Lignin peroxidase mediated biotransformations useful in the biocatalytic production of vanillin

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Lignin peroxidase mediated biotransformations useful in the biocatalytic production of vanillin

Rimko ten Have

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 17 maart 2000
des namiddags te half twee in de Aula.

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Stellingen

1 Dit proefschrift is binnen de levensduur van een bloem van de orchidee *Vanilla planifolia* te lezen (zie de voor- en achterzijde van het proefschrift).

2 Koduri *et al.* [1] beschrijven dat LiP compound II *p*-anisyl alcohol niet kan oxideren. Dat is echter in tegenspraak met hun eigen experiment waarmee de auteurs tonen dat 0,5 nmol LiP, in een optimaal geval, in staat is om 70 nmol *p*-anisyl alcohol te oxideren.

[1] Koduri, R.S., Tien, M. (1994) *Biochemistry* **33**:4225-4230

3 Tijdens de MiP-assay, zoals deze beschreven is door De Jong *et al.* [1], wordt niet alleen MiP gemeten, maar ook LiP (indien aanwezig) want de aanwezigheid van EDTA in het assaymengsel verhindert de oxidatie van 2,6-dimethoxyfenol door LiP geenszins [2].

[1] De Jong, E. (1993) Proefschrift Landbouwuniversiteit Wageningen. Pagina 130.

[2] Ten Have, R. Ongepubliceerd resultaat.

4 De schematische voorstelling van de polymerisatie van fenol door HrP [1] kan onmogelijk juist zijn omdat er een vijfwaardig C-atoom, een oligomeer met een fenylgroep i.p.v. een fenolgroep en een meta-gekoppeld oligomeer worden gevormd.

[1] Joo, H., Yoo, Y.J., Dordick, J.S. (1998) *Korean J. Chem. Eng.* **15**:362-374

5 Het is op z'n minst opvallend dat Aladdin niet dadelijk zijn eerste wens verzilverde voor véél meer wensen.

6 Na zeventien jaar biochemische studies met ligninolytische enzymen snapt men nog steeds geen bal van lignineafbraak door wit-rot schimmels in de natuur.

7 Het publiek dat voor de lol naar paalzitten kijkt is vermoedelijk veel gekker dan paalzit(s)ters zelf.

8 Omdat de tijdsduur van de witlofwortelincubaties ontbreekt, zoals deze beschreven zijn door Piet *et al.* [1,2], is het onvoldoende mogelijk te controleren of de experimentele opzet juist was.

[1] Piet, D.P., Franssen, M.C.R., De Groot, AE. (1996) *Tetrahedron* **52**:11273-11280

[2] Piet, D.P., Schrijvers, R., Franssen, M.C.R., De Groot, AE. (1995) *Tetrahedron* **51**:6303-6314

9 Het is slordig dat Blaakmeer *et al.* [1] niet beschrijven hoe een cruciaal en mislukt veldexperiment werd uitgevoerd met, voor het koolwitje, ovipositieremmende stoffen. Vooral omdat nu onduidelijk blijft welke zijde van de koolbladeren bespoten werd met de ovipositieremmende en tevens fotoinstabiele stof.

[1] Blaakmeer, A., Van der Wal, D., Stork, A., Van Beek, T.A., De Groot, AE., Van Loon, J.J.A.(1994) *J. Nat. Prod.* **57**:1145-1151

10 De opvatting van Adams [1]:"Een aanzienlijk deel van het heelal valt te deduceren aan de hand van een stuk taart" is juist.

[1] Adams, D. (1984) The hitchhikers guide to the galaxy. Harmony Books, New York, U.S.A.

11 Het werk dat beschreven is in het proefschrift met de titel:"Lignin peroxidase mediated biotransformations useful in the biocatalytic production of vanillin", door R. ten Have is gebaseerd op een waargebeurd verhaal.

Contents

1 General introduction

VANILLA

Vanilla is one of the most valuable flavours in the food industry. The flavour is marketed as a dilute ethanol extract of cured unripe *Vanilla* fruits. Commercial growers must pollinate the flowers, that last only a single day, by hand in order to obtain the valuable fruits. This cultivation method requires intensive labour since there is no clear evidence of a natural pollinator [23], so every flower needs special attention. Orchids of the genus *Vanilla* are mainly cultivated in the Malagasy Republic, the Comoro Islands and La Réunion, together accounting for 70 % of the world's supply of natural vanilla.

More than 170 volatile aromatic compounds are identified in *Vanilla* fruits of which 4-hydroxy-3-methoxybenzaldehyde (vanillin, **1** in Scheme 1.1), is the most abundant compound [24], accounting for 2-3 % of the cured bean mass [23]. Other predominant constituents of the vanilla aroma are e.g. 4-hydroxybenzoic acid (**2**), 4-hydroxybenzaldehyde (**3**), and 4-hydroxybenzyl-alcohol (**4**) [24]. In unripe fruits, these constituents are mainly present as non-volatile glycosides, which lack the characteristic vanilla flavour. By curing these unripe fruits, enzymatic hydrolysis of the glycosides occurs [23], releasing the volatiles.

VANILLIN

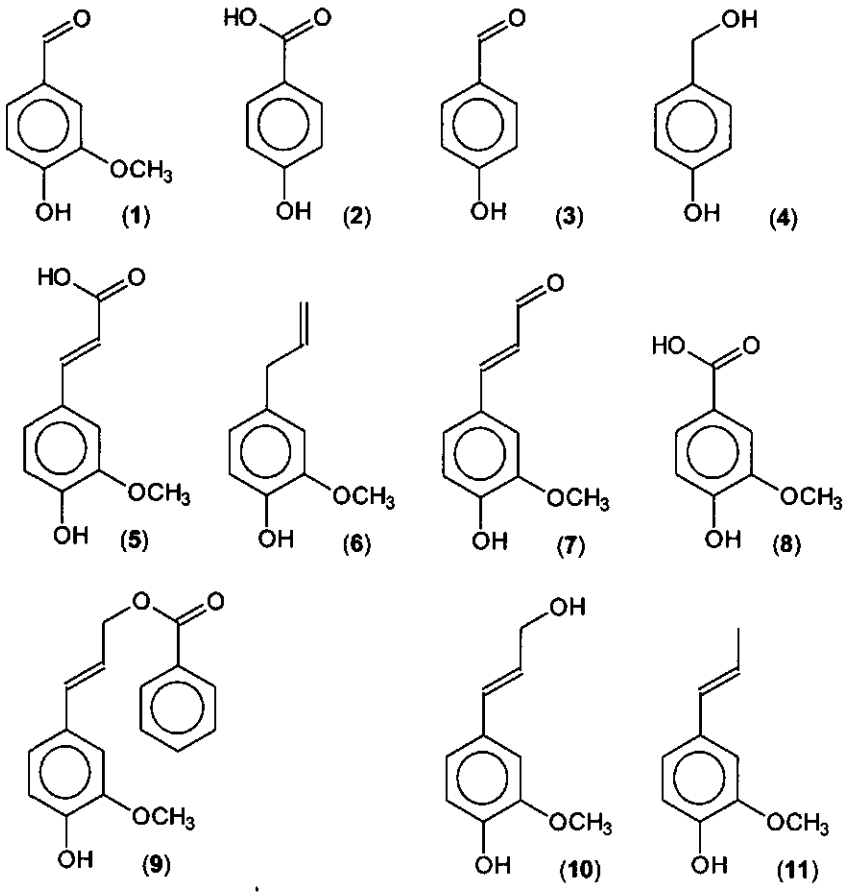
Recently, consumers prefer natural flavours like vanillin. Therefore, the food, pharmaceutical, and cosmetic industries [2] try to use natural vanillin in their products. The same compound may also be produced in a chemical manner. The feedstock for this chemical production is lignin. This is a heterogeneous polymer that occurs in wood and is also a waste product of the pulp and paper industry [7,11]. The chemically produced vanillin is marketed for a price of $ 15 kg^{-1} [21]. However, vanillin with the label "natural" has a substantially higher value owing to the consumer demand.

Legislation on foodstuffs allows the origin of natural vanillin to vary from *Vanilla* fruits [23] to the extracellular fluid of cultured cells from e.g. *Vanilla planifolia* [6,33], bacteria and fungi [3,21,26], or even incubation mixtures in which only enzymes [20,22] are applied.

VANILLIN PRODUCTION BY CELLS FROM *VANILLA PLANIFOLIA*

Plant cell culture processes are quite expensive. The break-even point is as high as $ 1,500 per kilogram of compound produced. Nevertheless, several research groups considered this possibility because every cell of the orchid contains the genetic information necessary to produce the constituents of vanilla [6].

Ferulic acid, 3-(4'-hydroxy-3'-methoxyphenyl)-2-propenioc acid (**5**), has been used in most cases since it appeared to be a good vanillin precursor [6,33]. Enzymes in the aerial root of *Vanilla* species have been applied to convert ferulic acid into vanillin. In this system charcoal was used to bind vanillin. In this manner product inhibition or further metabolism was prevented. The aerial root applied in this system produced up to 400 $mg.kg^{-1}$ dry weight material which is roughly 35-fold higher than the initial vanillin concentration in the roots [33].



SCHEME 1.1 **Structural formulas of several compounds referred to in the text.**

It is proposed that the ferulic acid consumed in this process is first esterified to form its CoA-derivative which then undergoes β-oxidation yielding vanillin [3,33].

FERMENTATIVE VANILLIN PRODUCTION

From an industrial point of view, vanillin production could be interesting using a cheap natural bulk substrate, such as the clove component

1-(4'-hydroxy-3'-methoxyphenyl)-2-propene (eugenol, **6**), as the starting material. This substrate has been fed to a mutant strain of the bacterium *Arthrobacter globiformis* that does not produce the enzyme aldehyde dehydrogenase. The strain first converts eugenol into 1-(4'-hydroxy-3'-methoxyphenyl)-propenal (coniferaldehyde, **7**) which is subsequently converted into vanillin by a treatment with mild base [3].
An alternative starting material is the extremely abundant phenolic compound ferulic acid [26]. The fungus *Aspergilles niger* [21], and resting cells of the yeast *Rhodotorula rubra* [15] have been used to transform ferulic acid into 4-hydroxy-3-methoxybenzoic acid (vanillic acid, **8**). The latter acid may be reduced to vanillin by another fungus, *Pycnoporus cinnabarinus*. About half of the vanillic acid consumed was converted into vanillin. In an optimal case, vanillin accumulated to a concentration of 560 mg/L [21].

VANILLIN PRODUCTION BY ENZYMES

The first method of vanillin production by enzymes is indirect. The predominant component (65 %) of Siam resin is 1-(4'-hydroxy-3'-methoxyphenyl)-3-benzoate-1-propene (coniferyl benzoate, **9**) which is treated with pig liver esterase. This has been shown to yield 1-(4'-hydroxy-3'-methoxyphenyl)-propen-3-ol (coniferyl alcohol, **10**) which is used as a substrate for horse liver alcohol dehydrogenase to form coniferaldehyde [20]. As described before, only a simple treatment of coniferaldehyde with mild base is enough to make vanillin [3].
Lipoxygenase from soybeans (Soy LOX) has been successfully applied in converting 1-(4'-hydroxy-3'-methoxyphenyl)-propene (isoeugenol, **11**) and coniferyl benzoate to vanillin. Typically, isoeugenol (100 g/L) was converted for 10-15 %, yielding 10-15 g vanillin [22]. In the presence of O_2, Soy LOX converts fatty acids, and probably also isoeugenol and coniferyl benzoate into hydroperoxides which subsequently undergo scission of the alkyl chain [8] resulting in vanillin formation.

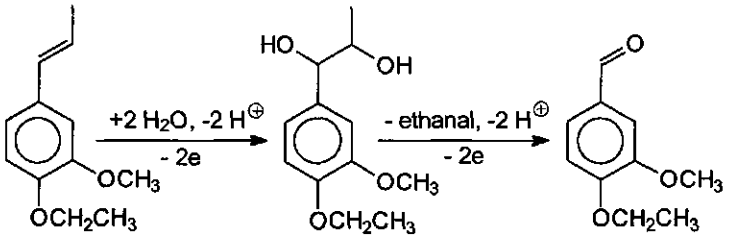
WHITE-ROT FUNGI AND LIGNIN OXIDIZING ENZYMES

White-rot fungi are the best degraders of lignin. This woodcomponent is made by the random polymerization of three monomeric phenolics of which coniferyl alcohol (**10**) is one.
The degradation of lignin to smaller fragments, such as vanillin [11], involves the enzymatically-initiated oxidation of the polymer and the subsequent chemical decomposition of the reactive intermediates to smaller fragments [28]. The enzymes used for vanillin production from lignin are non-specific and extracellular peroxidases and oxidases. The most important peroxidases are manganese peroxidase (MnP) and lignin peroxidase (LiP). MnP oxidizes Mn^{2+}, that is present in wood, to Mn^{3+} which acts as a diffusible oxidant, capable of oxidizing phenolic regions in lignin. MnP generally is not able to oxidize non-phenolic aromatic compounds directly [9]. The majority of moieties in lignin, however, are non-phenolic [1].

LiP is a much more potent peroxidase, capable of oxidizing non-phenolic aromatic compounds with a calculated ionization potential (IP) up to 9.0 eV [30]. The IP is defined as the energy needed to remove one electron from the highest occupied molecular orbital in these aromatic substrates. For this reason LiP is the preferable enzyme to use for this Ph.D.-project.

LIGNIN PEROXIDASE

LiP catalyzes the wanted C_α-C_β cleavage of alkyl side chains in non-phenolic aromatic compounds. This cleavage results in the formation of the corresponding benzaldehyde. An example of this reaction is the cleavage of 1-(4'-ethoxy-3'-dimethoxyphenyl)-propene which has been proposed to occur via a diol intermediate (see Scheme 1.2) [25].



SCHEME 1.2 C_α-C_β cleavage of 1-(4'-ethoxy-3'-dimethoxyphenyl)-propene by LiP based on the proposal by Renganathan et al. [25]

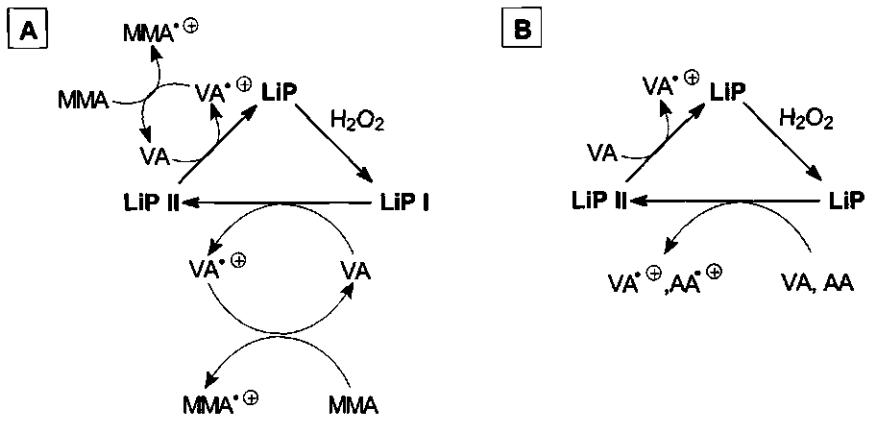
CHARACTERISTICS OF LIP

LiP from the white-rot fungus *Phanerochaete chrysosporium* has been described for the first time by Tien et al. in 1983 [31]. Additional studies showed that the activity is divided over a series of glycosylated isozymes with molecular masses ranging from 38 to 43 kDa. Each isozyme contains 1 mol of iron in the heme per mol of protein [9]. The LiP activity is generally measured spectrophotometrically by detecting the formation of 3,4-dimethoxybenzaldehyde (veratraldehyde, VAD) from 3,4-dimethoxybenzylalcohol (veratryl alcohol, VA) at pH 3.0. In the absence of O_2, this conversion requires the presence of 1 mol H_2O_2 for each mol of VAD produced [10].

ENZYMATIC ACTION OF LIP

In the catalytic cycle (see Scheme 1.3), native LiP is oxidized by H_2O_2 forming LiP compound I. Abstraction of one electron by LiP compound I from a suitable substrate like VA results in the formation of LiP compound II and a veratryl alcohol radical cation ($VA^{\cdot+}$ [12]). Also LiP compound II oxidizes VA by one electron closing the cycle. LiP compound II is less potent than compound I and therefore longer available to react with H_2O_2. This reaction yields an inactive form of LiP, LiP compound III [28,29].

Harvey et al. [13] proposed in 1986 that VA may act as a mediator during the oxidation of substrates that are difficult to oxidize such as 4-methoxybenzylalcohol (*p*-anisyl alcohol, AA) and 2-(4'-methoxyphenyl)-2-hydroxyethanoic acid (4-methoxymandelic acid, MMA).



SCHEME 1.3 **A) consumption of MMA via redox mediation by VA. B) consumption of AA in the presence of VA. VA closes the catalytic cycle.**

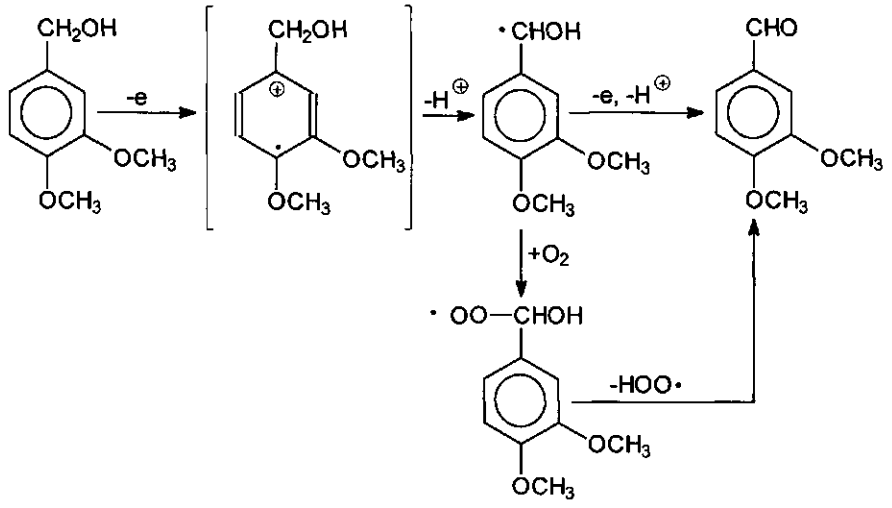
Addition of a little amount of VA enhanced the conversion of AA and MMA at least 4 and 15-fold, respectively. In this process LiP generates $VA^{\bullet+}$ which in its turn oxidizes AA or MMA. This proposal has been reexamined and proved to be correct for MMA [32]. In the case of AA, however, Koduri et al. [19] concluded that VA's stimulating effect on the AA consumption could be explained by VA closing the catalytic cycle. Their proposal implies that AA would only be converted by LiP compound I. The remaining LiP compound II, which is inert towards AA, is than reduced by VA (see Scheme 1.3 B). This mechanism, however, leaves the enhanced conversion of AA (4 mol of AA per mol of VA [13]) unexplained because according to Koduri's theory [19] only a maximal consumption of 1 mol of AA per mol of VA would be expected.

CHEMICAL DECOMPOSITION OF LIP GENERATED RADICAL CATIONS

There are several routes in which $VA^{\bullet+}$ may decompose. The main route to the predominant oxidation product VAD (70 %, [27]) involves VA-radical formation by the loss of a proton. The radical may be oxidized further by LiP yielding VAD. An alternative reaction route is the addition of O_2 to the VA radical to form a VA peroxyl radical which may decompose to VAD and superoxide (see Scheme 1.4) [28].

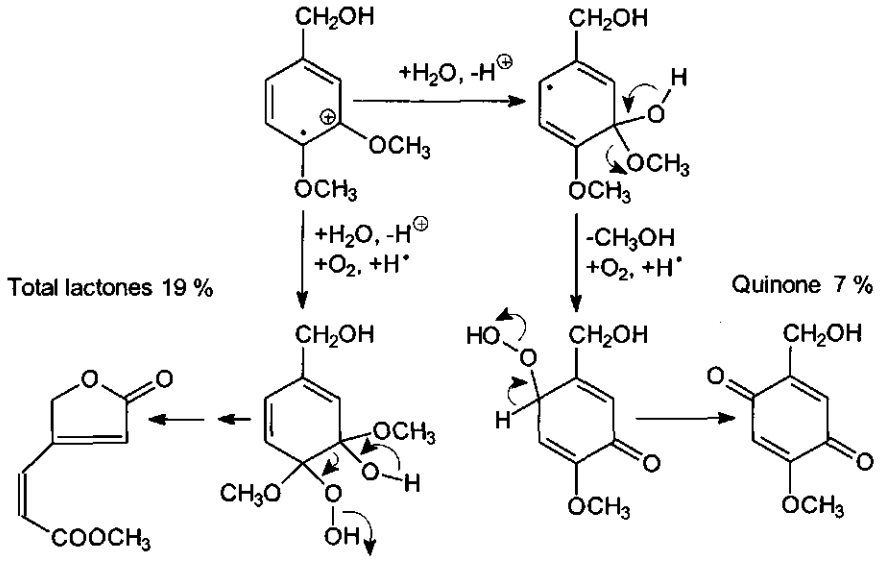
Peroxyl radicals may react in several manners. Among those are hydrogen abstraction, addition to double bonds, and decomposition via a tetroxide. Hydrogen abstraction by peroxyl radicals ($ROO^{\bullet}$) results in the formation of hydroperoxides (ROOH) [16]. These have been proposed to be

intermediates in side product formation from VA (see Scheme 1.5) [10] and the VA-methyl ether [27].



SCHEME 1.4 **The involvement of O_2 in VAD formation from VA by LiP.**

Such hydroperoxides have also been detected as products derived from the oxidation of sulfonated azo-dyes by LiP [4].

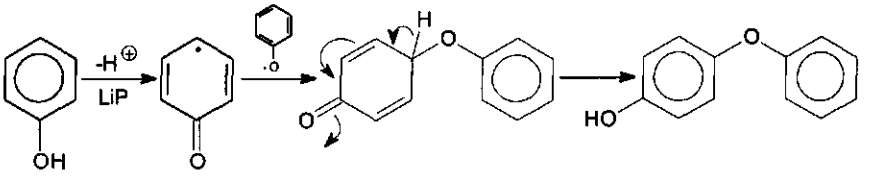


SCHEME 1.5 **Proposed hydroperoxides derived from VA and their involvement in the formation of side oxidation products.**

POLYMERIZATION OF PHENOLICS

The main disadvantage of applying LiP in vanillin production from cheap natural phenolics is the unwanted polymerization of the starting material. Upon oxidation of phenolics by LiP, phenoxy radicals are formed [14,26]. Resonance stabilizes the phenoxy radical and allows the unpaired electron to reach different sites. Together with the tendency of these radicals to couple, an enormous variety of oligomerisation products may be formed. The formation of oxidation products derived from several simple phenolics such as phenol [17], and 2-methoxyphenol (guaiacol) [5,14] and more complex phenolics as ferulic acid [26], and coniferyl alcohol [18] have been studied in detail.

Due to the coupling of two phenoxy radicals several dimers may be formed of which an example is depicted in Scheme 1.6. The dimers may be oxidized again, forming larger oxidation products [17].



SCHEME 1.6 Example of the LiP initiated dimerisation of phenol. The coupling of two phenoxy radicals results in an intermediate quinoid structure that undergoes tautomerisation, resulting in a dimer in which an ether group is present.

