezawa and Higuchi [6] when β-*O*-4 lignin model dimer was incubated with *P. chrysosporium*.

While monitoring the veratryl alcohol level in the ligninolytic cultures with thin layer and high pressure liquid chromatography we have systematically found another organic-soluble compound which accumulates in the ligninolytic cultures [7]. Here we give evidence that this compound is a ring cleavage product from veratryl alcohol and is produced both by intact cultures and by the ligninase preparation. To our knowledge this is the first report on aromatic ring cleavage reaction by the extracellular enzyme system from this fungus.

2. MATERIALS AND METHODS

Non-agitated cultures of *P. chrysosporium* (ATCC 24725) were grown in 100% oxygen atmosphere at 37°C in the presence or absence of 1.5 mM veratryl alcohol [8]. At given intervals 3 replicate cultures were filtered through glass fibre filter. The filtrate was extracted 3 times with dichloromethane. The extract was evaporated under nitrogen, resolubilized in methanol and the products were separated by high pressure liquid chromatography with Kontron HPLC-System 600 (Kontron AG, Switzerland). The separation column was 4.6 mm i.d. × 125 mm long, filled with RP 18 (Nucleosil C 18, 5 μm). Elution was carried out by increasing methanol gradient in water and the products were detected at 254 nm.

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00145793/85/$3.30

For large scale production of the organic soluble accumulating compounds 2 × 200 culture flasks were allowed to grow 65–75 h with 1.5 mM veratryl alcohol. After extraction with dichloromethane the organic soluble compounds were purified twice on preparative TLC (Kiesel-gel 60 F_{254}, Merck). Developing solvent was first benzol/EtOAc = 3:1 (v/v) (once) and then CH_2Cl_2 (4 times). The isolated purified compounds with an R_f value of 0.47 in the first solvent system and 0.08 and 0.12 in the second system were analyzed by mass spectrometry (MS), proton nuclear magnetic resonance (^{1}H-NMR) and infrared spectroscopy (IR).

In some experiments [^{14}C]glucose (Amersham, England; spec. act. 9.25 GBq·mmol^{-1}) was used. The accumulating organic soluble labelled compounds were separated by thin layer chromatography and counted with Packard TriCARB liquid scintillation spectrometer.

Extracellular ligninases of *P. chrysosporium* were produced in agitated C-limited conditions in the presence of 1.5 mM veratryl alcohol [9]. The enzymes were concentrated by ultrafiltration and further purified by ion exchange as described [9]. The concentrated enzyme was diluted to 12000 U/ml and stored at −20°C in 5 mM phosphate buffer, pH 7.25. The oxidation of veratryl alcohol was carried out in 100 mM Na-tartrate, pH 3.0, using 240 U/ml enzyme. One unit oxidised 1 nmol veratryl alcohol to veratraldehyde in a minute. The oxidation products were extracted with dichloromethane and separated by HPLC.

^{1}H-NMR spectra were obtained on 400 MHz nuclear magnetic resonance spectrometer (Bruker WH 400). The two unknown compounds were analyzed from 2 different cultures. The spectra indicated the presence of 2 isomers.

(i) ^{1}H-NMR ($CDCl_3$), δ (ppm), *cis*-isomer (IIIa): 30% (second sample: 47%), 3.78(3H,s,CH_3-OOC-), 5.25(2H,m,-CH_2-OOC-), 6.12(1H,d,J_{ab} = 12.41 Hz,H_a,*cis* double bond), 6.30(1H,m,H_c), 6.75(1H,dd,J_{ab} = 12.41 Hz, J_2 = 1–2 Hz,H_b,*cis* double bond). (ii) *trans* isomer (IIIb): 70% (second sample: 53%) 3.83(3H,s,CH_3-OOC-), 5.00(2H,m,-CH_2-OOC), 6.18(1H,d,J_{ab} = 16.43 Hz,H_a,*trans* double bond), 6.28(1H,m,H_c), 7.60(1H,dd,J_{ab} = 16.43 Hz,J_2 = 1–2 Hz,H_b,*trans* double bond).

Mass spectra were recorded on a Varian MAT CH5B. MS *m/z* (%): 168 (46,6, M^+), 139 (55,6), 137 (80,8), 136 (54,3), 124 (28,4), 111 (69,4), 109 (26,3), 81 (41,4), 80 (23,5), 79 (100), 59 (25,7), 53 (48,7), 52 (39,6), 51 (77,7), 50 (39,2), 39 (25,8), 29 (26,5), 27 (21,5). Only peaks with intensities higher than 20% of the base peak are listed.

Infrared spectra were obtained on Perkin Elmer infrared spectrometer 781. IR (cm^{-1}): 3050, 2950, 2920, 2850, 1800 (shoulder), 1790, 1760, 1745, 1720, 1635, 1600, 1570, 1450, 1430.