A polymer representing an average molecular weight between 5 and 15 kD has been identified as a guaiacol oxidation product. The proposed schematic structure of the polymer includes ortho-ortho and para-para (C-C) linked guaiacol moieties, with an occasional ortho-para link and some para-diphenoquinone structures [5].

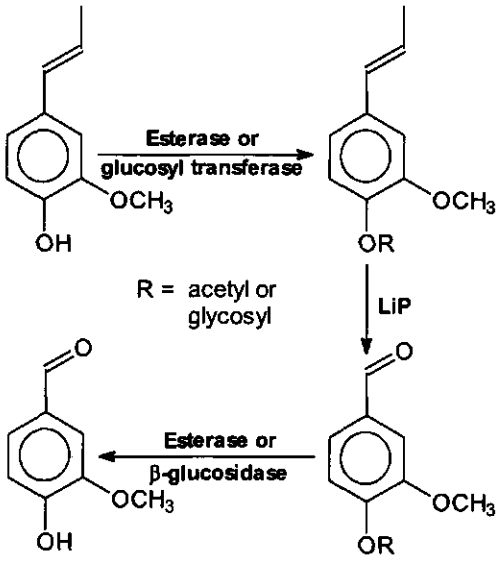
Coupling of the unpaired electrons at C_β of the ferulic acid derived phenoxy radical results in a side chain-coupling product. Besides this dimer much more different dimers have been identified [26].

In order to obtain synthetic lignin for biodegradation studies, coniferyl alcohol has been polymerised by a peroxidase resulting in an insoluble polymer. This polymer showed large resemblance with natural lignin [18].

To minimize these unwanted radical coupling reactions, protection of the phenolic group by easily removable groups has to be considered (see Scheme 1.7) [30]. An esterase or a glucosyl transferase would be necessary to modify the phenolic vanillin precursors. Thereafter, LiP may be used to cleave off the side chain. It is likely that this latter step will occur since than non-phenolic compounds as depicted in Scheme 1.2 are obtained which are readily cleaved by LiP to the corresponding benzaldehyde [24]. Finally, the protecting group would be removed by an esterase or a β-glucosidase.

OUTLINE OF THIS THESIS

This Ph.D.-study concentrated on biocatalytic conversions catalyzed by LiP that are useful in vanillin production. Chapter 2 shows the production of LiP by *Bjerkandera* sp. strain BOS55. The purification and characterization of two LiP isozymes are described in chapter 3. The predominant purified isozyme has been used to determine the IP-threshold value of non-phenolic aromatic compounds which are still oxidizable by LiP (chapter 4).



Scheme 1.7 **Possible route to produce vanillin from isoeugenol. First the phenolic vanillin precursor (here isoeugenol) is esterified or glycosylated by an esterase or a glucosyltranferase, respectively. Thereafter, LiP is used to cleave off the side chain. In the final step, the protecting group is removed by using an esterase for the ester or a β-glucosidase for the glucoside to obtain vanillin.**

An interesting finding was that isoeugenyl acetate was converted into vanillyl acetate in a reasonable molar yield. Therefore, the cleavage mechanism was studied in more detail as described in chapter 5. In Chapter 6, the cleavage mechanism of the methyl ether of isoeugenol is described which appeared to be quite distinct from the acetyl ester of isoeugenol.

LITERATURE

[1] Adler, E. (1977) *Wood Sci. Technol.* 11:169-218

[2] Bonnin, E.B., Lesage-Meessen, L., Asther, M., and Thibault, J-F (1999) *J. Sci. Food Agric.* 79:484-486

[3] Cheetham, P.S.J. (1993) *TIBTECH* 11:478-488

[4] Chivulka, M., Spadaro, J.T., and Renganathan, V. (1995) *Biochemistry* 34:7765-7772

[5] Crawford, R.L., Robinson, L.E., and Foster, R.D. (1981) *Appl. Environ. Microbiol.* **41**:1112-1116
[6] Dörnenburg, H., and Knorr, D. (1996) *Food Biotechnol.* **10**:75-92
[7] Fargues, C., Mathias, A., and Rodrigues, A. (1996) *Ind. Eng. Chem. Res.* **35**:28-36
[8] Gardner, H.W. (1991) *Biochim. Biophys. Acta* **1084**:221-239
[9] Gold, M.H., and Alic, M. (1993) *Micriobiol. Rev.* **57**:605-622
[10] Haemmerli, S.D., Schoemaker, H.E., Schmidt, H.W.H., and Leisola, M.S.A. (1987) *FEBS Lett.* **220**:149-154
[11] Hagedorn, S., and Kaphammer, B. (1994) *Annu. Rev. Microbiol.* **48**:773-800
[12] Harvey, P.J., Schoemaker, H.E., Bowen, R.M., and Palmer, J.M. (1985) *FEBS Lett.* **183**:13-16
[13] Harvey, P.J., Schoemaker, H.E., and Palmer, J.M. (1986) *FEBS Lett.* **195**:242-246
[14] Harvey, P.J., Gilardi, G-F, Goble, M.L., and Palmer, J.M. (1993) *J. Biotechnol.* **30**:57-69
[15] Huang, Z., Dostal, L., and Rosazza, J.P.N. (1993) *J. Biol. Chem.* **268**:23954-23958
[16] Ingold, K.U. (1969) *Acc. Chem. Res.* **2**:1-9
[17] Joo, H., Yoo, Y.J., and Dordick, J.S. (1998) *Korean J. Chem. Eng.* **15**:362-374
[18] Kirk, T.K., Connors, W.J., Bleam, R.D., Hackett, W.F., and Zeikus, J.G. (1975) *Proc. Nat. Acad. Sci. USA* **72**:2515-2519
[19] Koduri, R.S., and Tien, M. (1994) *Biochemistry* **33**:4225-4230
[20] Leffemberg, C., Gautier, A., and Ohleyer, E. (1992) *Appl. Biochem. Biotechnol.* **37**:43-52
[21] Lesage-Meesssen, L., Haon, M., Delattre, M., Thibault, J-F, Colonna, B., and Asther, M., (1997) *Appl. Microbiol. Biotechnol.* **47**:393-397
[22] Markus, P.H., Peters, A.L.J., and Roos, R. (1992) Eur. Pat. 0 542 348 A2
[23] Prince, R.C., and Gunson, D.E. (1994) *TIBS* **19**:521-521
[24] Ranadive, A.S. (1992) *J. Agric. Food Chem.* **40**:1922-1924
[25] Renganathan, V., Miki, K., and Gold, M.H. (1985) *Arch. Biochem. Biophys.* **241**:304-314
[26] Rosazza, J.P.N., Huang, Z., Dostal, L., Volm T., and Rousseau, B. (1995) *J. Ind. Microbiol.* **15**:457-471
[27] Schmidt, H.W.H., Haemmerli, S.D., Schoemaker, H.E., and Leisola, M.S.A. (1989) *Biochemistry* **28**:1776-1783
[28] Schoemaker, H.E. (1990) *Recl. Trav. Chim. Pays-Bas* **109**:255-272
[29] Schoemaker, H.E., and Leisola, M.S.A. (1990) *J. Biotechnol.* **13**:101-109
[30] ten Have, R., Rietjens, I.M.C.M., Hartmans, S., Swarts, H.J., and Field, J.A. (1998) *FEBS Lett.* **430**:390-392
[31] Tien, M., and Kirk, T.K. (1983) *Science* **221**:661-663
[32] Tien, M., and Ma, D. (1997) *J. Biol. Chem.* **272**:8912-8917
[33] Westcott, R.J., Cheetham, P.S.J., and Barraclough, A.J. (1994) *Phytochemistry* **35**:135-138

2 Interference of peptone and tyrosine with the lignin peroxidase assay

Rimko ten Have, Sybe Hartmans, and Jim A. Field

The N-unregulated white-rot fungus, *Bjerkandera* sp. strain BOS55, was cultured in 1 L peptone-yeast extract medium to produce lignin peroxidase (LiP). During the LiP assay the oxidation of veratryl alcohol (VA) to veratraldehyde (VAD) was inhibited due to tyrosine present in peptone and yeast extract.

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INTRODUCTION

The N-unregulated white-rot fungus *Bjerkandera* sp. strain BOS55 produces several extracellular enzymes to cause extensive degradation of lignin. Among these is lignin peroxidase (LiP) that uses veratryl alcohol as both a cofactor and substrate. Veratryl alcohol (VA) can participate in the catalytic cycle of LiP which involves two succesive one-electron oxidations of VA. Veratraldehyde is predominantly formed through a one-electron-oxidized intermediate, the veratryl radical cation ($VA^{\cdot+}$) [5,6]. $VA^{\cdot+}$ can oxidize other compounds such as phenols [2,3,7] and consequently it is reduced back to VA.
Veratryl alcohol is commonly added to cultures of white-rot fungi to increase the LiP activity, but in the case of *Bjerkandera* spp., LiP is only produced at high levels when these fungi are cultured on a high-nitrogen medium and not under nitrogen-limiting conditions. The best results are obtained with mycological peptone and yeast extract [4,9].

In this study BOS55 was cultured on 1 L peptone-yeast extract medium to obtain large amounts of LiP. We observed that the LiP activity could be greatly enhanced by precipitating the LiP proteins with $(NH_4)_2SO_4$. Since this indicated that medium components interfered with the LiP assay, we decided to determine which components in the culture broth were responsible for this effect.

MATERIALS AND METHODS

BOS55 was grown on standard peptone-yeast extract medium minus manganese [8,9]. Polyurethane foam cubes, that were completly covered with the fungus, were used as an inoculum. This inoculum was prepared in a Petri dish containing 15 mL of growth medium (without 2,2-dimethylsuccinate and VA) and five polyurethane foam cubes (length : width : height = 1:1:1 cm, ρ= 35 kg/m^3). One foam cube was used per culture.
BOS55 was grown statically in the dark at 30°C in 1 L medium in an Erlenmeyer flask (5 L) that contained a magnetic stirring bar. Passive aeration was assured by a beaker which was put up side down on top of the Erlenmeyer flask in stead of using cotton wool plugs. Before sampling the culture fluid was stirred, a 10 mL sample was then was removed and stored at -20°C. This storage procedure did not affect the LiP activity.

LiP proteins in the crude samples were precipitated by addition of $(NH_4)_2SO_4$ (80%, 0 °C) followed by centrifugation (10,000 *g*, 15 min, 0 °C). The pellet was dissolved in twice the original volume demineralized water. The LiP activity was measured in this sample and compared to a sample to which the $(NH_4)_2SO_4$ was not added.
The LiP activity then was measured spectrophotometrically (λ = 310 nm, T= 30 °C) by monitoring the production of VAD (ε= 9300 M^{-1} cm^{-1} [11]). The reaction mixture (0.5 mL) contained 50 mM tartrate (pH 3.0), 2 mM veratryl alcohol and 50 µL sample. The LiP activity was corrected for

interferences prior to the addition of H_2O_2 (0.5 mM [10]), which was used to initiate the LiP assay. The LiP activity is expressed in U/L (1 U forms 1 μmol VAD·min^{-1}).
The effect of peptone, L-tyrosine and L-phenylalanine (Acros, Geel, Belgium) on the LiP activity was studied using a partially purified preparation of LiP from *P. chrysosporium* (100,000 U/L), obtained from Tienzyme, Inc. (State College, PA, U.S.A). In the assay mixture this LiP was diluted 4000 and 8000 times. Also a purified LiP preparation from *Bjerkandera* sp. strain BOS55 was used for these experiments and for recording the VIS-spectra of the heme region.

RESULTS

In an experiment aimed at increasing LiP production on a 1L scale, *Bjerkandera* sp. strain BOS55 was cultured on peptone-yeast medium [8,9]. During the course of the experiment, LiP in the extracellular culture fluid was assayed by measuring the formation of veratraldehyde (VAD) from VA. However, unlike assays performed with semipurified preparations of LiP, the formation of VAD showed a biphasic pattern. Figure 2.1, line b shows a typical example of the LiP assay with the extracellular fluid collected on day 14. There is an initial slow increase in the E_{310} lasting about 70 s in the first period (I), followed by a second period (II) in which the increase in E_{310} was significantly higher.

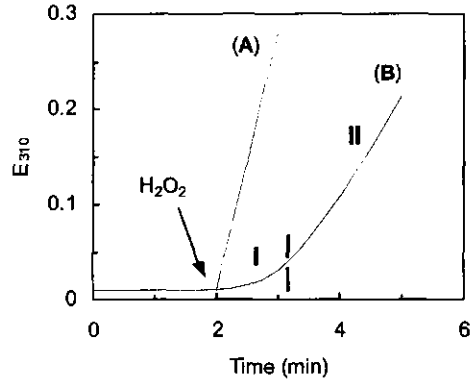

FIGURE 2.1 A typical VAD formation pattern observed during the LiP assay with a resuspended 80% $(NH_4)_2SO_4$ protein precipitate (line a), and with extracellular culture fluid (line b) sampled on day 14 of a 1 L culture of *Bjerkandera* sp. strain BOS55. Line b shows the biphasic character consisting of phase (I), the lag phase, and phase (II) which was used to calculate the LiP activity.

This second slope was used to calculate the LiP activity. However, if LiP in the extracellular culture fluid was precipitated with 80% $(NH_4)_2SO_4$ and resuspended, the LiP assay (line a) typically showed a minimized lag phase and the rate of VAD formation was significantly higher than the

maximal rate observed with the crude sample. From these observations we concluded that compounds in the culture fluid inhibited LiP during the assay.

To study this effect as a function of culture age, both the crude extracellular culture fluid and the $(NH_4)_2SO_4$ precipitate were assayed over a period of 30 days. Figure 2.2A shows that the LiP activity in the precipitated samples first appeared on day 8, reaching a maximum activity of approximately 600 U/L on day 14 after which it remained constant until the end of the experiment (day 30). In contrast, in the crude samples no activity could be measured before day 12. From day 14 to day 18, LiP was inactivated to some extent. From day 20 onward, the LiP activity in the crude samples and in the treated samples were comparable. These findings together suggested that medium components were responsible for the interference of the LiP assay.

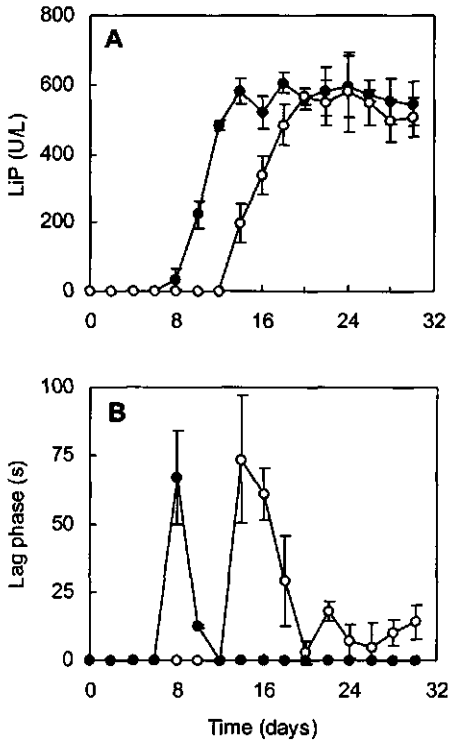

FIGURE 2.2 Panel A shows LiP activity (U/L) measured directly in the extracellular culture fluid of *Bjerkandera* sp. strain BOS55 (○) and in the resuspended 80% $(NH_4)_2SO_4$ protein precipitate (●) versus culture time (days). Panel B shows the lag phases observed during the LiP assay of the extracellular culture fluid (○) and the resuspended 80% $(NH_4)_2SO_4$ protein precipitate (●). The measurements are an average of three parallel cultures ± SEM.

Figure 2.2B shows the duration of the lag phase as a function of cultivation time. In the untreated samples, long lag phases were observed

between day 14 to 18 which were proportional to the observed losses of LiP activity. During this period the precipitated samples showed a constant LiP activity and no lag phase.

Several medium components were tested like 2,2-dimethylsuccinate and the BIII mineral solution, but they did not inhibit LiP, leaving peptone and yeast extract as the most logical components in the medium to study. For these studies, a commercially available partially purified LiP preparation from *Phanerochaete chrysosporium* was used. The same experiments were also carried out using a purified LiP preparation from *Bjerkandera* sp. BOS55. The results (not shown) were the same as those obtained with LiP from *P. chrysosporium*.

Both yeast extract and peptone were found to inhibit LiP, but yeast extract inhibited LiP to a much lower extent. Furthermore, it occurs only as a minor medium component (1 g/L) when compared to peptone (5 g/L) and for this reason we decided to study the inhibition of peptone in more detail.

The effect of peptone at different concentrations on the activity of the semipurified LiP was tested at two enzyme concentrations in the assay mixture, 13.7 and 27.4 U/L. Figure 2.3 shows that complete inhibition occured at 39 and 78 mg/L peptone for the low and high LiP concentrations, respectively.

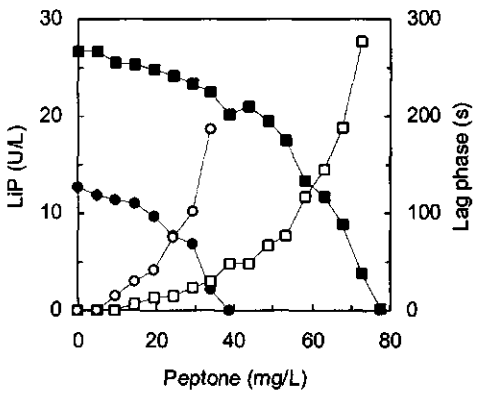

FIGURE 2.3 The effect of peptone (mg/L) on LiP from *Phanerochaete chrysosporium* (27.4 U/L (■) and 13.7 U/L (●) in the assay mixture). The open circles and squares show the lag phases as observed during the LiP assay.

Increasing LiP inhibition was associated with increasing lag phase duration, which is similar to the pattern observed in the fungal culture (Figure 2.2A and B).

To explain the inhibitory effect of peptone, we examined the inhibitory effect of L-tyrosine on LiP because the pattern of inhibition showed clear similarities to that reported for other phenolic compounds [2,3,7]. We observed a similar pattern as observed in Figure 2.3 (not shown) and found that LiP was completely inhibited at 10 and 18 μM L-tyrosine for the low and high LiP concentrations, respectively. As a control, we tested

the effect of the non-phenolic analogue of L-tyrosine, L-phenylalanine. The latter did not inhibit LiP.
Peptone and L-tyrosine appeared to inhibit LiP by the same mechanism as evidenced by the fact that in both cases the relationship between the decrease in LiP activity and lag phase was linear and highly correlated (see Figure 2.4).

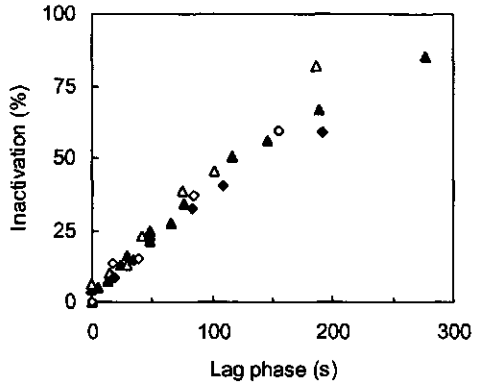

FIGURE 2.4 The relationship between the observed decrease in LiP activity (%) and the lag phase. In the presence of peptone (triangle, y= 0.344x + 5.095, R^2= 0.9579) and L-tyrosine (rectangle, y= 0.334x + 3.226, R^2= 0.9641) using data from both LiP concentrations 27.4 U/L (closed figures) and 13.7 U/L (open figures).

To study this mechanism in more detail we recorded VIS-spectra of the LiP heme group during the assay. Figure 2.5 shows spectra recorded seven minutes after addition of H_2O_2. After this time, the spectra did not change substantially. The LiP heme group absorbs light in the region between 380 - 430 nm (Fig. 2.5 A).

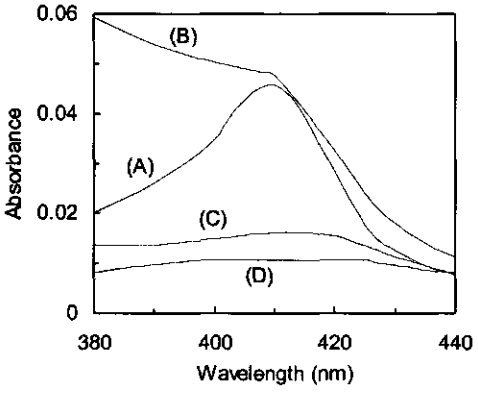

FIGURE 2.5 VIS-spectra of LiP from *Bjerkandera* sp. strain BOS55 taken after seven minutes. (A) just with water and without H_2O_2, (B) in the presence of veratryl alcohol and H_2O_2, (C) veratryl alcohol + L-tyrosine and H_2O_2, and (D) just with H_2O_2.

When LiP is incubated with just H_2O_2 the heme peak in the VIS-spectrum disappears (Fig. 2.5 D). This is an indication for bleaching of the heme. In the presence of both VA and H_2O_2 we observed a temporary shift of the heme peak to 418 nm. This indicated formation of LiPII/III. After seven minutes the heme peak was back in its original position (Fig. 2.5 B). This illustrated that VA protects LiP from irreversible inactivation by H_2O_2. Incubation of L-tyrosine, VA, H_2O_2 and LiP reduced the absorbance of the heme substantially (Fig. 2.5 C). This is explained by irreversible bleaching of the heme by H_2O_2, since almost the same thing happened in the presence of just H_2O_2 (Fig. 2.5D).

DISCUSSION

The mechanism of VAD production by LiP involves the formation of a veratryl radical cation ($VA^{\cdot+}$) which is an essential intermediate. During the lag phase $VA^{\cdot+}$s, generated by LiP, are very effectively reduced back to VA by phenolics until the latter are completely oxidized [3,7]. The oxidation of the phenolics and subsequent reduction of $VA^{\cdot+}$s is observed as a lag phase in which VAD is not produced. This mechanism explains the observed lag phase as observed in our experiments.
The rate limiting step in the catalytic cycle of LiP is the reduction of LiPII back to native LiP. LiPII can easily react with H_2O_2 forming inactive LiPIII. $VA^{\cdot+}$s can overcome accumulation of LiPIII since they revert it back to active ferric LiP [1]. In the presence of phenolics, however, the $VA^{\cdot+}$s are reduced and as a consequence of this, LiP compound III accumulates during the lag phase [3]. LiPIII can react with excess H_2O_2, resulting in irreversible inactivation of the enzyme by bleaching of the heme [2]. This theory fits well with our findings that the heme peak in the VIS-spectrum disappears during incubation of LiP with either VA+L-tyrosine+H_2O_2 or just H_2O_2 alone.

Thus, L-tyrosine present in peptone and yeast extract inhibits the oxidation of VA to VAD by LiP from both *P. chrysporium* and *Bjerkandera* sp. strain BOS55, and a secondary consequence is the inactivation of LiP by H_2O_2 resulting in an underestimation of the LiP activity.
There is also indirect evidence that peptides containing tyrosine inhibit LiP since the long lag phase observed at day 8 (see Figure 2b) implies that some peptides, precipitated with $(NH_4)_2SO_4$, also inhibited LiP. The observed lag phase was not due to the presence of free tyrosine, because amino acids are not precipitated using this method.

LITERATURE

[1] Barr, D.P., and Aust, S.D. (1994) *Arch. Biochem. Biophys.* **311**:378-382.
[2] Chung, N., and Aust, S.D. (1995) *Arch. Biochem. Biophys.* **316**:733-737.
[3] Chung, N., and Aust, S.D. (1995) *Arch. Biochem. Biophys.* **316**:851-855.
[4] Kaal, E.E.J., de Jong, E., and Field, J.A. (1993) *Appl. Environ. Microbiol.* **59**:4031-4036.

[5] Khindaria, A., A.T. Grover, and Aust, S.D. (1995) *Biochemistry* **34**:6020-6025.
[6] Khindaria, A., I. Yamazaki, and Aust, S.D. (1995) *Biochemistry* 34:16860-16869.
[7] Koduri, R.S., and Tien, M. (1995) *J. Biol. Chem.* **270**:22254-22258.
[8] Mester, T., E. de Jong, and Field, J.A. (1995) *Appl. Environ. Microbiol.* **61**:1881-1887.
[9] Mester, T., M. Peña, and Field, J.A.. (1996) *Appl. Microbiol. Biotechnol.* **44**:778-784.
[10] Tien M., and Kirk, T.K. (1988) *Meth. Enzymol.* **161B**:238-248.
[11] Tien, M., and Kirk, T.K. (1983) *Science* **221**:661-663.

3 Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55

Rimko ten Have, Sybe Hartmans, Pauline J.M. Teunissen, and Jim A. Field

The white-rot fungus *Bjerkandera* sp. strain BOS55 excretes at least seven lignin peroxidase (LiP) isozymes. Two of these, LiP-2 and LiP-5 (Mw 40-42 kD), were purified to homogenity. Both isozymes had the same N-terminal amino acid sequence which showed strong homology with LiP isozymes produced by other white-rot fungi. The kinetics of both isozymes were similar. LiP-5 oxidized veratryl alcohol optimally only in the presence of H_2O_2 near pH 3.0 (16.7 U/mg) and LiP-2 did this below pH 2.5 (33.8 U/mg). Also at normal physiologal pH's for fungal growth (pH 5.0-6.5) both isozymes were still active. Further characterization of LiP-2 and LiP-5 revealed that the K_m for H_2O_2 strongly decreased with increasing pH. As a result of this the catalytic efficiency (TN/K_m), calculated on the basis of the K_m for H_2O_2, in the oxidation of veratryl alcohol was constant over wide pH range.

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INTRODUCTION

White-rot fungi can degrade nature's second most abundant polymer, lignin. This polyaromatic polymer gives woody plants their strength. White-rot fungi excrete peroxidases and H_2O_2-generating enzymes which work together to initiate lignin oxidation [2,3]. The most potent peroxidase, having the capability to oxidize substrates with a high ionisation potential, is lignin peroxidase (LiP) [9]. This enzyme uses small aromatic fungal metabolites such as veratryl alcohol (VA) [15,22], and 2-chloro-1,4-dimethoxybenzene [17,18] as a cofactor [19].

White-rot fungi have been used to degrade a large variety of organic pollutants such as polycyclic aromatic hydrocarbons (PAH's). A comparison of the PAH-degrading ability of several strains of white-rot fungi revealed that *Bjerkandera* sp. strain BOS55 was superior [4]. The latter strain also was shown to be among the best biobleachers [16]. *Bjerkandera* sp. BOS55 excretes LiP and manganese peroxidase (MnP) as the major oxidative enzymes [5,15]. This study concentrates on the purification and characterization of two lignin peroxidase isozymes from strain BOS55.

MATERIALS AND METHODS

Bjerkandera sp. strain BOS55 was cultured on 1 liter nitrogen-rich glucose medium (5 g/l mycological peptone) as described earlier [5]. As an inoculum 10 agar plugs with BOS55 were used. After 20 days of culturing in the dark at 30°C, the extracellular culture fluid was harvested.

LiP ACTIVITY MEASUREMENT

The LiP activity was measured by recording the increase in absorbance at 310 nm (T= 30°C) owing to the oxidation of veratryl alcohol (VA) to veratraldehyde (VAD) by LiP (ε= 9,300 M^{-1} cm^{-1}). The reaction mixture contained 100 mM sodium tartrate pH 3.0, 2 mM VA, LiP, and H_2O_2 (0.5 mM) which was used to initiate the reaction.

The K_m for H_2O_2 was determined with 2 mM VA in the incubation mixture. The K_m for VA and 2-chloro-1,4-dimethoxybenzene was determined using 0.5 mM H_2O_2 in the incubation mixture. The formation of the predominant product 2-chloro-1,4-benzoquinone from 2-chloro-1,4-dimethoxybenzene was measured at a wavelength of 255 nm (T= 30°C) using a molar extinction coefficient of 16,900 M^{-1} cm^{-1}.

Inactivation of LiP by H_2O_2 was studied by incubating LiP, sodium tartrate buffer pH 3.0 or 5.0, and 0.125 mM H_2O_2 at 30°C. To analyze the remaining LiP activity the complete mixture was mixed with VA (final concentration 2 mM) and H_2O_2 (final concentration 0.5 mM).

ARYL ALCOHOL OXIDASE (AAO) AND MANGANESE PEROXIDASE (MnP) MEASUREMENTS

AAO activity was measured at pH 6.0 in 100 mM sodium acetate buffer using VA as a substrate. Formation of VAD was followed at a wavelenght of 310 nm.

MnP activity was determined at a wavelenght of 468 nm by measuring the oxidation of 2,6-dimethoxyphenol (ε= 49,600 M^{-1} cm^{-1}). The incubation conditions were: 100 mM malonate buffer pH 4.5, 2 mM $MnSO_4$, 0.5 mM H_2O_2, 0.5 mM 2,6-dimethoxyphenol, and sample.

PURIFICATION OF LIGNIN PEROXIDASE

The culture fluid was filtered through cheese cloth to remove fungal mycelium. At 0°C, $(NH_4)_2SO_4$ was added to the filtrate to 85 % saturation. The precipitated crude LiP fraction was recovered by centrifigation (10,000 x *g*, 0°C, 10 minutes). The pellet was resuspended in demi water and dialysed several times against 20 mM succinate pH 3.5 for two days at room temperature prior to using a FPLC cation exchange column (column material: Source 15S, Pharmacia, column dimensions: h= 7.0 cm, d= 1.5 cm). LiP was eluted using a salt gradient (0-400 mM NaCl in 30 minutes in 20 mM succinate pH 3.5, 3.0 ml/min). The LiP fraction was dialysed several times against 10 mM NaAc pH 6.0 at room temperature. The different LiP isozymes present in this fraction were separated using a FPLC anion exchanger (column material: Source 15Q, Pharmacia, column dimensions: h= 6.0 cm, d= 0.5 cm). The applied salt gradient increased from 10 mM to 450 mM NaAc pH 6.0 in 45 minutes at a flowrate of 1.0 ml/min.

Two collected LiP fractions were pure LiP isozymes as confirmed by the symmetrical peak eluting from the FPLC Source 15Q column. Furthermore, these fractions gave one single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 10 % acrylamide). The protein content was determined using the standard Lowry method with BSA as a standard.

N-TERMINAL SEQUENCE

The first 25 amino acids of the N-terminal sequence of both LiP isozymes were determined by Dr. R. Amons (Sylvius Laboratoria, Leiden, The Netherlands).

RESULTS

PURIFICATION OF LIP ISOZYMES

The purification results in Table 1 show that the culture broth contains a high proportion of contaminating peptides and proteins. Ammonium sulphate precipitation reduces this amount considerably and also removes peptides which interfere with the LiP assay [5]. This results in an apparent increase of total LiP activity of a factor 3.

During dialysis at pH 3.5, LiP was not as stable as anticipated, explaining the loss of activity observed after this step. In the cation exchange step, only lignin peroxidase (LiP) isozymes appear to bind to the column. The mixture of LiP isozymes is eluted as one single peak.

TABLE 3.1 **Purification of two lignin peroxidase isozymes from the extracellular culture broth (CB) of *Bjerkandera* BOS55. AS= $(NH_4)_2SO_4$ precipitation, D= dialysis, CE= cation exchange column, and AE= anion exchange column.**

Step	Volume (ml)	Activity (U)	Protein (mg)	Spec. Act. (U/mg)	Recovery (%)	Pur. fact.
CB	1860	446	5494	0.1	-	1
AS	48.4	1388	739	1.9	100	19
D	65.5	511	530	1.0	36.8	10
CE	4.64	166	32.1	5.2	12.0	52
AE LiP-2	4.6	28.3	1.7	16.7	2.0	167
LiP-5	4.6	25.7	1.4	18.2	1.9	182

An anion exchange column was used to separate the different LiP isozymes. Figure 1 shows the elution profile obtained with a FPLC Source 15Q column.

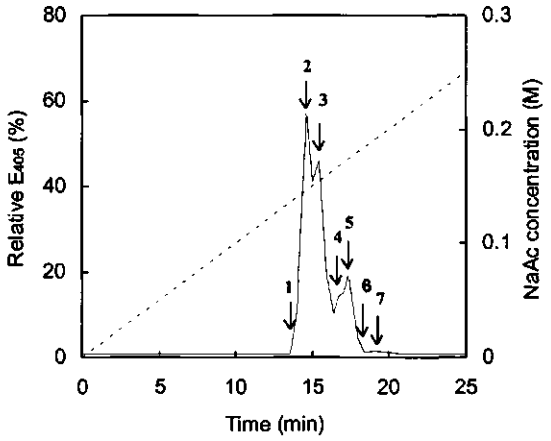

FIGURE 3.1 **The elution profile of LiP isozymes (1-7) produced by *Bjerkandera* sp. strain BOS55 from a Source 15Q FPLC column. The dashed line shows the salt gradient.**

The extinction at 405 nm shows the presence of seven heme proteins which were designated as LiP isozymes by measuring the H_2O_2 dependent veratryl alcohol (VA) oxidizing acitivity. The collected fractions LiP-2 and LiP-5 showed no contamination of aryl alcohol oxidase (AAO) and/or manganese peroxidase (MnP). Furthermore, both isozymes gave one band on SDS-PAGE. Finally, more than 1 mg of each pure LiP isozyme was obtained which was sufficient for further characterization.

CHARACTERIZATION OF LIP ISOZYMES

Both isozymes (LiP-2 and LiP-5) have the same molecular weight of approximately 40 to 42 kD. Their A_{407}/A_{280} ratio were 5.3 and 4.2, respectively. The VIS-spectrum showed a strong absorbance at 407 nm

and weak absorbances at 508 and 638 nm which is characteristic for native LiP isozymes.
Figure 3.2 shows that LiP-5 oxidizes VA optimally near pH 3.0. The decrease in V_{max} at pH values below 3.0 suggests that this isozyme denaturates under these conditions.

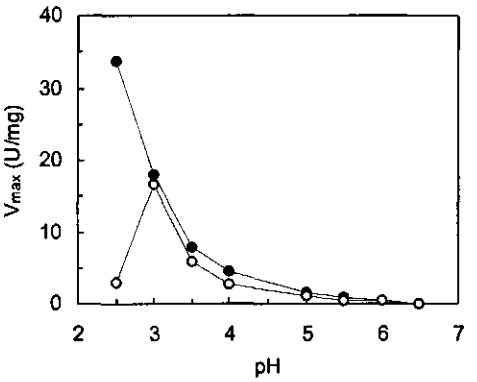

FIGURE 3.2 The effect of the pH on the V_{max} (U/mg) of LiP oxidizing VA studied using both LiP-2 (●) and LiP-5 (○). Experimental conditions: 2.0 mM VA, 100 mM sodium tartrate pH 3.0-5.0 / 100 mM sodium acetate pH 5.0-6.5, H_2O_2= 0.5 mM, and T= 30°C.

The pH-optimum of LiP-2 is even below pH 2.5 (33.8 U/mg). Both isozymes could still oxidize VA at high pH values of 5.0 to 6.5 (V_{max} of LiP-2: 1.7 - 0.1 U/mg) which are of relevance in relation to normal fungal culture conditions. This differs from LiP from *Phanerochaete chrysosporium* which had no appreciable activity at pH values greater than 5.0 [20].

Table 3.2 shows that the K_m of LiP-2 and LiP-5 for veratryl alcohol (VA) increases by raising the pH from 3.0 to 5.0.

TABLE 3.2 The kinetic parameters, K_m (μM) and V_{max} (U/mg) determined at different pH's using two fungal metabolites veratryl alcohol (VA) and 2-chloro-1,4-dimethoxybenzene. Conditions: Tartrate buffer (100 mM, pH 3.0-5.0), 0.5 mM H_2O_2, T=30°C.

	Bjerkandera BOS55 LiP-2				*Bjerkandera* BOS55 LiP-5			
	Veratryl alcohol		2-Cl-1,4-DMB		Veratryl alcohol		2-Cl-1,4-DMB	
pH	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
3.0	89	15.9	n.d.	n.d.	59	13.9	318	2.0
4.0	123	7.5	106	0.4	107	5.6	198	0.5
5.0	190	1.5	-	-	131	0.9	-	-

n.d.: no data, and -: 2-chloro-1,4-dimethoxybenzene was not oxidized by LiP at pH 5.0.

The data suggests that VA binding to LiP is stronger at pH 3.0 than at pH 5.0. Since the turnover number (TN) of LiP-2 decreases from 11.1 s^{-1} at pH 3.0 to 1.1 s^{-1} at pH 5.0 and the K_m increases, the catalytic efficiency

(TN/K_m) decreases from 1.2 x 10^5 at pH 3.0 to 5.8 x 10^3 M^{-1} s^{-1} at pH 5.0. These values are in the same range as those reported for purified LiP from *Phanerochaete chrysosporium* [20]. Both LiP isozymes also oxidized the fungal metabolite 2-chloro-1,4-dimethoxybenzene. The K_m of this compound at pH 4.0 was in the same range as that for VA, but the V_{max} was about 10 to 20-fold lower.

Figure 3.3 shows that the K_m for H_2O_2, measured using both the predominant isozyme (LiP-2) and the minor isozyme (LiP-5), strongly depends on the pH.

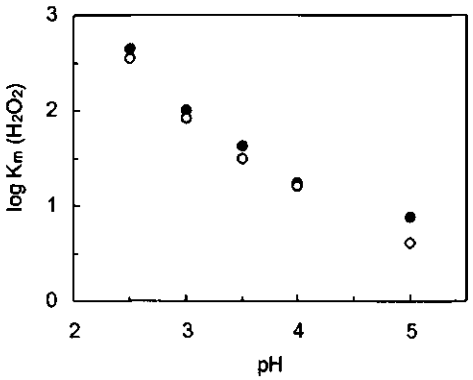

FIGURE 3.3 The logarithm of the K_m for H_2O_2 of LiP-2 (●) and LiP-5 (○) at different pH's. Conditions: 2.0 mM VA, 100 mM sodium tartrate pH 3.0-5.0, and T=30°C.

The linear relationship between log K_m and pH suggest that a single acid group is responsible for the binding of H_2O_2. At pH 2.5 the K_m (442 μM) of LiP-2 is about 60 times higher than at pH 5.0 (K_m= 7.5 μM). Comparison of the K_m values at pH 4.0 (LiP-2= 17.2 μM, and LiP-5= 15.7 μM) with literature data shows that these values are similar to those reported for LiP H1 and H8 produced by *Phanerochaete chrysosporium*. The K_m for H_2O_2 of other *P. chrysosporium* LiP isozymes are higher, but in the same range [3].
The catalytic efficiency of LiP using H_2O_2 (TN/K_m) during the oxidation of VA is constant in a broad pH range from 3.0 to 5.0 (values were between 1.2 to 2.6 x 10^5 M^{-1} s^{-1}) which is comparable to LiP produced by *P. chrysosporium* [20].

INACTIVATION OF LIP BY H_2O_2

Lignin peroxidase uses H_2O_2 for its catalysis, but it is also known to be inactivated by H_2O_2 [21]. Figure 3.4 shows that at pH 3.0 LiP-2 is almost instantly inactivated in the absence of VA whereas, at pH 5.0 activity can still be detected after 15 minutes. The VIS-spectrum of the heme region (not shown) showed that LiPII and/or LiPIII [23] were present at least

20 minutes at pH 5.0. At pH 3.0, however, the heme was completely bleached within 3 minutes.

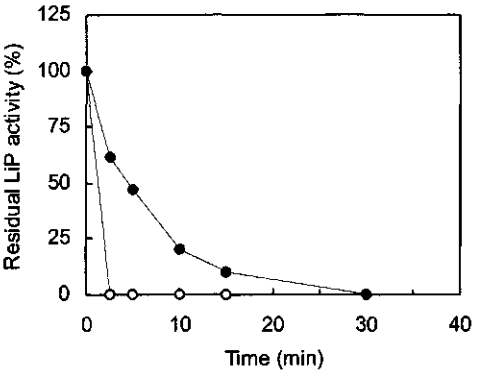

FIGURE 3.4 Inactivation of the major isozyme LiP-2 at pH 3.0 (○) and 5.0 (●) in the presence of 0.125 mM H_2O_2 at 30°C. Residual activity was measured by mixing the incubated LiP with a solution of 2 mM VA, 0.5 mM H_2O_2 and 100 mM sodium tartrate pH 3.0.

N-TERMINAL AMINO ACID SEQUENCE COMPARISON

The N-terminal sequences were determined to allow comparison with literature N-terminal sequences of LiP isozymes produced by other fungi see Table 3.3).

TABLE 3.3 Comparison of the N-terminal amino acid sequence of LiP-2 and LiP-5 with those reported for selected LiP-isozymes produced by other white-rot fungi. BOS55= *Bjerkandera* sp. strain BOS55, B.a.= *Bjerkandera adusta*, P.c.= *Phanerochaete chrysosporium*, T.v.= *Trametes versicolor*.

LiP isozyme	N-terminal sequence	Literature
BOS55 LiP-2	V-A-C-P-D-G-R-H-T-A-I-N-A-A-C-C-N-L-F-T-V-R-D-D-I	this work
BOS55 LiP-5	V-A-C-P-D-G-R-H-T-A-I-N-A-A-C-C-N-L-F-T-V-R-D-D-I	this work
B.a. LPO-3	V-A-C-P-D-G-R-N-T-A-I-N-A	[11]
B.a. LPO-1	V-A-C-P-D-G-K-N-T-A-I-N-A-A-C-C-S-L-F-T-A-R-D-D-I	[11]
B.a. LPO-2	V-A-C-P-D-G-K-N-T-A-I-N-A	[11]
P.c. H1	V-A-C-P-?-G-V-H-T-A-S-N-A-A-C-C-A	[7]
P.c. H2	V-A-C-P-D-G-V-H-T-A-S-N-A-A-C-C-A	[7]
T.v. LP12	V-A-C-P-D-G-V-N-T-A-T-N-A-A-C-C-Q-L-F-A-V-R-E-D-L	[6,8]

The two sequences for LiP-2 and LiP-5 were identical, and showed a high degree of homology with the N-terminal sequence of the three LiP isozymes, LPO-1, LPO-2, and LPO-3, produced by *Bjerkandera adusta* [12]. Especially LPO-3 showed a high degree of homology; only one amino acid was different when the 12 N-terminal amino acids are compared.

DISCUSSION

Two different LiP isozymes have been purified to homogenity. The N-terminal region showed high homology towards LiP isozymes produced by

other white-rot fungi especially to those produced by *Bjerkandera adusta* [12].

Both VA and H_2O_2 are extremely important in physiological oxidation reactions catalyzed by LiP. Native LiP reacts with H_2O_2 forming LiP compound I which can indirectly oxidize substrates by first oxidizing VA to the corresponding radical cation ($VA^{\cdot+}$). The latter can in turn oxidize phenolics and organic acids [1,13]. Furthermore, VA may act as a substrate for LiP compound II allowing LiP to complete its catalytic cycle [10]. The combination of these two roles makes VA important in the LiP catalyzed oxidation reactions necessary for the degradation of polycyclic aromatic hydrocarbons (PAH's) and lignin.
Bjerkandera sp. strain BOS55 produces H_2O_2, but the physiological concentration is very low. In liquid media with BOS55, the H_2O_2 concentration is relatively constant at 5 μM H_2O_2 [14]. For *P. chrysosporium* similar values have been reported [21]. These low H_2O_2 levels suggest that the fungal peroxidases should be very efficient in using their H_2O_2 at physiological pH's. As can be seen in Figure 3.3 the K_m for H_2O_2 at pH 5.0 are 7.5 μM (LiP-2) and 4.1 μM (LIP-5), respectively. In the whole range of pH-values between 3.0 and 5.0 the catalytic efficiency of both LiP-2 and LiP-5, using H_2O_2 during the oxidation of VA, is more or less constant.
The logarithm of the K_m for H_2O_2 was found to be linearly dependent on the pH. This relation suggests that one ionizable group is involved in the binding of H_2O_2.

At pH 5.0 to 6.5, far away from the pH-optimum, VA is still oxidized by both LiP isozymes, meaning that under normal liquid BOS55 culture conditions the excreted LiP isozymes are also active. This suggests that LiP-2 and LiP-5 can participate in the extracellular degradation of organic pollutants such as PAH's.

The fungal metabolite 2-chloro-1,4-dimethoxybenzene [17,18] was a LiP substrate at pH values below 4.0. At pH 5.0, however, no appreciable activity could be detected. For this reason current research concentrates on the physiological role of 2-chloro-1,4-dimethoxy-benzene and other chlorinated aromatic compounds.

LITERATURE

[1] Barr, B.P., Shah, M.M., Grover, T.A., and Aust, S.D. (1992) *Arch. Biochem. Biophys.* **298**:480-485.
[2] Cai, D., and Tien, M. (1991) *J. Biol. Chem.* **266**:14464-14469.
[3] Farell, R.L., Murtagh, K.E., Tien, M., Mozuch, M.D., and Kirk,T.K. (1989) *Enzyme Microb. Technol.* 11:322-328.
[4] Field, J.A., de Jong, E., Feijoo-Costa, G., and de Bont, J.A.M. (1992) *Appl. Environ. Microbiol.* **58**:2219-2226.