3. RESULTS

P. chrysosporium was grown in the presence of [^{14}C]glucose for 70 h. The major organic soluble accumulating labelled compounds found in the culture filtrate were veratryl alcohol (60%), veratraldehyde (5–10%) and two unknown metabolites sometimes comprising up to 30% of the total extractable compounds. Veratryl alcohol was the first accumulating substance appearing in the cultures after 43–45 h of growth. The two unknown compounds were detected by HPLC after 47–50 h of growth. In the presence of 1.5 mM initial veratryl alcohol the unknown compounds appeared earlier and reached a higher concentration than without exogenously added alcohol. Further evidence that both of these compounds were obtained from veratryl alcohol was given when the oxidation of veratryl alcohol was

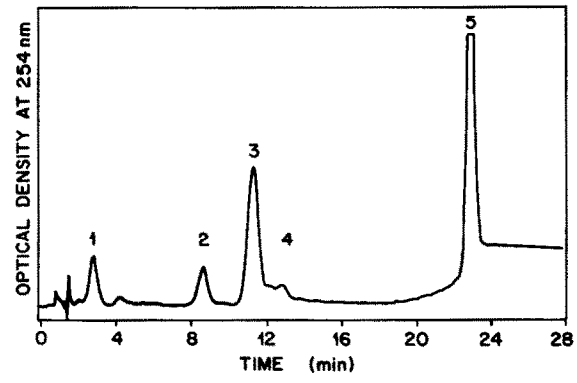

Fig.1. Separation of the oxidation products from veratryl alcohol by HPLC after 65 h incubation in the presence of ligninase and H_2O_2. (1) Unknown compound, (2) and (3) *trans*- and *cis*-isomers of the ring cleavage product, (4) veratryl alcohol and (5) veratraldehyde.

carried out with the purified ligninase preparation. 73% of the oxidation products were recovered as aldehyde (fig.1). Three other main products were obtained after 65 h incubation. Two of them were identical with the unknown compounds previously extracted from the culture filtrate.

IR spectra and ^{1}H-NMR spectra of the purified compounds showed the absence of an aromatic ring. Mass spectral analysis of the compounds gave a molecular mass of 168 Da to both compounds and ^{1}H-NMR suggested the presence of two isomers. A tentative structure and formation mechanism of these compounds according to MS and ^{1}H-NMR analysis are given in fig.2. In cultures the *cis*-isomer was always detected first and it reached its maximal concentration 10–15 h earlier than the *trans*-isomer. This indicates that the *cis*-isomer is the primary ring cleavage product.

4. DISCUSSION

Several different oxidation reactions by the extra-cellular oxidative enzymes of *P. chrysosporium* have been reported [10]. The crude enzyme preparation contains several heme proteins [11] which so far are only partially characterized. Umezawa and Higuchi [6] showed that an aromatic ring cleavage reaction occurs when a dimeric β-*O*-4 lignin substructure is incubated with ligninolytic *P. chrysosporium*. They also suggested that the organism has two different degradation pathways for this model dimer, one of them involving aromatic ring cleavage [12].

In this study we have isolated from ligninolytic cultures of *P. chrysosporium* two compounds which according to spectroscopic analysis are two isomers of an aromatic ring cleavage product from veratryl alcohol. The compounds start to accumulate in the ligninolytic cultures about 5 h later than veratryl alcohol. Their concentration could be increased by exogenous addition of veratryl alcohol and they were also detected as minor oxidation products when the alcohol was oxidised to aldehyde by the extracellular ligninase preparation.

Whether the ring cleavage product is formed by the action of the major ligninase proteins or whether a so far non-identified protein is responsible for this reaction is presently not known. It seems that analogous to β-*O*-4 dimer degradation [12] also veratryl alcohol has two degradative pathways, one leading to formation of veratraldehyde (fig.2A), the other one involving aromatic ring cleavage (fig.2B). We are presently optimizing the production of the ring cleavage compound as well as trying to prove its molecular structure and formation mechanism by isotopic studies and by chemical synthesis of the compound.

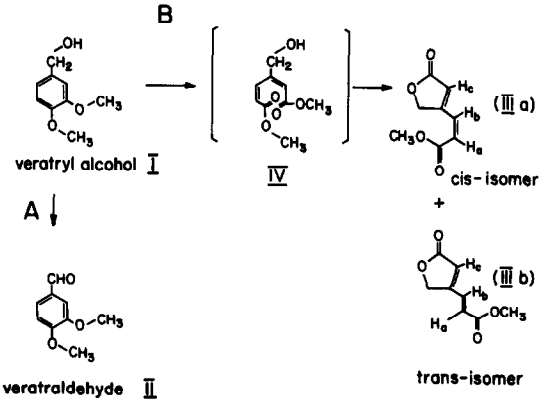

Fig.2. Proposed structure and formation mechanism for the *cis*- and *trans*-isomers from the aromatic ring cleavage of veratryl alcohol.

ACKNOWLEDGEMENT

We are grateful to Dr H. Schmidt from Givaudan AG for carrying out the spectroscopic analysis and for helping us to interpret the obtained data.

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