[5] ten Have, R., Hartmans, S., and Field, J.A. (1997) *Appl. Environ. Microbiol.* **63**:3301-3303.
[6] Johansson, T., and Nyman, P.O. (1993) *Arch. Biochem. Biophys.* **300**:49-56.
[7] Johnson, T.M., Pease, A., Li, J.K.K., and Tien, M. (1992) *Arch. Biochem. Biophys.* **296**:660-666.
[8] Jönsson, L., Karlsson, O., Lundquist, K., and Nyman, P.O. (1989) *FEBS Letters* **247**: 143-146.
[9] Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammar, B., and Kirk, T.K. (1990) *Biochem. J.* **268**:475-480.
[10] Khindaria, A., Yamazaki, I., and Aust, S.D. (1995) *Biochemistry* **34**:16860-16869.
[11] Kimura, Y., Asada, Y., Oka, T., and Kuwahara, M. (1991) *App. Microbiol. Biotechnol.* **35**:510-514.
[12] Kimura, Y., Asada, Y., and Kuwahara, M. (1990) *Appl. Microbiol. Biotechnol.* **32**:436-442.
[13] Koduri, R.S., and Tien, M. (1995) *J. Biol. Chem.* **270**:22254-22258.
[14] Mester, T., Gimaraes, C., Field, J.A., and Hilhorst, R. (1997) *Tappi J.* (in press).
[15] Mester, T., de Jong, E., and Field, J.A. (1995) *Appl. Environ. Microbiol.* **61**:1881-1887.
[16] Moreira, M.T., Feijoo, G., Lema, J., Field, J.A., and Sierra-Alvarez, R. (1996) *Biotechn. Techn.* **10**:559-564.
[17] Spinnler, H.E., de Jong, E., Mauvais, G., Semon, E., and le Quere, J.L. (1994) *Appl. Microbiol. Biotechnol.* **42**:212-221.
[18] Swarts, H.J., Verhagen, F.J.M., Field, J.A., and Wijnberg, J.B.P.A. (1996) *Phytochemistry* **42**:1699-1701.
[19] Teunissen, P.J.M., and Field, J.A. (1997) *Appl. Environ. Microbiol.* (submitted).
[20] Tien, M., Kirk, T.K., Bull, C., and Fee, J.A. (1986) *J. Biol. Chem.* **261**:1687-1693.
[21] Tonon, F., and Odier, E. (1988) *Appl. Environ. Microbiol.* **54**:466-472.
[22] Valli, K., Wariishi, H., and Gold, M.H. (1990) *Biochemistry* **29**:8535-8539.
[23] Wariishi, H., and Gold, M.H. (1990) *J. Biol. Chem.* **265**:2070-2077.

4

Calculated ionisation potentials determine the oxidation of vanillin precursors by lignin peroxidase

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In view of the biocatalytic production of vanillin, this research focussed on the lignin peroxidase (LiP) catalyzed oxidation of natural occuring phenolic derivatives: *O*-methyl ethers, *O*-acetyl esters, and *O*-glucosyl ethers. The ionisation potential (IP) of a series of model compounds was calculated and compared to their experimental conversion by LiP, defining a relative IP threshold of approximately 9.0 eV. Based on this threshold value only the *O*-acetyl esters and glucosides of isoeugenol and coniferyl alcohol would be potential LiP substrates. Both *O*-acetyl esters were tested and were shown to be converted to *O*-acetyl vanillin in molar yields of 51.8 and 2.3 %, respectively.

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INTRODUCTION

Producing vanillin from natural precursors, using enzymatic or fermentative processes is of growing interest. Many natural precursors like eugenol, coniferaldehyde, and ferulic acid have been considered to produce the aromatic aldehyde [3,10,11]. All of these have an alkyl side chain which has to be cleaved to form vanillin.

Fungal lignin peroxidase (LiP) can cleave similar alkyl side chains in lignin and in lignin model compounds [9,15,17] and may therefore have potential applications in the production of vanillin. Incubation of underivatised phenolic vanillin precursors like coniferyl alcohol [14] with LiP and H_2O_2 yield unwanted lignin like polymers. When the phenolic group is protected, like in *O*-ethyl isoeugenol, C_α-C_β cleavage occurs yielding the corresponding benzaldehyde [13]. This suggests that protection of the phenolic OH-group makes polymerization a less important event in the overall reaction mechanism.

LiP's ability to oxidize a series of methoxybenzenes to the corresponding radical cations has been shown to be limited by the experimentally determined ionisation potential (IP) [8]. The IP value is a measure for the ease to abstract one electron from the highest occupied molecular orbital (HOMO). Above a certain IP threshold value the methoxybenzenes were no longer oxidized by LiP [8].

Here we use calculated IP values as a tool to predict the susceptibility of phenolic derviatives: *O*-methyl ethers, *O*-acetyl esters, and glucosides to oxidation by LiP. Especially the *O*-acetyl esters and glucosides are interesting potential vanillin precursors since the acetyl and glucosyl moieties can be enzymatically removed.

MATERIALS AND METHODS

LIGNIN PEROXIDASE (LIP)

LiP-2 was purified from the extracellular culture broth of *Bjerkandera* sp. strain BOS55 according to ten Have et al. [6].

INCUBATION MIXTURES

The incubation reactions were performed in a HPLC vial and contained: 100 mM sodium succinate pH 3.0, 0.5 mM substrate, 1.0 mM H_2O_2, 2.0 mM 1,4-dimethoxybenzene, and LiP 1000 U/l (V_{tot}= 250 µl). The mixture was incubated for 10 minutes at 20 °C. Subsequently 500 µl acetonitrile was added which stopped the enzymatic activity completely. Samples were capped and analysed with HPLC according to ten Have et al [5].

COMMERCIALLY AVAILABLE CHEMICALS

O-methyl isoeugenol, 3,4-dimethoxyphenylacetone and 3,5-dimethoxy-cinnamic acid were obtained from Aldrich (Milwaukee, USA). Homoveratryl alcohol, *p*-methyl eugenol, *O*-methyl ferulic acid, cinnamyl alcohol, homoveratric acid, 3-methoxycinnamic acid, cinnamaldehyde and cinnamic acid were obtained from Acros (Geel, Belgium). *O*-acetyl

isoeugenol and *O*-acetyl eugenol were obtained from Roth (Karslruhe, Germany).

SYNTHESIZED CHEMICALS

GC-MS analyses were carried out on a HP5973 quadrupole MS coupled to a HP6890 gas chromatograph equipped with a fused silica capillary column (HP-5MS, 30 m x 0.25 mm i.d., film thickness: 0.25 μm). Carrier gas and flow: He at 1.0 ml/min. Injector temperature 220°C; temperature program: 70-250°C at 7°C/min, hold 5 min. EIMS were obtained at 70 eV. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Bruker AC-E 200 spectrometer at 200 MHz and 50 MHz, respectively in $CDCl_3$.

SYNTHESIS OF *O*-METHYL CONIFERYL ALCOHOL

O-methyl coniferyl alcohol was synthesized from *O*-methyl ferulic acid according to literature [1,2]. EIMS *m/z* 194 $[M]^+$ (58), 165(10), 151(100), 138(59), 119(16), 107(13), 91(36), 77(29), 65(12), 55(15), 39(7). ^{1}H-NMR δ 1.67 (1H, br. s), 3.86 (3H, s), 3.87 (3 H,s), 4.29 (1H, d, *J*= 5.5 Hz), 6.22 (1H, dt, *J*= 15.9, 5.8 Hz), 6.53 (1H, d, *J*= 15.8 Hz), 6.85 (1H, d, *J*= 8.7 Hz), 6.93 (1H, s). ^{13}C-NMR δ 55.8(q), 55.9(q), 63.8(t), 108.7(d), 111.0(d), 119.6(d), 126.5(d), 129.7(s), 131.1(d), 2x 149.0(s).

SYNTHESIS OF *O*-METHYL CONIFERYL ALDEHYDE

O-methyl coniferyl aldehyde was synthesized from *O*-methyl coniferyl alcohol according to literature [1]. EIMS *m/z* 192 $[M]^+$ (88), 177(22), 161(100), 149(24), 133(14), 121(24), 103(19), 91(35), 77(43), 63(15), 51(10). ^{1}H-NMR δ 3.88 (3H, s), 3.89 (3 H,s), 6.57 (1H, dd, *J*= 7.8, 15.7 Hz), 6.87 (1H, d, *J*= 8.3 Hz), 7.04 (1H, d, *J*= 2.0 Hz), 7.13 (1H, d, *J*= 8.3 Hz), 7.38 (1H, d, *J*= 15.8 Hz), 9.62 (1H, d, *J*= 7.8 Hz). ^{13}C-NMR δ 55.9(q), 56.0(q), 109.8(d), 111.1(d), 123.5(d), 126.7(d), 127.0(s), 149.3(s), 151.9(s), 152.9(d), 193.6(d).

SYNTHESIS OF *O*-ACETYL CONIFERYL ALCOHOL

O-acetyl coniferyl alcohol was synthesized from *O*-acetyl eugenol (ICN, Zoetermeer, The Netherlands) according to literature [16]. EIMS *m/z* 222 $[M]^+$ (6), 180 (74), 152 (8), 137 (100), 124 (63), 103 (9), 91 (20), 77 (14), 65 (9), 55 (9), 43 (27). ^{1}H-NMR δ 2.18 (1H, br. s), 2.28 (3 H,s), 3.79 (3H, s), 4.25 (1H, d, *J*= 3.5 Hz), 6.25 (1H, dt, *J*= 15.9, 5.5 Hz), 6.52 (1H, d, *J*= 15.8 Hz), 6.97 (1H, m). ^{13}C-NMR δ 20.7(q), 55.8(q), 63.4(q), 110.1(d), 119.1(d), 122.8(d), 129.0(d), 130.1(d), 135.9(s), 139.2(s), 151.1(s), 169.2(s).

SYNTHESIS OF *O*-ACETYL CONIFERYL ALDEHYDE

O-acetyl coniferyl aldehyde was synthesized from *O*-acetyl coniferylalcohol according to literature [1]. EIMS *m/z* 220 $[M]^+$ (3), 178 (100), 161 (20), 147 (42), 135 (31), 118 (14), 107 (17), 89 (10), 77 (19), 63 (8), 51 (14), 43 (43). ^{1}H-NMR δ 2.32 (3H, s), 3.86 (3 H,s), 6.65 (1H, dd, *J*= 8.1, 11.6 Hz), 7.14 (3H, m), 7.43 (1H, d, *J*= 15.9 Hz), 9.68 (1H, d, *J*= 7.6 Hz). ^{13}C-NMR δ 20.7(q), 56.0(q), 111.4(d), 121.9(d), 123.5(d), 128.7(d), 132.9(s), 142.4(s), 151.6(s), 151.9(d), 168.7(s), 193.5(d).

CALCULATION OF IONISATION POTENTIALS

Ionisation potentials were calculated on a Silicon Graphics Indigo computer using Spartan version 5.0 (Wave function, Inc., Irvine, California). A semi-empirical molecular orbital method, applying the AM1 Hamiltonian was used. Closed shell calculations were performed using the Restricted Hartree Fock method. Geometries were optimized for all bond lengths, bond angles and torsion angles. These calculated ionisation potentials are those for molecules in a vacuum.

The IP strongly depends on the three dimensional structure of a molecule. Therefore, the IP of at least 4 and maximally 8 conformations have been calculated. The average value of these ± standard error of the mean (SEM) are presented.

RESULTS

DETERMINATION OF THE IONISATION POTENTIAL THRESHOLD

Several nonphenolic compounds, characterized by an aromatic ring and an alkyl side chain, were incubated with LiP. Table 4.1 shows the observed relationship between the substrate consumed and the calculated ionisation potential (IP). The results presented show that compounds with a calculated IP lower than 9.0 eV were all converted by LiP; whereas, all model compounds with an IP above this threshold value were not.

TABLE 4.1 The calculated ionisation potential (in eV ± SEM) of various commercially available compounds and the qualitive outcome of the incubation of these compounds with LiP. A "+" indicated that the compounds is transformed by LiP. See for incubation conditions Materials and Methods.

Compound	Substrate (+/-)	IP (eV)
O-methyl isoeugenol	+	8.41 ± 0.04
O-methyl coniferyl alcohol	+	8.58 ± 0.05
O-methyl eugenol	+	8.64 ± 0.07
homoveratryl alcohol	+	8.66 ± 0.03
3,4-dimethoxyphenylacetone	+	8.84 ± 0.05
O-methyl ferulic acid	+	8.87 ± 0.05
O-methyl coniferaldehyde	+	8.92 ± 0.11
homoveratric acid	+	8.99 ± 0.08
cinnamyl alcohol	–	9.00 ± 0.04
3,5-dimethoxycinnamic acid	–	9.16 ± 0.03
3-methoxycinnamic acid	–	9.29 ± 0.03
cinnamaldehyde	–	9.39 ± 0.02
cinnamic acid	–	9.53 ± 0.05

NATURAL PHENOLICS AND THEIR DERIVATIVES

Direct incubation of phenolics with LiP and H_2O_2 yield unwanted polymers [4,14,18]. Literature data on *O*-ethyl isoeugenol show that the latter is cleaved by LiP to the corresponding benzaldehyde [13]; whereas, it was observed that isoeugenol was polymerized to a white precipitate. Therefore, we studied protected phenolic derivatives: *O*-methyl ethers, *O*-acetyl esters, and glucosides (see Figure 4.1).

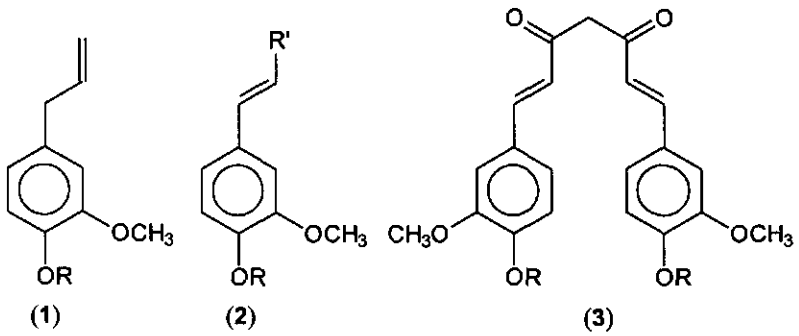

FIGURE 4.1 Structural formulas of eugenol (1, R=H), isoeugenol (2, R=H, R'= CH_3), coniferyl alcohol (2, R= H, R'= CH_2OH), coniferaldehyde (2, R= H, R'= CHO), ferulic acid (2, R= H, R'= COOH), and curcumin (3, R= H). In O-methyl ethers R= methyl, for O-acetyl esters R= acetyl, and for glucosides R= glucosyl.

Table 4.2 presents the calculated IP values for the various phenolics and derivatives, showing that the IP values for the *O*-acetyl esters and the glucosides are significantly higher than those of the phenolics and the *O*-methylated analogues.

TABLE 4.2 The calculated ionisation potential (± SEM in eV) of several phenolic compounds and their derivatives.

Compound	Phenol	*O*-methyl ether	*O*-acetyl ester	Glucoside
eugenol	8.73 ± 0.06	8.64 ± 0.07	9.17 ± 0.07	9.14 ± 0.05
isoeugenol	8.46 ± 0.04	8.41 ± 0.04	8.81 ± 0.06	8.76 ± 0.02
ferulic acid	8.95 ± 0.05	8.87 ± 0.05	9.33 ± 0.06	9.23 ± 0.06
coniferyl alcohol	8.60 ± 0.06	8.58 ± 0.05	8.91 ± 0.05	8.92 ± 0.04
coniferaldehyde	8.86 ± 0.05	8.92 ± 0.11	9.21 ± 0.07	9.15 ± 0.05
curcumin	8.78 ± 0.02	8.64 ± 0.04	9.17 ± 0.05	no data

This trend was found in all cases. Protection of the phenolic OH-group with an acetyl or a glucosyl moiety increases the IP of the compounds beyond 9.0 eV, suggesting that they are not substrates for LiP. Only the *O*-acetyl esters and glucosides of isoeugenol and coniferyl alcohol have IP values below 9.0 eV.

C_α-C_β CLEAVAGE OF *O*-METHYL ETHERS AND *O*-ACETYL ESTERS

The results presented so far, allow us to predict which compound will be a LiP substrate. The first step in the C_α-C_β cleavage reaction is the removal of one electron, forming a radical cation intermediate. This reactive species may undergo spontaneous addition of water or loss of a proton which are essential for the C_α-C_β cleavage reaction required to obtain the benzaldehyde derivatives. The C_α-C_β cleavage was studied by measuring the formation of 3,4-dimethoxybenzaldehyde (VAD) from five O-methyl ethers. Table 4.3 shows these LiP substrates were significantly cleaved to VAD by LiP, suggesting that predicted LiP substrates with an alkyl side chain of three carbon atoms undergo C_α-C_β cleavage.

This hypothesis is further evidenced by the results in Table 4.4 showing that both *O*-acetyl isoeugenol and coniferyl alcohol are consumed and converted to *O*-acetyl vanillin to varying extents in the presence of 1,4-dimethoxybenzene (1,4-DMB).

TABLE 4.3 The average molar VAD yield (%), calculated as the amount of VAD formed per mole substrate converted, obtained after incubation of different substrates (0.5 mM). For incubation conditions see Materials and Methods.

Substrate	Conversion (%)	Molar VAD yield (%)
O-methyl ferulic acid	60 ± 0.3	54.3 ± 3.2
O-methyl isoeugenol	100 ± 0	53.2 ± 3.0
O-methyl coniferaldehyde	12 ± 1.0	24.7 ± 1.6
O-methyl coniferylalcohol	100 ± 0	11.7 ± 0.1
O-methyl eugenol	100 ± 0	2.6 ± 0.1

It should be noted that the cofactor 1,4-DMB was required for extensive LiP oxidation of the *O*-acetyl esters. Without 1,4-DMB the conversion of *O*-acetyl isoeugenol and coniferyl was 3.8 and 6.5 %, respectively. Such a stimulating effect was not observed in the case of the *O*-methyl ethers (data not shown).

TABLE 4.4 The conversion (%) of various *O*-acetyl esters and the molar yield of *O*-acetyl vanillin (product, %), calculated as mole product per mole substrate converted. Values are an average of two duplicate run incubations. See for incubation conditions Materials and methods.

Substrate	Conversion (%)	Molar *O*-acetyl vanillin yield (%)
O-acetyl isoeugenol	97.3	51.8
O-acetyl coniferyl alcohol	59.8	2.3
O-acetyl eugenol	0.0	0.0
O-acetyl coniferaldehyde	0.0	0.0

The other acetyl esters, *O*-acetyl eugenol and coniferaldehyde, were not converted by LiP (Table 4.4). This was as expected based on the calculated IP values which are above the relative threshold value of 9.0 eV.

DISCUSSION

It has been shown that the experimentally determined ionisation potential (IP) of small methoxybenzenes correlates with the susceptibility to oxidation by lignin peroxidase (LiP) [8]. The results of the present study extend this model with quantum mechanically calculated IP values. Using nonphenolic aromatic compounds with an alkyl side chain, an IP threshold value of 9.0 eV was obtained which allowed us to screen potential vanillin precursors.

The calculated IP values of the *O*-acetyl esters and glucosides were significantly higher than those of the corresponding phenolics and the *O*-methyl ethers, probably due to the electron withdrawing effect of the *O*-acetyl and *O*-glucosyl groups, decreasing the electron density in the aromatic ring.

In contrast to the *O*-methyl ethers, the conversion of the *O*-acetyl esters of isoeugenol, and coniferyl alcohol was significantly enhanced in the presence of 1,4-dimethoxybenzene (1,4-DMB), suggesting that both *O*-acetyl esters were poor substrates for LiP compound II. Without 1,4-DMB, LiP compound II accumulates and may react with H_2O_2 to form the inactive LiP compound III [12]. In the presence of 1,4-DMB (IP=8.55 eV) compound II is effectively reduced back to native LiP, completing the catalytic cycle and thus enhancing the total turnover of the enzyme. The results also suggest a redox potential difference of 0.1-0.2 eV between the more potent LiP compound I and compound II.
The conversion of a series of phenolic derivatives, *O*-methyl ethers, to the C_α-C_β cleavage product 3,4-dimethoxybenzaldehyde by LiP, allowed us to hypothesize that similar compounds with an IP < 9.0 eV would undergo the same C_α-C_β cleavage reaction upon incubation with LiP. *O*-acetyl isoeugenol and coniferyl alcohol both were indeed converted to *O*-acetyl vanillin. Renganathan *et al.* [13] have proposed that the C_α-C_β cleavage mechanism of *O*-ethyl isoeugenol occurs via a diol intermediate. The oxidation of the double bond in the alkyl side to a diol might also occur in the case of the *O*-acetyl esters studied here. There are, however, strong indications that also superoxide* plays an important role in the C_α-C_β cleavage mechanism, suggesting the formation of instable hydroperoxides during LiP catalysis [5].

Future research will concentrate on studying the C_α-C_β cleavage mechanism in more detail in order to explain the difference in yield of 3,4-dimethoxybenzaldehyde between the *O*-methyl ethers of isoeugenol and coniferyl alcohol. This could give ideas how to increase the low molar *O*-acetyl vanillin yield of 2.3 % from *O*-acetyl coniferyl alcohol.

LITERATURE

[1] Bürgi, C., Liu, G., and Rüedi, P. (1993) *Helv. chim. Acta* **76**:1901-1915
[2] Freudenberg, K., and Schuhmacher, G. (1954) *Chemische Berichte* **12**:1882-1887.
[3] Hagedorn, S., and Kaphammer, B. (1994) *Annu. Rev. Microbiol.* **48**:773-800
[4] Harvey, P.J., and Palmer, J.M. (1990) *J. Biotechnology* **13**:169-179
[5] ten Have, R., Franssen, M.C.R., and Field, J.A. (1998) Submitted
[6] ten Have, R., Hartmans, S., Teunissen, P.J.M., and Field, J.A. (1998) *FEBS Lett.* 422: 391-394.

* This statement was based on the observed inhibition of the molar VAD yield from the isoeugenyl methyl ether by the superoxide scavengers superoxide dismutase (SOD), tetranitromethane (TNM) and Mn^{2+}. However, additional experiments showed that the inhibition of SOD was not due to the enzymatic acitivity of SOD (not shown). Furthermore, the inhibition of TNM was explained by a difference in the H_2O_2 concentration rather than by a superoxide scavenging effect (not shown). Finally, Mn^{2+} may serve several roles in LiP catalyzed reactions of which scavenging superoxide is only one. Taken these comments together, it is not likely that superoxide is directly involved in C_α-C_β cleavage (see Chapter 6).

[7] Joshi, D.K, and, Gold, M.H. (1996) *Eur. J. Biochem.* **237**:45-57
[8] Kersten, P.J., Kalyanaraman, B., Hammel, K.E, Reinhammer, B., and Kirk, T.K. (1990) *Biochem. J.* **268**:475-480
[9] Kirk, T.K., and Farell, R.L. (1987) *Annu. Rev. Microbiol.* **41**:465-505
[10] Koseki, T., Ito, Y., Furuse, S., Ito, K., and Iwano, K. (1996) *J. Ferment. Bioeng.* **82**: 46-50
[11] Leffemberg, C., Gautier, A., and Ohleyer, E. (1992) *Appl. Biochem. Biotech.* **37**:43-52
[12] Renganathan, V., and Gold, M.H. (1986) *Biochemistry* **25**:1626-1631
[13] Renganathan, V., Miki, K., and Gold, M.H. (1985) *Arch. Biochem. Biophys.* **241**:304-314
[14] Sarkanen, S., Razal, R.A, Piccariello, T., Yamamoto, E., and Lewis, N.G. (1991) *J. Biol. Chem.* **266**:3636-3543
[15] Schoemaker, H.E., Harvey, P.J., Bowen, R.M., and Palmer, J.M. (1985) *FEBS Lett.* **183**:7-12
[16] Steglich, W., and Zechlin, L. (1978) *Chemische Berichte* **111**:3939-3948
[17] Tien, M., and Kirk, T.K. (1983) *Science* **24**:661-663
[18] Wallace, G., and Fry, S.C. (1995) *Phytochemistry* **39**:1293-1299

5 Veratryl alcohol-mediated oxidation of isoeugenyl acetate by lignin peroxidase

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The mechanism of the veratryl alcohol (VA) mediated oxidation of isoeugenyl acetate (IEA) by lignin peroxidase (LiP), and the subsequent spontaneous C_α-C_β cleavage of isoeugenyl acetate to vanillyl acetate were studied. IEA oxidation only occurred in the presence of VA. IEA probably did not bind to LiP as evidenced by an unaffected K_m for VA in the presence of IEA, and by the fact that a 10-time molar excess of the unreactive IEA counterpart, eugenyl acetate, did not affect the IEA oxidation rate. IEA was very efficient in recycling VA. Up to 34 moles of IEA were oxidized per mole VA. Formation of the predominant VA oxidation product, veratraldehyde (VAD), was postponed untill IEA was almost completely oxidized. Together these findings suggest that IEA was oxidized by $VA^{\bullet+}$ rather than directly by LiP. Thus, VA functioned as a redox mediator during IEA oxidation which is rather remarkable due to the high calculated ionization potential of 8.81 eV.

Regardless of the presence of O_2 approximately 2 moles of IEA were consumed per mole H_2O_2 which indicated that IEA was enzymatically oxidized by one electron to the putative radical cation ($IEA^{\bullet+}$). After formation of $IEA^{\bullet+}$, a series of O_2-dependent chemical reactions were responsible for C_α-C_β cleavage to the major oxidation product vanillyl acetate as evidenced by the observation that a N_2 atmosphere did not inhibit IEA oxidation, but almost completely inhibited vanillyl acetate formation. GC-MS analyses revealed that under an air atmosphere also 1-(4'-acetoxy-3'-methoxyphenyl)-2-propanone, 1-(4'-acetoxy-3'-methoxyphenyl)-1-hydroxy-2-propanone, and 1-(4'-acetoxy-3'-methoxyphenyl)-2-hydroxy-1-propanone were formed. The formation of the latter two was diminished under a N_2 atmosphere.

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INTRODUCTION

Lignin peroxidase (LiP) is an important extracellular enzyme produced by white-rot fungi participating in the degradation of lignin [1]. LiP functions together with the cofactor veratryl alcohol (VA) which protects the enzyme from inactivation by H_2O_2 [2], but also acts as a redox mediator during the oxidation of large [3,4], or other oxidizable molecules [5-7] which do not readily bind with the enzyme and have a lower ionization potential (IP) than VA. The IP value indicates the ease to abstract an electron from the highest occupied molecular orbital. Among peroxidases, LiP is the most potent [8,9] since it can oxidize aromatic compounds with calculated IP values up to 9.0 eV [10].

VA, or 1,4-dimethoxybenzene strongly enhance the turnover of LiP during oxidation of poor substrates with a relatively high IP such as *p*-anisyl alcohol (AA) [5] and isoeugenyl acetate (IEA) [10]. AA is oxidized by LiP compound I, but is almost inert towards LiP compound II [6]. VA functions as an effective reductant for LiP compound II, enabling the closure of the catalytic cycle during AA oxidation, which minimizes inactivation by H_2O_2. Since AA poorly inhibits the consumption of VA [5], direct redox mediation by VA is unlikely. According to Goodwin et al. [11] other compounds with a high IP should show oxidation kinetics like AA in the presence of VA. Surprisingly, preliminary data indicated that IEA (IP identical to AA) was very efficient in inhibiting VA oxidation, suggesting different oxidation kinetics when compared to AA.

Also the C_α-C_β cleavage mechanism of IEA to vanillyl acetate [10] was investigated. This reaction is of relevance in relation to the biocatalytic conversion of phenyl propanoid structures to their corresponding benzaldehydes.

MATERIALS AND METHODS

CHEMICALS

Veratryl alcohol (VA), veratraldehyde (VAD), and vanillyl acetate were obtained from Aldrich (Bornem, Belgium). Isoeugenyl acetate (IEA), and eugenyl acetate (EA) were obtained from Roth (Karlsruhe, Germany). Cinnamyl alcohol was obtained from Acros (Geel, Belgium), and benzyl alcohol was obtained from Merck (München, Germany).

LiP PREPARATION

In order to obtain the LiP isozyme mixture used throughout the experiments, *Bjerkandera* sp. strain BOS55 was cultured on 1 L nitrogen-rich glucose medium (5 g/L mycological peptone) as described before [12]. After 20 days of culturing in the dark, the extracellular fluid was harvested. The preparation of ammonium sulfate precipitate occurred as described before [13]. The resuspended precipitate was dialyzed (two times 1 M NaAc pH 6.0, dilution factor = 625), centrifuged at 10,000 *g* for 10 minutes at 2 °C and subsequently applied on a preparative anion exchange column (DEAE, d = 2.6 cm, h = 1.0 cm). After another dialysis

step (10 mM NaAc pH 6.0, dilution factor = 300) the sample was applied on a FPLC anion exchange column (Source Q, d = 1.5 cm, h = 7.0 cm) to fractionate the LiP isozymes.

STANDARD INCUBATION CONDITIONS

The incubation mixture (V_{tot}= 250 µL) contained: 1 mM IEA, 0.5 mM VA, 0.5 mM H_2O_2, 100 mM succinate buffer pH 3.0, and LiP 0.36 U/ml (1 U oxidizes 1 µmol VA.min^{-1} at pH 3.0). IEA was added from a stock solution in acetone. Therefore, each incubation mixture contained 5.2 % (v/v) acetone. The reaction, performed at 23 °C under air, was initiated upon addition of LiP and stopped by introducing 500 µl 15 mM vitamin C after varying periods of time (10, 20, 40, 60, 80, 100, 120, 150, 180, 240, 300, 450, and 600 s). Generally, the data obtained during the first 100 s was used to calculate initial rates. Control experiments were performed by omitting LiP, VA, or by using inactivated LiP (obtained by boiling for 20 min.) and were incubated for the maximal duration of the experiment.

During an experiment on comparing incubations under an atmosphere of N_2, air, or O_2, the standard incubation mixtures were flushed for 10 minutes with corresponding gas prior to addition of IEA. The reaction was subsequently initiated upon addition of a thoroughly flushed LiP preparation.

In an experiment studying the effect of cinnamyl alcohol, and benzyl alcohol, each compound was added, from a stock solution in acetone, to the standard incubation mixture till a concentration of 1 mM was reached.

In an experiment studying the effect of 1 mM eugenyl acetate on the oxidation of IEA, an IEA concentration of 0.1 mM was used. The experiment was performed by applying standard conditions.

The conversion of IEA is calculated based on the elimination of IEA using the starting concentration as a reference. Negligible elimination of IEA occurred in the abiotic controls. The molar yield is expressed as the ratio between product formed and substrate consumed.

H_2O_2 CONSUMPTION

H_2O_2 was measured spectrophotometrically by following the oxidation of ABTS at a wavelength of 420 nm by horseradish peroxidase (HRP). The cuvet (V_{tot}= 1 mL) contained 0.1 M Na_3PO_4 pH 6.0, 0.29 mM ABTS, 1 U/mL HRP, and 150 µl sample. The H_2O_2 concentration was determined using a standard curve.

O_2 CONSUMPTION

A standard incubation mixture with a total volume of 3 mL was prepared. The dissolved oxygen concentration was measured at 23 °C and measured using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). Water flushed with air was used to calibrate the signal corresponding to a saturated dissolved O_2 concentration (0.267 mM).

HPLC ANALYSIS

The samples were analyzed using a HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany) equipped with a HP1100 pumping station, an HP1040M series II photo diode array detector, and a HP9000-300 data processor. HPLC chromatograms were recorded at 220, 280, and 310 nm. A reversed phase C18 column (200 mm x 3 mm filled with ChromoSpher C18-PAH 5 µm particles, Chrompack) was used. Compounds were eluted using a linear water/ acetonitrile gradient starting at 10 % acetonitrile and increased to 100 % acetonitrile in 15 minutes. The flow was 0.4 ml/min and the temperature was held constant at 30°C. Compounds were identified by comparison of retention times and UV-spectra, obtained after HPLC analysis, with standards. Concentrations of identified peaks were determined by using external standards.

HEAD SPACE ANALYSIS

For the determination of ethanal a standard incubation was performed in a closed HPLC vial. After the reaction was completed, the samples were placed in a waterbath with a temperature of 30 °C. Control experiments did not contain LiP, or H_2O_2 and were incubated for the same period of time. The gas phase (100 µl) was analyzed using a Hewlett Packard 6890 gas chromatograph equipped with a CP-sil 19CB wood fused silica column (30 m x 0.32 mm). For two minutes the oven temperature was held constant at 40°C thereafter it raised till 80°C (5°C/min). The carrier gas was N_2 (flow 1 ml/min). Ethanal was identified by a matching retention time, compared with an ethanal standard. Quantification of ethanal occurred using external standards.

GC-MS CONDITIONS AND PREPARATION OF THE SAMPLES

For analysis of oxidation products in the liquid phase, a standard incubation experiment was performed on a 2.5 mL scale.

After 10 minutes of incubation, the reaction was stopped with 5 mL 15 mM vitamine C. This mixture was extracted twice with ethyl acetate. The combined organic layers were dried using brine and after separation concentrated under a N_2 stream. Control experiments did not contain H_2O_2.

GC-MS analyses were carried out on a HP5973 quadrupole MS coupled to a HP6890 gas chromatograph (Hewlett Packard) equipped with a fused silica capillary column (HP-5MS, 30 m x 0.25 mm i.d., film thickness: 0.25 µm). Carrier gas and flow: He at 1.0 ml/min. Injector temperature 220°C; temperature program: 70-250°C at 7°C/min, hold 5 min. EIMS were obtained at 70 eV.

Since conversion of VA was independent of the presence of O_2, VA was used as an internal standard.

Vanillyl acetate was identified by comparing the GC retention time, and mass spectrum with a vanillyl acetate standard.

SUPEROXIDE ASSAY

As a result of the presence of superoxide, tetranitromethane (TNM, 1 mM) was reduced to the yellow trinitromethane anion which was measured spectrophotometrically at a wavelength of 350 nm (ε=13,500 [14]).

CALCULATION OF IONISATION POTENTIALS

Ionisation potentials were calculated as described before [10].

STATISTICAL PROCEDURE

average values are presented ± the standard error of the mean (SEM or $\sigma_{n-1}/\sqrt{n}$).

RESULTS

VERATRYL ALCOHOL AS A REDOX MEDIATOR

Veratryl alcohol (VA) was essential for the oxidation of isoeugenyl acetate (IEA, see Fig. 5.1a).

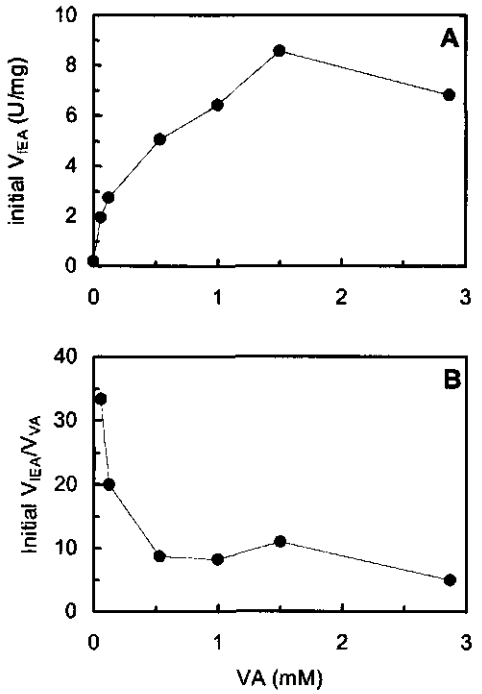


FIGURE 5.1 Panel A shows the initial rate (U/mg) in which IEA is consumed by LiP as a function of the applied VA concentration. Panel B shows the ratio between the initial IEA and VA consumption rate.

Without it, no consumption of IEA occurred. In the VA concentration range from 0 to 1 mM, the initial IEA oxidation rate was stimulated. In

the presence of excess VA, beyond a VA concentration of 1 mM, the IEA oxidation rate slightly increased. The K_m for VA of 0.18 mM was not affected by the presence of IEA. This indicated that competition for the catalytic site between IEA and VA was minimal. Also similar maximal rates were observed in which either VA was directly oxidized, or IEA was oxidized in the presence of VA. During the oxidation of IEA, VA was effectively recycled, which was especially efficient at low VA concentrations (see Fig. 5.1b). Upon applying a VA concentration of 0.04 mM, the rate ratio V_{IEA}/V_{VA} indicated that initially 34 mole of IEA were converted per mole of VA. At higher VA concentrations, the VA recycling effectiveness sharply declined reaching an average value of 8 mole of IEA per mole VA molecule in the VA concentration range from 0.5 to 2.9 mM.
At a constant VA concentration of 0.5 mM, the initial IEA oxidation rate increased in the IEA concentration range from 0 to 0.4 mM (see Fig. 5.2). From an IEA concentration of 0.4 mM onwards, a constant initial oxidation rate of approximately 6 U/mg was reached which is similar to the velocity in which the isozyme mixture oxidized 0.5 mM VA alone. This observation indicates that the consumption of IEA is limited by the oxidation of VA.

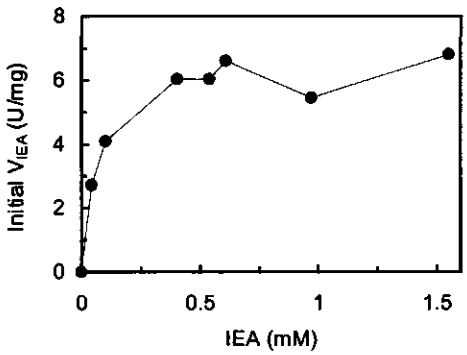


FIGURE 5.2 The initial rate in which LiP consumes varying IEA concentrations at a constant VA concentration of 0.5 mM.

During the period of time in which IEA is almost completely oxidized, the formation of VAD was postponed. These lag phases were roughly proportional to the applied IEA concentration. Figure 5.3 shows a typical example of a lag phase with a duration of 130 s using an IEA concentration of 0.5 mM in the incubation mixture.

ONE-ELECTRON OXIDATION OF IEA

The evidence that indicates that the initial oxidation of IEA is due to an enzymatic one electron oxidation step is given in Figure 5.4. The linear relationship ($R^2 = 0.993$) between IEA consumed and H_2O_2 added shows that 1 mole of H_2O_2, representing two oxidation equivalents, is used to oxidize approximately 2 mole of IEA. This corresponds with 1 mole of IEA

per oxidation equivalent. After the total reaction time, H_2O_2 was measured and was shown to be almost completely (>97 %) consumed (result not shown).

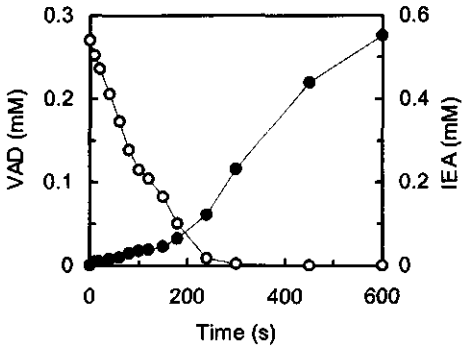


FIGURE 5.3 **A typical example of a lag phase observed in VAD formation (●) during IEA consumption (O).**

A similar stoichiometry between IEA and H_2O_2 was found under a N_2 atmosphere (result not shown).

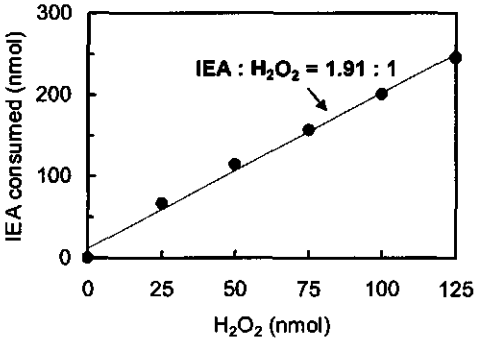


FIGURE 5.4 **The total IEA conversion (nmol) at different amount of H_2O_2 (nmol).**

THE FORMATION OF VANILLYL ACETATE FROM IEA

The C_α-C_β cleavage mechanism of 1-(4'-acetoxy-3'-methoxyphenyl)-propene (IEA) was studied in more detail. Preliminary data suggested that a distinct mechanism is involved in comparison with a mechanism proposed by Gold et al. [15] for a similar compound 1-(4'-ethoxy-3'-methoxyphenyl)-propene (EMPP). In the cited study, EMPP was consumed directly by LiP. A diol was detected as an EMPP oxidation product which was proposed to be the precursor prior to C_α-C_β cleavage to the corresponding aldehyde [15]. However in the case of IEA, such a diol would not likely be an important intermediate in the formation of the major oxidation product

vanillyl acetate, since the calculated ionisation potential (IP) of such a diol was 9.19 ± 0.07 eV (n= 5) which is above the threshold value of 9.0 eV observed for LiP substrates [10].
Figure 5.5a depicts that the consumption of IEA under N_2, and air was similar, indicating that the LiP activity was independent of the presence of oxygen. Remarkably, the presence of oxygen appeared to be essential for vanillyl acetate formation.

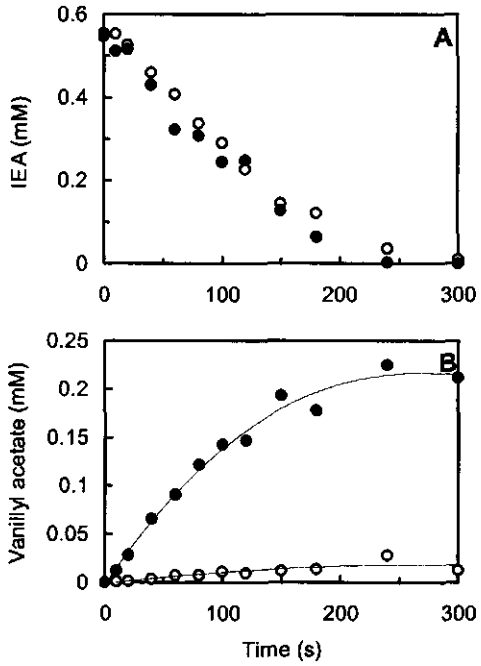


FIGURE 5.5 Panel A shows the effect of an air (●), and a N_2 (○) atmosphere on the consumption of isoeugenyl acetate in time by LiP. Panel B shows the production of vanillyl acetate under a N_2 (○), and an air atmosphere (●) in time. Different from standard incubation conditions an IEA concentration of 0.5 mM was applied.

Figure 5.5b shows that in comparison with the normal formation of vanillyl acetate under air, an N_2 atmosphere almost completely prevented vanillyl acetate formation. Table 5.1 shows that the molar vanillyl acetate yield was enhanced 1.5 fold by applying an O_2 atmosphere in comparison with a normal air atmosphere.

TABLE 5.1 The effect of the atmosphere applied on the molar vanillyl acetate yield. Average values at similar conversions 0.4-0.8 mM are presented (n=4). Standard incubation conditions were used.

Atmosphere	Molar vanillyl acetate yield ± $\sigma_{n-1}/\sqrt{n}$ (%)
Oxygen	54.0 ± 2.1
Air	37.0 ± 1.7
Nitrogen	2.5 ± 0.6

During a standard incubation, oxygen was clearly consumed as depicted in Figure 5.6A. As a result of the rapid O_2 consumption, the molar vanillyl acetate yield typically decreased during the course of the reaction a shown in Figure 5.6B. Only traces of oxygen were converted to superoxide (result not shown), suggesting that O_2 itself was incorporated into a reactive intermediate derived from IEA.

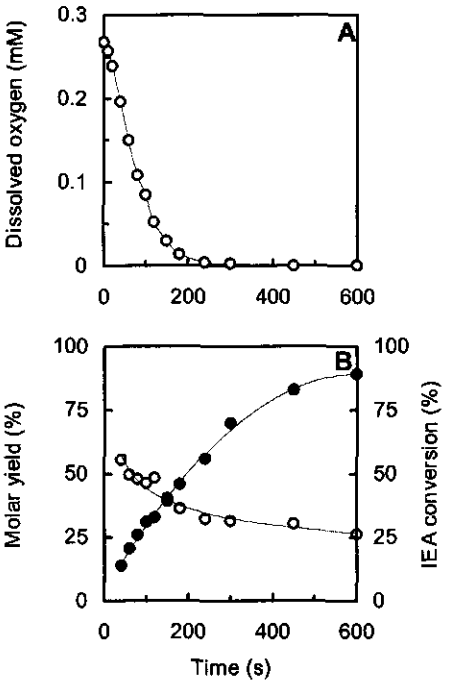


FIGURE 5.6 **Panel A shows the dissolved O_2 concentration (mM) during the reaction as obtained during a BOM experiment. Panel B shows the molar vanillyl acetate yield (%) and the IEA conversion (%) during the reaction.**

THE FORMATION OF SIDE PRODUCTS FROM IEA

GC-MS analysis showed that under an air atmosphere at least 21 products were formed. Besides the obvious vanillyl acetate (peak area 30.1 %) and unreacted IEA (peak area 19.9 %), also 3 other products related to IEA were identified as shown in Table 5.2. The products were identified on the basis of their fragmentment ion pattern and by comparison with mass spectra of similar compounds in literature [16] and in the NIST98 mass spectral data base. The first product, showing a molecular ion peak ($[M]^+$) at m/z 222, was tentatively identified as 1-(4'-acetoxy-3'-methoxyphenyl)-2-propanone. The two remaining compounds showed both a $[M]^+$ at m/z 238 and a similar fragmentation pattern indicating that the two compounds are isomers. On the basis of distinct differences in the mass spectra, the first isomer was tentatively identified as 1-(4'-acetoxy-3'-methoxyphenyl)-1-hydroxy-2-propanone (**5**) and the second isomer was identified as 1-(4'-acetoxy-3'-methoxyphenyl)-2-hydroxy-1-propanone (**3**).

TABLE 5.2 **Additional products formed under an air atmosphere from IEA during a standard incubation. The presented peak area is calculated as the percentage from products that may originate from IEA. Products were assigned on the basis of characteristic fragment ion peaks in the mass spectrum.**

Products	Area %	*m/z* (EIMS)
1-(4'-acetoxy-3'-methoxyphenyl)-2-propanone	5.4	222 (2), 180 (27), 137 (100), 122 (5), 94 (4), 43 (15)
1-(4'-acetoxy-3'-methoxyphenyl)-1-hydroxy-2-propanone (**5**)	2.5	238 (2), 195 (21), 153 (100), 151 (30), 125 (15), 110 (5), 93 (40), 65 (13), 43 (20)
1-(4'-acetoxy-3'-methoxyphenyl)-2-hydroxy-1-propanone (**3**)	12.1	238 (1), 196 (13), 151 (100), 123 (8), 108 (3), 93 (3), 65 (3), 45 (4), 43 (9)
Unidentified products	30	

Under an N_2 atmosphere the formation of the isomers **3** and **5** was reduced by a factor 24 and 235, respectively. The ketone, 1-(4'-acetoxy-3'-methoxyphenyl)-2-propanone, was produced 3.5 times less. Instead of these products, trace amounts of some additional dimeric products were observed in the $[M]^+$ range at m/z 360 to 426.

Formation of vanillyl acetate from IEA implies that a C2-fragment must have been cleaved off. Head space analyses by GC showed that ethanal was produced. HPLC analysis of the same sample indicated that one mole ethanal was formed per mole vanillyl acetate.

Together, the evidence suggest that intermediate peroxyl radicals are formed prior to vanillyl acetate formation. Peroxyl radicals may add to double bonds or abstract an benzylic hydrogen atom [17]. These reactions were not important in the formation of vanillyl acetate since cinnamyl alcohol, and benzyl alcohol were not converted in the complete reaction mixture.

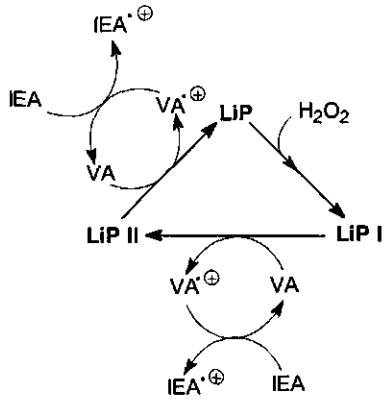
DISCUSSION

THE MEDIATOR ROLE OF VERATRYL ALCOHOL

The presence of veratryl alcohol (VA) was shown to be essential for the oxidation of isoeugenyl acetate (IEA) by lignin peroxidase (LiP). This observation suggests that IEA is a poor substrate for LiP compound II. In the literature, several examples have been described for compounds that are almost inert towards LiP compound II like *p*-anisyl alcohol (AA) [5,18] and 4-methoxymandelic acid (MMA) [6]. The presence of a good compound II reductant like the cofactor VA, generally enhances the oxidation of the substrate by closing the catalytic cycle as in the case of AA [5] or by mediating the oxidation as in the case of MMA [6]. However, it is also possible to oxidize AA by redox mediation as shown by Teunissen et al. [19] by using 2-chloro-1,4-dimethoxybenzene as a cofactor.

If VA only serves to close the catalytic cycle, than maximally 1 mole of terminal substrate would be expected to be oxidized per mole of VA

consumed. This is clearly not the case for IEA, since up to 34 moles of this substrate were consumed per mole of VA.
In part based on the work by Koduri et al. [5], Goodwin et al. [11] stated that terminal substrates having high IP values like AA would be expected to have similar oxidation kinetics with LiP in the presence of VA. The calculated IP value of anisyl alcohol (8.81 ± 0.02 eV) and IEA (8.81 ± 0.06 eV [10]) are the same. However, these compounds clearly show different oxidation kinetics, of which very efficient VA recycling during IEA oxidation is the most important difference.
VA probably served as a redox mediator in the oxidation of IEA (see Scheme 5.1). Besides the extraordinary ratio between IEA and VA consumed of 34 to 1, the mediation theory explains why IEA was oxidized while it did not seem to enter the catalytic site. This is evidenced by an unchanged K_m for VA in the presence of IEA, and by the fact that a 10-fold excess of the inert [10] IEA counterpart, eugenyl acetate (EA), did not affect the IEA oxidation rate. Furthermore, high VA concentrations up to 2.9 mM did not inhibit the initial IEA oxidation rate. Inhibition would be expected if VA served to close the catalytic cylce since VA would compete for LiP compound I in that case.
As shown in Scheme 5.1, IEA was probably only oxidized by $VA^{\cdot+}$.



SCHEME 5.1 VA functioning as a redox mediator during the oxidation of IEA by LiP.

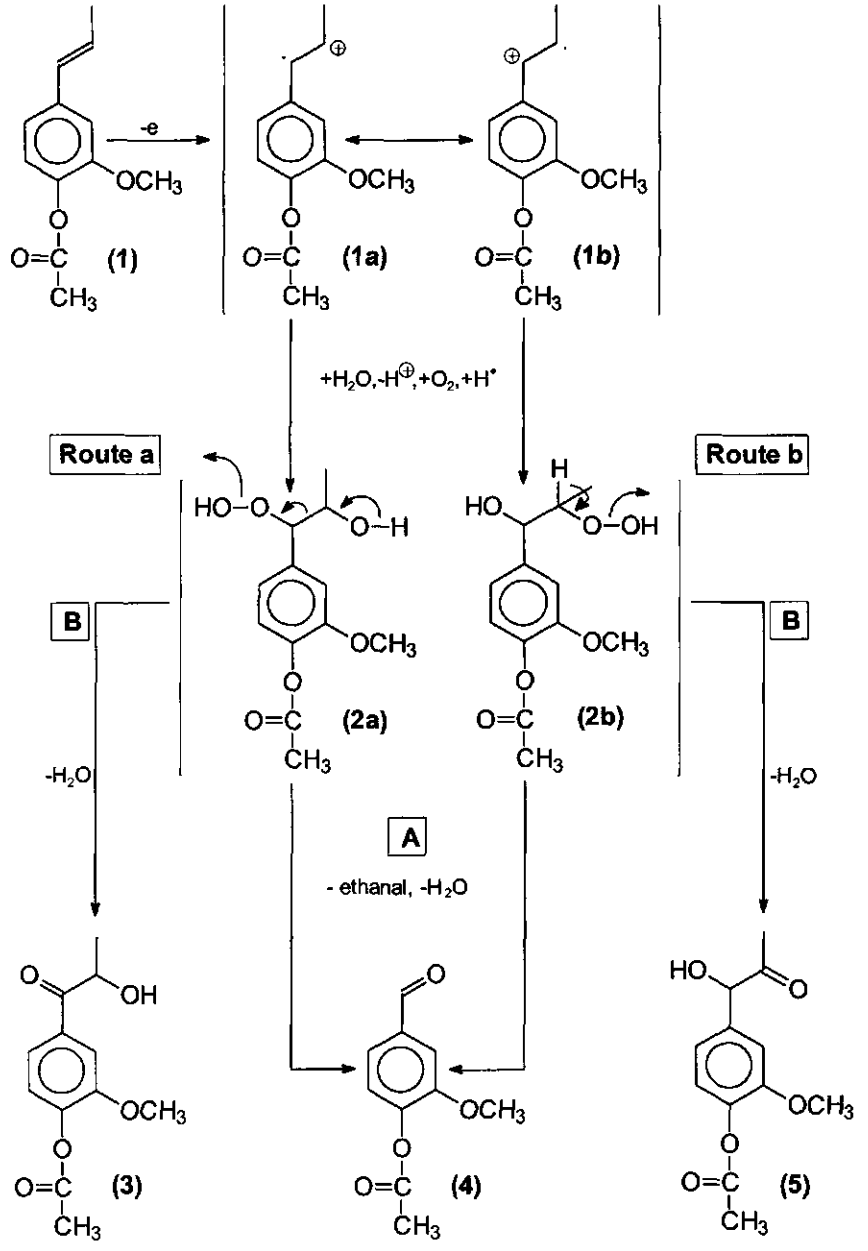
Direct IEA oxidation by LiP compound I, is expected to be of less importance since the calculated IP value of IEA (8.81 ± 0.06 eV, n= 8 [10]) exceeds that of VA (8.67 ± 0.06 eV, n= 5). Using literature data on the LiP compound I reduction rate of AA 2.3 x 10^3 M^{-1} s^{-1} and of VA 1.5 x 10^5 M^{-1} s^{-1} [5] it is demonstrated that a small difference in calculated IP values (0.06-0.22 eV) result in a large effect in the LiP compound I reduction rate. VA has also been described to mediate the oxidation of MMA which, like IEA, has a higher IP than VA if it is present in the protonated form. Tien et al. [6] proposed that oxidation of MMA occurs via abstraction of one electron from the deprotonated form of MMA. The presence of the negative

charge in MMA results in a calculated IP which is much lower than that of VA.
In conclusion, compounds with a higher calculated IP value than VA are also oxidized by redox mediation, indicating that also other factors than just the IP value are important in explaining the mediation phenomenon. A possible factor involved is the LiP-$VA^{\bullet+}$ complex [20,21] which would be expected to accumulate more for terminal substrates which have no binding with LiP. According to the Nernst equation, a higher $VA^{\bullet+}$ concentration would enhance the abillity to oxidize terminal substrates with a high IP.

THE FORMATION OF VANILLYL ACETATE FROM IEA

Formation of vanillyl acetate was shown to require only a one electron oxidation step of IEA. If formation of vanillyl acetate would have occurred via an intermediate diol, as proposed for the direct LiP-substrate 1-(4'-ethoxy-3'-methoxyphenyl)-propene [15], four single electron oxidation steps would have been necessary. In addition such a diol, 1-(4'-acetoxy-3'-methoxyphenyl)-1,2-propanediol, was shown to have a calculated IP beyond the IP threshold value of 9.0 eV [10] and would not be converted by LiP.
A possible mechanism involved in vanillyl acetate and side product formation is pointed out in Scheme 5.2. Several typical peroxyl radical reactions like: addition to double bonds, or benzylic hydrogen abstraction [17] were considered and, on the basis of several experiments, omitted from the C_α-C_β cleavage mechanism involved in vanillyl acetate formation from IEA. This strongly narrowed the number of possible mechanims.
IEA (**1**) is enzymatically oxidized to the corresponding radical cation $IEA^{\bullet+}$ which is stabilized by several resonance structures. The positive charge may be either present on C_β (**1a**), or C_α (**1b**) and the unpaired electron on C_α (**1a**), or C_β (**1b**) owing to the sp^2 hybridization of C_α and C_β. The subsequent step is the reaction of $IEA^{\bullet+}$ with water, followed by addition of oxygen forming a putative peroxyl radical. The formation of peroxyl radicals is a common event in LiP catalyzed reactions as shown in the literature [1]. These peroxyl radicals are suggested to each abstract a hydrogen atom, which may originate from the buffer, yielding the instable hydroperoxides **2a** and **2b**. Several examples in the literature indicate that also hydroperoxides may be formed owing to LiP activity [22,23]. Route A in Scheme 5.2 shows that heterolytic cleavage of **2a** or **2b**, in analogy to a mechanism proposed by Schmidt et al. [23], yields vanillyl acetate (**4**) and ethanal.
Route B in Scheme 5.2 depicts how spontaneous heterolytic cleavage of **2a**, and **2b** results in the observed compounds 1-(4'-acetoxy-3'-methoxyphenyl)-1-hydroxy-2-propanone (**5**), and 1-(4'-acetoxy-3'-methoxyphenyl)-2-hydroxy-1-propanone (**3**). Formation of the compound 1-(4'-acetoxy-3'-methoxyphenyl)-2-propanone can be rationalized by two successive one electron oxidation steps of IEA (not shown).
It is well known that only a minimal amount of enzymatically catalyzed oxidation steps is necessary to initiate O_2-dependent spontaneous

chemical reactions [1]. Without any O_2 present, C_α-C_β cleavage did not occur. Instead yields of dimerized products were increased, suggesting that other oligomeric products were formed as well. This hypothesis implies that O_2 may be important in overcoming oligomerization reactions.



SCHEME 5.2 **The proposed mechanism involved in vanillyl acetate formation from IEA by LiP. For details see text.**

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LITERATURE

[1] Schoemaker, H.E. (1990) *Recl. Trav. Chim. Pays-Bas* **109**:255-272
[2] Tonon, E., and Odier, E. (1988) *Appl. Envrion. Microbiol.* **54**:466-472
[3] Teunissen, P.J.M., and Field, J.A. (1998) *FEBS Lett.* **439**:219-223
[4] Harvey, P.J., and Candeias, L.P. (1995) *Biochem. Soc. Trans.* **23**:262-267
[5] Koduri, R.S., and Tien, M. (1994) *Biochemistry* **33**:4225-4230
[6] Tien, M.,and Ma, D. (1997) *J. Biol. Chem.* **272**:8912-8917
[7] Harvey, P.J., Schoemaker, H.E., and Palmer, J.M. (1986) *FEBS Lett.* **195**:242-246
[8] Popp, J.L., and Kirk T.K. (1991) *Arch. Biochem. Biophys.* **288**:145-148
[9] Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammer B., and Kirk, T.K. (1990) *Biochem. J.* **268**:475-480
[10] ten Have, R., Rietjens, I.M.C.M., Hartmans, S., Swarts, H.J., and Field, J.A. (1998) *FEBS Lett.* **430**:390-392
[11] Goodwin, C.D., Aust, S.D., and Grover, T. A. (1995) *Biochemistry* **34**:5060-5065
[12] ten Have, R., Hartmans, S., and Field, J.A. (1997) *Appl. Environ. Microbiol.* **63**:3301-3303
[13] ten Have, R., Hartmans, S., Teunissen, P.J.M., and Field, J.A. (1998) *FEBS Lett.* **422**: 391-394
[14] Alsheikhly, M.I., and Simic, M.G. (1986) In *Superoxide and superoxide dismutase in chemistry, biology and medicine* (Rotilio, G. ed). pp 32-34. Elsevier Science Publishers B.V., Amsterdam, The Netherlands
[15] Gold, M.H., Kuwahara, M., Chiu, A.A., and Glenn, J.K. (1984) *Arch. Biochem. Biophys.* **234**:353-362
[16] Bovicelli, P., and Sanetti, A. (1996) *Tetrahedron* **52**:10969-10978
[17] Ingold, K.U. (1968) *Acc. chem. res.* **2**:1-9
[18] Valli, K., Wariishi H., and Gold, M.H. (1990) *Biochemistry* **29**:8535-8539
[19] Teunissen, P.J.M., Sheng, D., Reddy, G.V.B., Moënne-Loccoz, P., Field, J.A., and Gold, M.H. (1998) *Arch. Biochem. Biophys.* **360**:233-238
[20] Khindaria, A., Yamazaki, I., and Aust, S.D. (1996) *Biochemistry* **35**:6418-6424
[21] Candeais, L.P., and Harvey, P.J. (1995) *J. Biol. Chem.* **270**:16745-16748
[22] Chivulka, M., Spadaro, J.T., and Renganathan, V. (1995) *Biochemistry* **34**:7765-7772
[23] Schmidt, H.W.H., Haemmerli, S.D., Schoemaker, H.E., and Leisola, M.S.A. (1989) *Biochemistry* **28**:1776-1783

6

C_α-C_β cleavage of 1-(3',4'-dimethoxyphenyl)-propene by lignin peroxidase from *Bjerkandera* sp. strain BOS55

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In view of the biocatalytic conversion of plant phenolics to vanillin, lignin peroxidase (LiP) has been used to study the C_α-C_β cleavage of the propylene side chain in 1-(3',4'-dimethoxyphenyl)-propene (DMPP), a derivative of isoeugenol, to 3,4-dimethoxy-benzaldehyde (VAD). Under an air atmosphere, LiP oxidized DMPP to VAD (27.8 %), 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA, 8.7 %), and at least 17 unidentified products after 10 minutes of incubation. Dissolved oxygen was rapidly consumed during DMPP conversion and one third was converted to superoxide, which indirectly provided LiP with additional H_2O_2 by dismutation. The remaining two thirds of the consumed oxygen was involved in C_α-C_β cleavage of DMPP to VAD and in self-propagating chemical reactions stimulating the consumption of DMPP. The latter was confirmed by using the well-known peroxyl radical reductant, Mn^{2+}, which severely inhibited DMPP consumption under air, but did not affect the DMPP consumption under N_2. The presence of Mn^{2+} also decreased the molar VAD yield, indicating that VAD was also formed during these self-propagating chemical reactions. Another VAD producing route was found by incubating the DMPP oxidation product, DMPA with LiP. Under air VAD was formed in a molar yield of 29.7 %. In the absence of O_2, the C_α-C_β cleavage of DMPA to VAD was strongly inhibited. These conditions predominantly resulted in the formation of side chain coupling products (dimers). The cleavage of DMPA to VAD is proposed to occur via an unstable hydroperoxide intermediate which may alternatively decompose to the tentatively identified 1-(3',4'-dimethoxyphenyl)-1,2-propane-dione.

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INTRODUCTION

Lignin is a substituted phenylpropanoid polymer which is most extensively decomposed by white-rot fungi. These fungi excrete several peroxidases that depolymerize lignin by oxidation of the phenyl moieties leading to the splitting of β-O-4 linkages, and C_α-C_β bonds in the side chain. Lignin peroxidase (LiP) is largely responsible for these reactions [1] which could be deduced from the oxidation products derived from small aromatic compounds, representing lignin model compounds [1,2]. The enzyme oxidises aromatic compounds by one-electron to the corresponding radical cation [1,3,4] which may undergo various non-enzymatic reactions like addition of water [1,5], or splitting off a proton to form a radical [1]. The formation of a radical enables various additional reactions such as addition of superoxide ($HOO^\bullet$) [1,6,7], radical coupling [8], or peroxyl radical formation owing to the reaction with O_2 [9]. These reactions are mainly responsible for the large variety of oxidation products.

LiP has been used to study its potential in the biocatalytic conversion of plant phenolics to vanillin. In a previous study we indicated that 1-(4'-acetoxy-3'-methoxyphenyl)-propene (isoeugenyl acetate, IEA) was converted into 4-acetoxy-3-methoxybenzaldehyde (vanillyl acetate) in a reasonable molar yield of 51.8 % [10]. Also C_α-C_β cleavage of a structurally related compound 1-(4'-ethoxy-3'-methoxyphenyl)-propene (EMPP) by LiP has been reported [2,12]. It was proposed that EMPP is first converted into a diol intermediate which is thereafter oxidized again by LiP resulting in C_α-C_β cleavage to 4-ethoxy-3-methoxybenzaldehyde [2,12]. Oxygen appeared to be involved in this reaction since it has been shown to be incorporated in the diol at C_β [12].

In the case of IEA the presence of 3,4-dimethoxybenzylalcohol (veratryl alcohol, VA) was essential for IEA consumption by LiP. The studied C_α-C_β cleavage reaction to vanillyl acetate was largely dependent on O_2 and required only a single one-electron oxidation step. Based on this information and on the tentative identification of side products, unstable hydroperoxides have been proposed to be the intermediates subjected to C_α-C_β cleavage [11].

Here, the consumption of 1-(3',4'-dimethoxyphenyl)-propene (DMPP) and the subsequent cleavage to 3,4-dimethoxybenzaldehyde (veratraldehyde, VAD) by LiP is evaluated. We report on an unique oxidation pattern of the substrate involving the formation of 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA), extensive superoxide generation and inhibition by Mn^{2+}.

MATERIALS AND METHODS

CHEMICALS

Veratryl alcohol (VA), veratraldehyde (VAD), 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA), 1-(3',4'-dimethoxyphenyl)-propene (DMPP), and tetranitromethane (TNM) were obtained from Aldrich (Bornem, Belgium).

INCUBATION CONDITIONS

Throughout the experiments, the purified LiP isozyme-2 (LiP) from *Bjerkandera* sp. strain BOS55 was used [13]. The substrate, DMPP, was added from a 40.4 mM stock solution prepared in acetone. A standard incubation mixture (V_{tot} = 250 µl) contained: 0.25 mM H_2O_2 and 0.48 mM DMPP. The used buffer and the LiP concentration varied and are described per experiment. The reaction was initiated by the addition of LiP and performed in at least duplicate. At varying incubation times the reaction was stopped by the addition of 500 µl acetonitrile. Control experiments were incubated for the maximal duration of the experiment and did not contain H_2O_2 or active LiP (inactivated by boiling).

The substoichiometric requirement for H_2O_2 was studied using 100 mM sodium tartrate pH 3.0, 0.57 µM LiP, and was performed in duplicate at room temperature. The incubation time was 10 minutes.

In an experiment studying the O_2 and DMPP consumption in relation to superoxide and VAD production, the reaction was performed at 30 °C in 100 mM sodium succinate pH 3.0. The LiP concentration was 0.053 µM. The experiments were performed in duplicate and stopped after 20, 40, 60, 90, 120, 150, 180, 240, 300, and 420 s.

The effect of Mn^{2+} under air was studied at room temperature using 100 mM sodium lactate pH 3.0/4.0 and at a LiP concentration of 0.085 µM. Initial turnovers were calculated by using data from duplicate incubations stopped after 20, 40, 60, 80, and 100 s. The molar VAD yield, found at the Mn^{2+} concentrations studied, is presented as the average of these five duplicate experiments.

In an experiment studying the effect of complete anaerobic conditions on the reaction without Mn^{2+}, the experiment was performed in an anaerobic hood (atmosphere: 96 % N_2 and 4 % H_2). This experiment was performed in triplicate in 100 mM succinate buffer pH 3.0 at a LiP concentration of 0.085 µM. The reaction was stopped after 100 s.

The effect of Mn^{2+} was also studied in the anaerobic hood. This experiment was performed in duplicate in 100 mM lactate buffer pH 3.0 at a LiP concentration of 0.085 µM. The reaction was stopped after 100 s.

The effect of 1500 units superoxide dismutase (SOD) from bovine erythrocytes (Sigma, Zwijndrecht, The Netherlands) was studied using 100 mM sodium lactate pH 3.0/4.5. The incubation time was 100 s. After terminating the reaction, SOD was precipitated by the addition of acetonitrile. Samples were therefore subjected to centrifugation (10,000 *g*) prior to HPLC analysis.

HPLC ANALYSIS

Samples were analysed using a HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany) equipped with a HP1100 pumping station, a HP1040M series II photo diode array detector, and a HP9000-300 data processor. HPLC chromatograms were recorded at 215, 280, and 310 nm. A reversed phase C18 column (200 mm x 3 mm filled with ChromoSpher C18-PAH 5 µM particles, Chrompack) was used. Compounds were eluted using a linear water/ acetonitrile gradient which started at 10 %

acetonitrile and increased to 100 % acetonitrile in 15 minutes. The flow was 0.4 ml/min and the temperature was held constant at 30°C. VAD and DMPP concentrations were determined using an external standard.

GC-MS CONDITIONS AND PREPARATION OF THE SAMPLES

For analysis of oxidation products, incubations were performed on a 2.5 mL scale in a test tube. The following conditions were applied: 100 mM sodium succinate pH 3.0, 0.085 μM LiP, 0.25 mM H_2O_2, and 0.48 mM DMPP or 0.50 mM DMPA. The acetone content was 1.2 v/v %. After 10 minutes of incubation, the reaction was stopped with 5 mL 15 mM vitamin C. Thereafter, the internal standard, 3-fluoro-4-methoxybenzaldehyde, was added whereafter the mixture was extracted twice with ethyl acetate. The combined organic layers were dried using brine and after separation concentrated under a N_2 stream. Control experiments were performed using boiled LiP.

GC-MS analyses were carried out on a HP5973 quadrupole MS coupled to a HP6890 gas chromatograph (Hewlett Packard) equipped with a fused silica capillary column (HP-5MS, 30 m x 0.25 mm i.d., film thickness: 0.25 μm). Carrier gas and flow: He at 1.0 ml/min. An injector temperature of 220°C was used. The temperature program started at 70 °C and raised till 250°C at 7°C/min. The final temperature was maintained for 5 min. Fragment ions were obtained at an electron impact of 70 eV.

The formation of VAD, and DMPA was confirmed by comparing the retention times (GC) and mass spectra with VAD and DMPA standards. Molar yields were calculated as mol of product formed per mol of DMPP converted.

SUPEROXIDE ASSAY

The formation of the yellow trinitromethane anion, formed as a reaction product from tetranitromethane (TNM) and superoxide, was measured spectrophotometrically at a wavelength of 350 nm (ε=13,500 M^{-1} cm^{-1} [14]) at 30 °C.

MEASUREMENT OF O_2

An incubation mixture containing 100 mM sodium succinate pH 3.0, 0.25 mM H_2O_2, 0.48 mM DMPP, and 0.053 μM LiP was prepared (V_{tot}= 3 mL). The O_2 consumption was measured at 30°C and measured using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, USA).

Determination of the residual LiP-activity

In order to study whether the presence of Mn^{2+} caused LiP inactivation, the residual LiP activity was measured after 100 s of incubation. This was done by transferring 100 μL sample from a standard incubation mixture (+/- 1 mM Mn^{2+}) to a mixture containing 50 mM succinate pH 3.0, 0.5 mM H_2O_2, and 4 mM ABTS. The colouring of ABTS was measured spectrophotometrically at a wavelength of 420 nm at room temperature.

MATHEMATICAL PROCEDURE

When DMPP consumption was measured during the reaction, a curve was obtained. An equation of the type $y = ax^3 + bx^2 + cx + d$ was used to describe the curve. The initial DMPP consumption rate was obtained by substituting the value zero for x in $y' = 3ax^2 + 2bx + c$.

STATISTICAL PROCEDURE

Average values are presented ± the standard error of the mean (SEM).

RESULTS

CONSUMPTION OF 1-(3',4'-DIMETHOXYPHENYL)-PROPENE BY LiP

LiP directly oxidized DMPP at a very high maximal velocity (V_{max}) of 130 $\mu mol.min^{-1}.mg^{-1}$ ($U.mg^{-1}$) in 100 mM sodium lactate buffered at pH 3.0. This value corresponds with a turnover frequency of 89 s^{-1}. The K_m for DMPP was 0.25 mM. Veratryl alcohol was not required for DMPP consumption.

GC-MS analysis of an ethyl acetate extract obtained from an incubation conducted under an air atmosphere revealed the presence of 5 discernible oxidation products. The two most predominant products (91.6 % of the product peak area) were identified as 3,4-dimethoxybenzaldehyde (VAD, 4 in Scheme 6.2) and 1-(3,4-dimethoxyphenyl)-2-propanone (DMPA, 2 in Scheme 6.2) and these were formed in molar yields of 27.8 and 8.1 %, respectively. The products not accounted for by GC-MS analysis (approx. 60 %) were probably not amendable to GC analysis.

The HPLC method indicated that additional products eluted after DMPP (t_r = 14.6 min.) from the column between 15 and 19 min. By acidifying the eluent (water) to pH 3.0, a total of 14 compounds were clearly detected in this region. Together these accounted for 35 % of the product peak area detected at a wavelength of 215 nm.

This study focuses on the GC-MS amendable compounds.

OXYGEN UPTAKE DURING DMPP CONSUMPTION

Figure 6.1A shows the consumption of DMPP at a LiP concentration of 0.053 μM. The initial consumption rate of 0.35 $nmol.s^{-1}$ is about 3 fold higher than the formation rate of VAD (0.11 $nmol.s^{-1}$). Figure 6.1B shows the uptake of O_2 during DMPP consumption, occurring at an initial rate of 0.21 $nmol.s^{-1}$. Oxygen was clearly involved in the formation of DMPP oxidation products as demonstrated by the formation of HOO• which was produced at a rate of 0.12 $nmol.s^{-1}$. Because O_2 is consumed faster than HOO• is formed, incorporation of O_2 into DMPP derived oxidation products would likely account for the difference. When incubations were carried out under completely anaerobic conditions, the molar VAD yield was at best two times lower than the molar yield under an air atmosphere (see Figure 6.2). This stressed the importance of O_2 for C_α-C_β cleavage. Also the rate of DMPP consumption was clearly inhibited by the absence of O_2. The possible involvement of HOO• in VAD formation was

studied by introducing the superoxide scavenger [15] superoxide dismutase (SOD) into the incubation mixture.

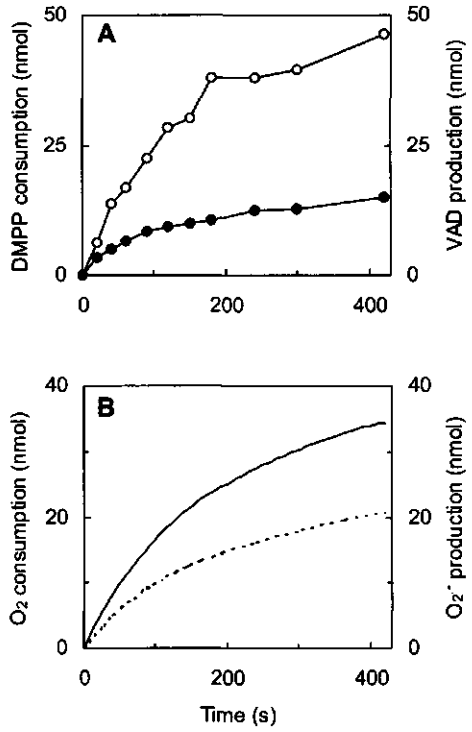


FIGURE 6.1 **Panel A shows the consumption of DMPP (○) by LiP (0.053 μM) and the simultaneous production of VAD (●). Panel B shows the consumption of oxygen (solid line) and the production of superoxide (broken line). The experiments were performed in 100 mM sodium succinate buffer pH 3.0 at 30 °C.**

Table 6.1 shows that the presence of 1500 Units SOD did not affect the conversion of DMPP nor the molar VAD yield. The same effect was observed at pH 4.5 (result not shown), indicating that HOO• was not involved in the C_α-C_β cleavage mechanism to VAD.

TABLE 6.1 **The effect of SOD (1500 Units) on the DMPP conversion by LiP (0.085 mM) and the molar VAD yield in lactate buffer pH 3.0 after an incubation time of 100 seconds. Average values of three incubations ± SEM are presented. The experiment was performed in 100 mM sodium lactate buffer pH 3.0 at room temperature.**

Extra added	DMPP conversion (%)	Molar VAD yield (%)
Nothing	39.7 ± 0.7	28.4 ± 1.2
SOD	41.4 ± 0.7	30.2 ± 0.5
Boiled SOD	41.5 ± 0.7	21.2 ± 0.1

EFFECT OF H_2O_2 CONCENTRATION

It is well known that HOO• rapidly dismutates at these low pH's forming H_2O_2 and O_2 [15]. This possibility was studied by introducing varying H_2O_2 amounts and allowing the reaction to run till completion. Figure 6.3A shows that the requirement for H_2O_2 was substoichiometric as evidenced by the maximal consumption of 13 mol of DMPP per mol of H_2O_2, which indicated that H_2O_2 was produced endogenously.

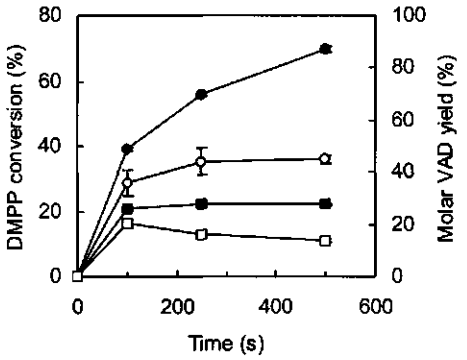


FIGURE 6.2 The conversion of DMPP (●) and the molar VAD yield (■) observed under air or in the complete absence of O_2 (○, □). The experiment was performed in triplicate in 100 mM succinate buffer pH 3.0 at room temperature.

Figure 6.3B shows that the molar VAD yield increases from 27.0 % in the presence of 6.5 nmol H_2O_2 (0.026 mM) to 40 % in the range from 62.5 to 125 nmol H_2O_2 (0.25-0.5 mM).

EFFECTS OF Mn^{2+}

An interesting observation was that the presence of Mn^{2+} caused a large inhibitory effect on the initial DMPP consumption rate and the molar yield of VAD. Since SOD had no inhibitory effect, the inhibition caused by Mn^{2+} could not be attributed to its HOO•-scavenging activity. This finding was studied in more detail since no comparable effect of Mn^{2+} on a LiP catalysed reaction has been reported up to now.

Figure 6.4A shows that the presence of Mn^{2+} caused a sharp decrease in the observed turnover frequency in the concentration range from 0 to 0.5 mM. From there on, only a slight decrease was noted. At a pH value of 3.0, 1 mM Mn^{2+} decreased the observed turnover frequency by a factor of 2.3. At pH 4.0, this factor was 3.8.

The nature of this inhibition was studied and was found to be non-competitive as evidenced by an unchanged K_m value and a decreased V_{max} in the presence of Mn^{2+} (result not shown). To study whether the presence of Mn^{2+} caused inactivation of LiP, the residual activity in samples incubated for 100 s with and without 1 mM Mn^{2+} was measured. No inactivation of LiP could be attributed to Mn^{2+} (result not shown). If instead of DMPP, VA was used as a substrate for LiP, no inhibition of

VA (0.1-2 mM) oxidation was observed at either pH 3.0 and 4.0 in the presence of Mn^{2+} (2 mM).

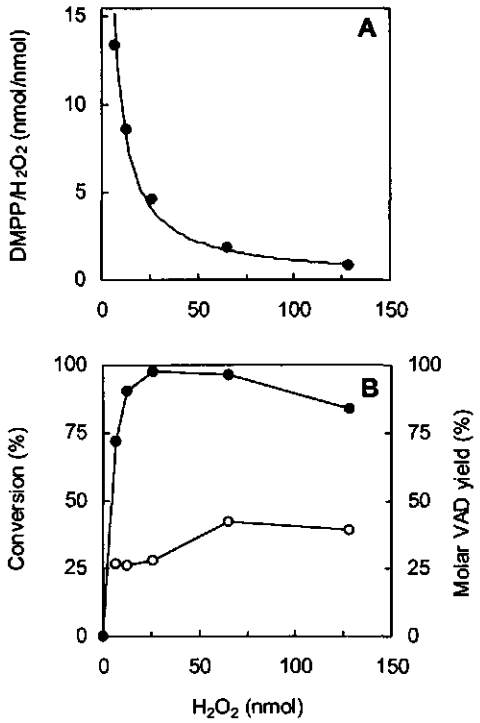


FIGURE 6.3 Panel A shows the ratio between consumed DMPP (nmol) and initially added H_2O_2 (nmol) (●) by LiP (0.57 μM). Panel B shows the DMPP conversion (●) and the molar VAD yield (○). The experiment was performed in 100 mM sodium tartrate buffer pH 3.0 at room temperature. The total reaction time was 10 minutes and the initial DMPP amount was 121 nmol (0.48 mM).

Table 6.2 shows the effect of Mn^{2+} on the DMPP consumption rate in the presence and in the absence of air. Mn^{2+} inhibits the DMPP consumption in the air incubation to a level which is comparable to that observed under anaerobic conditions.

TABLE 6.2 The effect of several incubation conditions on the DMPP conversion and the molar VAD yield. The experiments were performed in duplicate in 100 mM sodium lactate buffer pH 3.0 at room temperature and incubated for 100 s.

Condition	Normalized to the air incubation (%)	
	DMPP conversion	Molar VAD yield
Air	100	100
Air + 1 mM Mn^{2+}	56	45
Anaerobic hood	48	54
Anaerobic hood + 1 mM Mn^{2+}	41	22

When the effect of Mn^{2+} was studied under anaerobic conditions only very little inhibition of the DMPP consumption was observed. Therefore, Mn^{2+} eliminated the stimulating effect of O_2 on the DMPP consumption. Mn^{2+} (1 mM) also affected the molar yield of VAD. This yield decreased by 50 to 60 % under both aerobic and anaerobic incubation conditions (see Table 6.2 and Figure 6.4B).

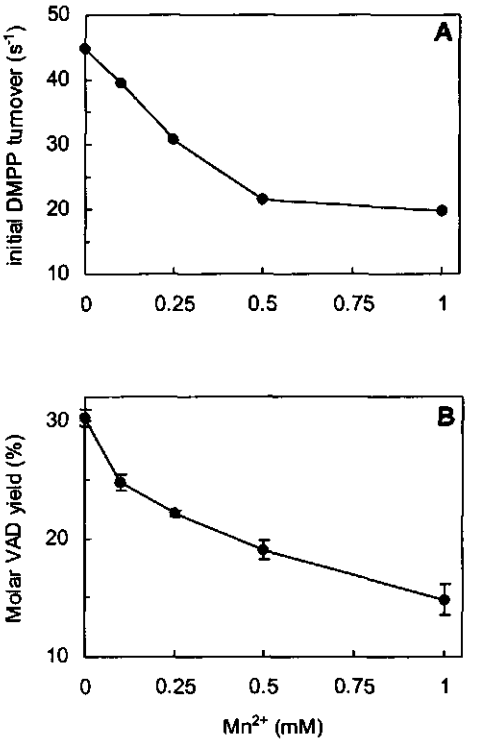


FIGURE 6.4 Panel A shows the observed turnover frequency (s^{-1}) of LiP (0.085 μM) in the presence of DMPP (0.48 mM) at varying Mn^{2+} concentrations. Panel B shows the average (n= 5) molar VAD yield (%) at varying Mn^{2+} concentrations. The experiment was performed in 100 mM lactate buffer pH 3.0 at room temperature.

DMPA AS AN INTERMEDIATE VAD PRECURSOR

Since DMPA was an intermediate of DMPP oxidation, it was tested as a substrate for LiP. Upon incubating DMPA (2 in Scheme 6.2) with LiP for a total reaction time of 10 minutes under air, 66.7 % of the initially added DMPA was converted.GC-MS analysis showed that VAD was formed at a molar yield of 29.7 %. Table 6.3 shows that some additional side products were also formed under aerobic conditions. The most pronounced side product could be tentatively identified as 1-(3',4'-dimethoxyphenyl)-propane-1,2-dione (DMPPD, **3** in Scheme 6.2) on the basis of its fragmentation pattern and by comparison with mass spectra in the

NIST98 mass spectral data base. As shown in Table 6.3, product formation was clearly different under anaerobic (N_2 flushing) conditions. In this case, two DMPA dimers with a $[M]^+$ at a m/z of 386 were the predominant products, whereas VAD and DMPPD were only minor products. The dimers (**5** in Scheme 6.2), formed in a 1.96 (t_r = 27.5 min.) to 1 (t_r = 29.9 min.) ratio showed almost identical mass spectra, indicating that these two dimers were stereoisomers. The predominant fragmentation peak at a m/z of 193 is rationalised by $[M\text{-}(DMPA\text{-}H^+)]^+$, suggesting that these dimers are side chain coupling products of DMPA.

TABLE 6.3 GC-MS analysis of compounds formed from DMPA under an air or N_2 atmosphere and the main fragmention peaks in the MS-spectrum of the tentatively identified compounds. The experiment was performed in 100 mM succinate buffer pH 3.0 at a LiP concentration of 0.085 μM.

Compound	Product peak area (%)		*m/z* (EIMS) of tentatively identified compounds
	Air	N_2	
VAD	61.5	5.3	
DMPPD	19.9	2.0	208 (2), 165 (100), 137 (10), 122 (9), 107 (6)
Dimer (t_r= 27.5 min.)	0.0	53.8	386 (8), 343 (2), 300 (6), 286 (10), 193 (100), 165 (19), 162 (15), 43 (23)
Dimer (t_r= 29.9 min.)	0.0	27.5	386 (6), 343 (3), 300 (5), 286 (8), 193 (100), 165 (23), 162 (15), 43 (22)
Unknown*	18.6	11.4	

*** Includes also unknown compounds that are not shared.**

DISCUSSION

This study on the oxidation of 1-(3',4'-dimethoxyphenyl)-propene (DMPP) by the lignin peroxidase isozyme-2 (LiP) from *Bjerkandera* sp. strain BOS55 shows that the C_α-C_β cleavage reaction involved in 3,4-dimethoxybenzaldehyde (VAD) formation strongly depends on the presence of oxygen. This finding was comparable with data on the veratryl alcohol (VA) mediated oxidation of 1-(4'-acetoxy-3'-methoxyphenyl)-propene (isoeugenyl acetate, IEA) [11]. Because IEA has a similar propylene side chain as DMPP, it was expected that both side chains were cleaved off by the same reaction mechanism. In view of this expectation, several findings on the oxidation of DMPP were surprising. In contrast to the IEA oxidation experiments [11], superoxide ($HOO^\bullet$) was extensively produced. Furthermore, Mn^{2+} was shown to affect the reaction of DMPP, but had no effect on that of IEA. Also VA was not essential for the consumption of DMPP as was observed for IEA [11].

CONSUMPTION OF DMPP BY LIP

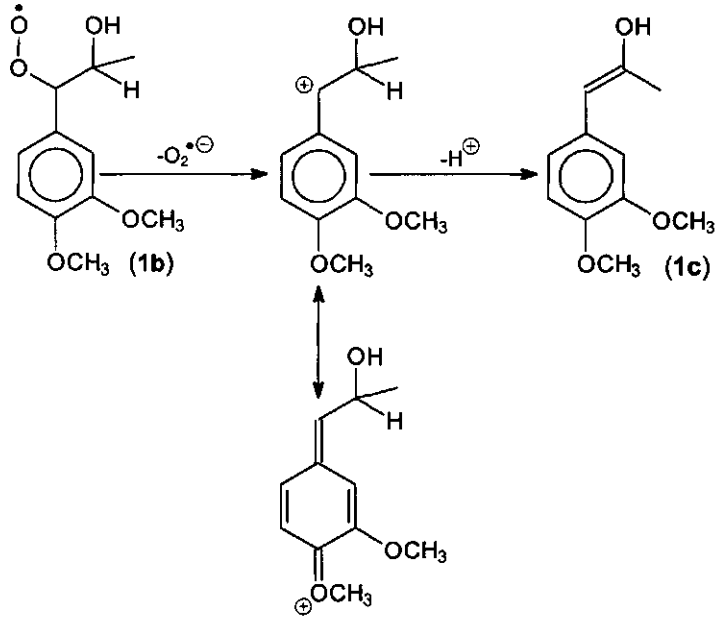
DMPP was found to be directly oxidized by LiP at a maximal velocity (V_{max}) of 130 $U.mg^{-1}$. This is much faster than the V_{max} in which, in theory, the VA-mediated consumption of IEA maximally may occur (16-17 $U.mg^{-1}$ [13]). DMPP may be consumed faster by LiP owing to its lower

calculated ionisation potential (IP) of 8.41 ± 0.04 eV in comparison with the IP of VA (8.67 ± 0.06 eV) [10]. However, there is also evidence that DMPP is also consumed by O_2-dependent self-propagating chemical reactions. This possibility is discussed in the following sections.

THE FORMATION OF SUPEROXIDE DURING CONSUMPTION OF DMPP BY LIP

The colour reaction of TNM during consumption of DMPP under an air atmosphere clearly demonstrates HOO• formation. The instable HOO• may have dismutated, forming O_2 and H_2O_2 [15]. Endogenous H_2O_2 production may explain the substoichiometric requirement for H_2O_2 during DMPP consumption as depicted in Figure 6.3A.

During consumption of IEA, HOO• was only formed at an extremely slow rate [11] whereas it was robustly formed during DMPP consumption (see Figure 6.1B). This difference is explained by the electron withdrawing effect of the acetoxy group in IEA which is not favourable for the elimination of HOO•. In the case of DMPP, HOO• production does occur owing to the electron donating methoxy group (attached to C4) which enhances the conversion to enol **1c** (see Scheme 6.1 and 6.2).



SCHEME 6.1 The methoxy present at C4 stimulates the elimination of superoxide owing to the resonance stabilized intermediate cation derived from 1b.

THE EFFECTS OF ANAEROBIC CONDITIONS

As depicted in Figure 6.2, anaerobic conditions resulted in at least a 2 fold lower molar VAD yield, stressing the importance of O_2 for C_α-C_β cleavage which has also been observed during vanillyl acetate formation from IEA [11]. The fact that still some VAD is formed under anaerobic conditions

may be attributed to the possibility that some O_2 has been formed during the reaction. LiP-derived radical cation intermediates may react with H_2O_2 forming HOO• [16] which dismutates to O_2 and H_2O_2 [15]. The presence of Mn^{2+} would minimize formation of O_2 resulting from the latter reaction because HOO• is then rapidly reduced to H_2O_2 [15]. This theory corresponded to the observation that under anaerobic conditions, 1 mM Mn^{2+} inhibited the molar VAD yield (see Table 6.2).

THE INHIBITORY EFFECT OF Mn^{2+} ON THE DMPP CONSUMPTION BY LiP

Mn^{2+} was found to inhibit both the DMPP consumption rate and the molar yield of VAD. This is unusual since in the case of the well-studied LiP substrate VA, addition of Mn^{2+} caused no inhibition (this study), but enhanced it [6,15].

Mn^{2+} may play several roles in inhibiting LiP catalysed reactions. It may reduce peroxyl radicals to their corresponding hydroperoxides [9], it may function as a superoxide scavenger [15], or it may function as an alternative reducing substrate for LiP compound I [16].

The role of Mn^{2+} as a HOO•-scavenger does not account for the observed inhibition since scavenging of HOO• with SOD did not cause a noteworthy inhibitory effect on either the DMPP consumption or the molar yield of VAD. The possibly extra endogenously generated H_2O_2 in the presence of Mn^{2+} did not result in inactivation of LiP since the residual LiP activity after 100 s of incubation was unaffected.

Furthermore, Mn^{2+} was not a competitive reducing substrate for LiP compound I because the presence of 2 mM Mn^{2+} did not affect the consumption rate of VA in the VA concentration range from 0.1 to 2 mM (result not shown).

The inhibitory effect of Mn^{2+} on the DMPP consumption was almost exclusively evident in the presence of oxygen. The extent of the inhibition reduced the activity of LiP to levels that were observed under anaerobic conditions. These observations point to Mn^{2+}-mediated reduction of intermediate peroxyl radicals. These are formed owing to the extremely fast reaction reaction between O_2 and an organic radical [1,9]. Also the O_2 balance indicates the consumption of O_2 by DMPP derived radical intermediates.

Peroxyl radicals may undergo self-propagating chemical reactions such as hydrogen atom abstraction or addition to double bonds. The described free radical reactions are well-known to be terminated by Mn^{2+} since it reduces the peroxyl radical to the corresponding hydroperoxide, overcoming further self-propagation chemical reactions [9]. The occurrence of these two chemical reactions in the presence of O_2 results in a faster net consumption of DMPP because it is consumed both chemically and enzymatically. In other words: the presence of Mn^{2+} shifts the reaction more towards enzymatic consumption of DMPP.

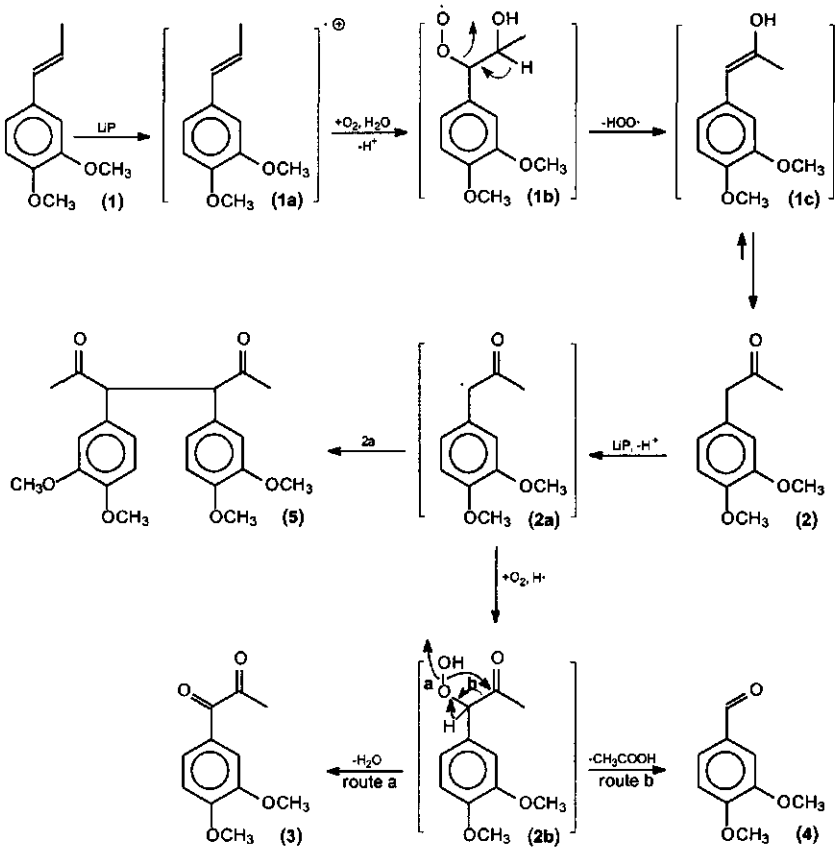
Mn^{2+} was also found to inhibit the molar VAD yield (mol of VAD per mol of DMPP converted). Because Mn^{2+} terminates the discussed self-propagating chemical reactions it is likely to assume that VAD has also

been formed as a result of these reactions. A possible VAD producing route owing to these reactions is the discussed in the following section.

PROPOSED REACTION MECHANISMS INVOLVED IN VAD FORMATION

The data on DMPP suggests that the C_α-C_β cleavage mechanism is distinct from that proposed for 1-(4'-ethoxy-3'-methoxyphenyl)-propene [2,12] since there is no involvement of a diol intermediate. None of the observed product peaks showed fragment ion peaks at a m/z of 212, 194, or 167 which would be expected in the case of a diol intermediate. Instead a major intermediate appeared to be DMPA.

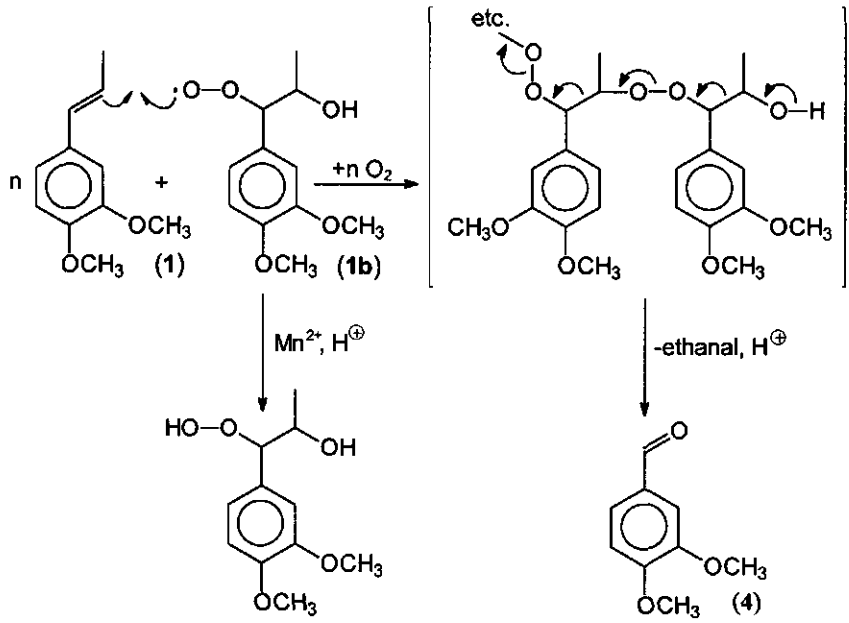
A C_α-C_β cleavage route of DMPP is depicted in Scheme 6.2. DMPP (**1**) is oxidized to a radical cation intermediate (**1a**) which may react with water and oxygen to form a putative peroxyl radical (**1b**).



SCHEME 6.2 Proposed reaction mechanism involved in the formation of VAD form DMPP catalysed by LiP. For details see text.

This radical may split off superoxide, giving the resonance-stabilised enol **1c** which is in equilibrium with 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA, **2**) of which we showed that it is used again as a substrate for LiP.

DMPA is oxidised by LiP to DMPA$^{\cdot+}$. After splitting off a proton, DMPA$^{\cdot}$ (**2a**) is formed. The latter radical may dimerise in O_2 deficient conditions to the tentatively identified stereoisomers with structure **5**. The presence of O_2, however, results in the formation of a peroxyl radical. By a radical disproportionation reaction the intermediate hydroperoxide (**2b**) is formed. Hydroperoxides have been proposed to be intermediates in the formation of side oxidation products from VA [1,6], but have also been detected as LiP oxidation products [17]. Spontaneous heterolytic cleavage of hydroperoxides has been proposed before [1,6,7,11]. Applying this proposal on **2b**, yields two possible cleavage routes, route a and b.
In route a, H_2O is cleaved off which results in the tentatively identified 1-(3',4'-dimethoxyphenyl)-1,2-propanedione.
In route b, the binding between C_α and C_β is cleaved resulting in VAD (4).
A possible way in which Mn^{2+} may interfere with VAD formation is by reducing the peroxyl radical **1b** to the corresponding anion [9]. In this manner it overcomes self-propagating chemical reactions in which **1b** couples with another DMPP molecule (see Scheme 6.3), or in which **1b** abstracts a hydrogen atom from a new DMPP molecule [9]. It is proposed that the O_2-dependent self-propagating chemical reactions result in the formation of unstable organic peroxides of which a possible structure is depicted in Scheme 6.3. These may decompose to form VAD and ethanal.



SCHEME 6.3 Proposed reaction mechanism involved in VAD formation during the hypothesized consumption of DMPP by O_2-dependent free radical reactions.

In comparison with previously conducted research on the cleavage mechanism of IEA, anaerobic conditions did not affect the substrate consumption [11], indicating that IEA solely was consumed via an enzymatic process.

ACKNOWLEDGEMENTS

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LITERATURE

[1] Schoemaker, H.E. (1990) *Recl. Trav. Chim. Pays-Bas* **109**:255-272

[2] Renganathan, V., Miki, K., and Gold, M.H. (1985) *Arch. Biochem. Biophys.* **241**:304-314

[3] Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammer B. and Kirk, T.K. (1990) *Biochem. J.* **268**:475-480

[4] Khindaria, A., Yamazaki, I. and Aust, S.D. (1995) *Biochemistry* **34**:16860-16869

[5] Joshi, D.K., and Gold, M.H. (1996) *Eur. J. Biochem.* **237**:45-57

[6] Haemmerli, S.D., Schoemaker, H.E., Schmidt, H.W.H., and Leisola, M.S.A. (1987) *FEBS Lett.* **220**:149-154

[7] Schmidt, H.W.D., Haemmerli, S.D., Schoemaker, H.E., and Leisola, M.S.A. (1989) *Biochemistry* **28**:1776-1783

[8] Huixian, Z., and Taylor, K.E. (1994) *Chemosphere* **28**:1807-1817

[9] Ingold, K.U. (1969) *Acc. Chem. Res.* **2**:1-9

[10] ten Have, R., Rietjens, I.M.C.M., Hartmans, S., Swarts, H.J., and Field J.A. (1998) *FEBS Lett.* **430**:390-392

[11] ten Have, R., de Thouars, R.G., Swarts, H.J., and Field, J.A. (1999) *Eur. J. Biochem.* **265**:1008-1014

[12] Andersson, L.A., Renganathan, V., Chiu, A.A., Loehr, T.M., and Gold, M.H. (1985) *J. Biol. Chem.* **260**:6080-6087

[13] ten Have, R., Hartmans, S., Teunissen, P.J.M., and Field, J.A. (1998) *FEBS Lett.* **422**:391-394

[14] Alsheikly, M.I., Simic, M.G. (1986) Superoxide and superoxide dismutase in chemistry, biology and medicine pp. 32-34 Elsevier Science Publishers B.V., Amsterdam, The Netherlands

[15] Bono, J.J., Goulas, P., Boe, J.F., Portet, N., and Seris, J.L. (1990) *Eur. J. Biochem.* **192**:189-193

[16] Barr, D.P., Shah, M.M., and Aust, S.D. (1993) *J. Biol. Chem.* **268**:241-244

[17] Chivulka, M., Spadaro, J.T., and Renganathan, V. (1995) *Biochemistry* **34**:7765-7772

7 Concluding remarks

LIGNIN PEROXIDASE

APPLICATION IN VANILLIN PRODUCTION

Concerning the current demand for natural additives, biocatalytic conversions useful in vanillin production by lignin peroxidase have been studied during this Innovative Oriented research Project (IOP, IKA94002). A logical step was to use oxidative enzymes of ligninolytic white-rot fungi since they produce vanillin during growth on their natural substrate. These fungi grow on wood and are able to degrade the wood component lignin, a random linked heterogeneous aromatic polymer. The main reason for degrading lignin is to obtain better access to polysaccharides in the wood fibers [7].

The oxidative enzymes involved in lignin degradation are either non-specific or use small redox mediators that may penetrate wood fibers, oxidizing lignin which is not within reach of large enzymes [7]. Among the oxidative enzymes are laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP). MnP oxidizes Mn^{2+}, that is naturally present in wood, to Mn^{3+} in the presence of H_2O_2. The Mn^{3+} formed may than oxidize phenolic moieties in lignin. LiP is a much more potent peroxidase capable of oxidizing both phenolic and non-phenolic substrates directly [6].

The LiP catalyzed oxidation of non-phenolic aromatic substrates with a propenyl side chain, as in potential vanillin precursors, result in the desired C_α-C_β cleavage reaction, yielding the corresponding benzaldehyde. Therefore, LiP was chosen for further studies on biocatalytic conversions useful in vanillin production.

PRODUCTION OF LIP BY *BJERKANDERA* SP. STRAIN BOS55

In this research the white-rot fungus *Bjerkandera* sp. strain BOS55 is used. While white-rot fungi generally produce ligninolytic enzymes in response to nitrogen starvation, a useful property of BOS55 is the fact that the fungus is nitrogen-unregulated which enables it to produce LiP in proportion to the biomass [4].

In chapter 2, the production of by BOS55 in batch cultures is described. After 14 days of culturing BOS55 at 30°C in the nitrogen-rich medium containing peptone, the LiP activity in the extracellular culture broth reached a maximum of 600 U/L. From that day on, the activity was stable for at least 16 days.

The presence of peptone resulted in stimulation of the LiP activity [4], but also in interference during the LiP assay. During the standard assay the oxidation of 3,4-dimethoxybenzyl alcohol (veratryl alcohol, VA) to 3,4-dimethoxybenzaldehyde (veratraldehyde, VAD) is followed spectrophoto-

metrically. After the addition of H_2O_2 the formation of VAD is normally measured as a steady increase in the extinction at a wavelenght of 310 nm. The presence of peptone, however, interfered with this pattern since VAD formation was delayed (lag phase). The interference was minimized by precipitating LiP with $(NH_4)_2SO_4$ and conducting the assay by using the resuspended LiP-pellet. Crude samples that showed a lag phase during the assay did not show it after the prepurification step. Furthermore, the maximal rate in which VAD was formed increased substantially if compared to the rate after the lag phase as observed by using crude samples.

The phenolic amino acid, tyrosine, present in peptone either free or in a peptide was responsible for the observed interference. As a possible explanation for the observed inhibition, the option that tyrosine was the preferable substrate for LiP is discussed. If tyrosine were a much better substrate for LiP than VA, a mixture of LiP, tyrosine, and H_2O_2 would not have caused bleaching of the heme. However, evident bleaching occurred, indicating that tyrosine was a very poor substrate for LiP.

Tyrosine may be consumed by LiP, but only in the presence of the redox mediator VA. This indirectly evidenced by a) the finding that inclusion of VA, to the above described mixture, overcomes bleaching of the heme and b) the presence of VA results in lag phases in VAD formation that are proportional to the tyrosine concentration.

Normally, LiP oxidizes VA to the radical cation ($VA^{\cdot+}$). The latter may decompose to the VA radical ($VA^{\cdot}$) which may be oxidized further by either O_2 or LiP, resulting in VAD. The observed lag phases indicate that essential intermediates in VAD formation are reduced back to VA. In agreement with the literature [2,5], it is proposed that $VA^{\cdot+}$ is reduced back to VA by tyrosine.

The results indicate that the use of N-rich medium may result in an underestimation of the LiP amount present in the culture broth.

PURIFICATION AND CHARACTERIZATION OF LIP FROM BOS55

The purification and characterization of LiP isozymes from BOS55 is described in chapter 3. *Bjerkandera* sp. strain BOS55 produces at least seven LiP isozymes of which two were purified. The molecular mass of the two purified isozymes (LiP-2 and LiP-5) ranged between 40 and 42 kD. Both showed an evident peak in the UV-VIS spectrum at 407 nm which indicated that these contained a heme as the prosthetic group. The isozymes oxidized the fungal metabolites [8,11] VA and 2-Cl-1,4-dimethoxy-benzenze in the presence of H_2O_2. From pH 5.0 to pH 3.0, the V_{max} of the two isozymes was more or less the same. A pronounced difference between LiP-2 and LiP-5 was the VA-oxidizing activity at pH 2.5. The V_{max} of LiP-5 strongly decreased whereas that of LiP-2 increased even further at that pH value. Since the physiological pH of the fungus is much higher than that of LiPs pH-optimum, the catalytic parameters of one of the isozymes was studied in detail over a pH range of 3.0 to 5.0. Interestingly, the affinity of LiP-2 for H_2O_2 largely with increasing pH (see Table 7.1). Also the inactivation by H_2O_2 was tested. It appeared that

LiP-2 was rapidly inactivated at pH 3.0 in the presence of 125 μM H_2O_2. At pH 5.0, however, complete inactivation took at least 10-fold longer.

TABLE 7.1 **The kinetic parameters of LiP-2: V_{max} (U/mg), and K_m (mM) for VA and H_2O_2 at different pHs.**

pH	V_{max} for VA (U/mg)	K_m for VA (μM)	K_m for H_2O_2 (μM)
3.0	15.9	89	100
4.0	7.5	123	17.2
5.0	1.5	190	7.5

The inactivation reaction between LiP and H_2O_2 results in the formation of LiP compound III, a complex between native LiP and superoxide [9]. If the inactivation were directly related to H_2O_2 than the inactivation would have occurred much faster at pH 5.0, in accordance with the much higher affinity for H_2O_2.
In relation to these findings, also the pH-dependent dismutation of superoxide to H_2O_2 and O_2 was considered. At a pH value of 5.0, this spontaneous reaction occurs almost at a maximum rate ($V= V_{max}$ at the pKa of 4.88). At lower pH values, near pH 3.0, this rate is roughly 45 fold lower [1]. This may suggest that dismutation of superoxide is more favourable at pH 5.0 than the formation of a LiP-superoxide complex.

Several extracellular enzymes of *Bjerkandera* sp. strain BOS55 produce H_2O_2 owing to this H_2O_2 may accumulate reaching a concentration of 5 μM [6]. The combined results concerning the inactivation owing to the presence of H_2O_2 and the low K_m for H_2O_2 at pH 5.0 indicate that the predominant fungal LiP isozyme (LiP-2) works very efficient at this normal physiological extracellular pH.

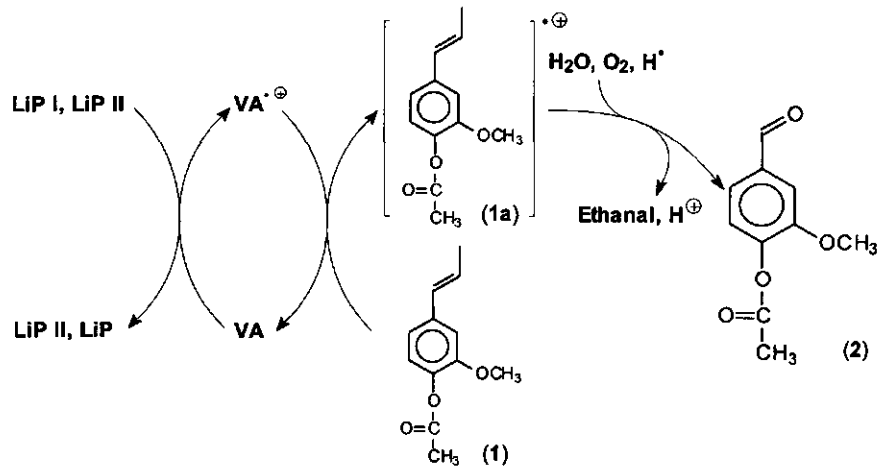
THE SUBSTRATE SPECTRUM OF LIP-2
There are several possible phenolic vanillin precursors available in nature such as eugenol, ferulic acid, coniferyl alcohol etc. To minimize unwanted polymerization of these phenolics owing to the enzymatic action of LiP, protection of the phenolic group was considered as a possible solution for this problem. Methyl ethers, acetyl esters, and glycosides were studied. This increased the number of possible vanillin precursors considerably. Since most of these derivatives are not commercially available and have to be synthesized, a model was developed which predicts if derivatives are LiP substrates. These predictions were required since synthesizing all possible derivatives for testing would not be practically feasible.
Several factors like e.g. size, binding, distance to the heme, and redox potential may be required in describing whether an aromatic compound will be oxidized by LiP. The model described in chapter 4, however, concentrates on the most important factor, which is the energy needed to remove one electron from the highest molecular orbital or the ionization potential (IP). A series of non-phenolic aromatic model compounds with increasing calculated IP values were tested as LiP substrates in order to

establish the IP threshold value. The model substrates were incubated with LiP-2 in the presence of the co-substrate 1,4-dimethoxybenzene (1,4-DMB). The presence of the co-substrate ensures that the system also consumes compounds that are very difficult to oxidize. In the catalytic cycle of LiP there are two oxidation states, LiP compound I and LiP compound II. The inclusion of 1,4-DMB in the reaction mixture aims at conversions that are just within the reach of the LiP compound I which has the highest oxidative power. The remaining LiP compound II may than be reduced to native LiP by 1,4-DMB. The results from this test system indicated that non-phenolic aromatic compounds with a calculated IP value up to 9.0 eV are oxidisable by LiP. Beyond this threshold value, conversion would not be expected.

Bulk natural precursors of vanillin with protecting groups could be selected for further study based on the IP-prediction that they would be substrates for LiP. From this evaluation, glucosides and acetyl esters of isoeugenyl and coniferyl alcohol were selected as promising vanillin precursors. However, of the derivatives tested only isoeugenyl acetate (IEA) was converted into a reasonable molar yield of vanillyl acetate. For this reason the C_α-C_β cleavage mechanism of IEA was studied in more detail.

1-(4'-ACETOXY-3'-METHOXYPHENYL)-PROPENE

The IP of 1-(4'-acetoxy-3'-methoxyphenyl)-propene (isoeugenyl acetate, IEA, 1) is 8.81 ± 0.06 eV which is clearly below the threshold value. IEA was only oxidized significantly in the presence of a cosubstrate either 1,4-dimethoxybenzene (chapter 4) or VA (chapter 5). The way in which IEA was consumed by LiP in the presence of VA was studied in more detail (chapter 5). The results indicate that VA served as a redox mediator between LiP and IEA. LiP first oxidized VA to the corresponding radical cation which in turn oxidized IEA (see Scheme 7.1).



SCHEME 7.1 **Consumption of IEA by LiP via redox mediation by VA and the subsequent proposed formation of vanillyl acetate.**

The evidence for redox mediation is based on the following observations: a) the oxidation of IEA did not occur in the absence of VA, b) VA was effectively recycled during IEA consumption, and c) the formation of VAD from VA was postponed until IEA was completely consumed.
The redox mediator assisted enzymatic reaction could result in the formation of a radical cation derived from IEA ($IEA^{\cdot+}$). The latter may react with water to form a radical which may subsequently react with oxygen. For this reason the effect of O_2 on the reaction products was studied.
An interesting observation was that the formation of vanillyl acetate from IEA was strongly dependent on the presence of O_2 as shown in Table 7.2. The enzymatic consumption of IEA, however, was independent of the presence of O_2.

TABLE 7.2 Effect of the atmosphere on the molar vanillyl acetate yield. The mean ± SEM (n= 3) is presented.

Atmosphere	Molar vanillyl acetate yield (%)
Nitrogen	2.5 ± 0.6
Air	37.0 ± 1.7
Oxygen	54.0 ± 2.1

The proposed mechanism of IEA consumption involves the formation of a VA radical cation which oxidizes IEA by one electron. In the proposed C_α-C_β cleavage mechanism of the propenyl side chain, $IEA^{\cdot+}$ is proposed to react with H_2O and O_2 to form peroxyl radicals. These may abstract a hydrogen atom to form instable hydroperoxide intermediates, that decompose to vanillyl acetate (2) and ethanal. The proposed occurrence of the hydroperoxides is indirectly evidenced by the O_2-dependent presence of alternative decomposition products (hydroxyketones).

1-(3',4'-DIMETHOXYPHENYL)-PROPENE

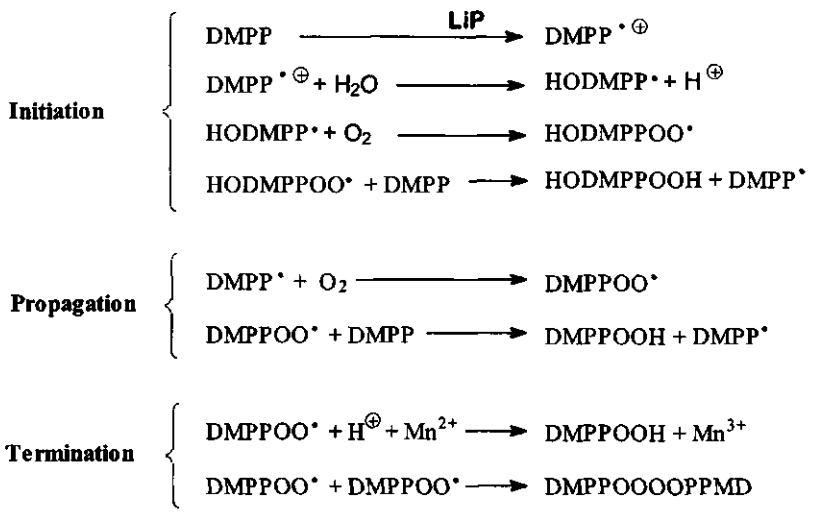
The substrate, 1-(3',4'-dimethoxyphenyl)-propene (DMPP, **3**) or the isoeugenol methyl ether has a calculated IP value of 8.41 ± 0.04 eV and was oxidized without the necessity of VA by LiP (chapter 6). Main GC-MS amendable DMPP oxidation products were 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA, **4**) and 3,4-dimethoxybenzaldehyde (VAD, **5**). In comparison with the findings described on IEA (chapter 5) several results on DMPP were unexpected. These were a minimal requirement of H_2O_2 for excessive DMPP consumption, and inhibitory effects of Mn^{2+} on both the DMPP consumption and the molar VAD yield. These findings were interpreted as characteristics of an unique consumption and C_α-C_β cleavage mechanism. Therefore, these were studied in detail.
Oxygen was consumed rapidly during consumption of DMPP, and only in part converted into superoxide. The latter dismutated, providing the reaction with additional H_2O_2. This explained the minimal H_2O_2 requirement of 1 mole per 13 mole of DMPP. The fact that the superoxide production rate did not fully account for the O_2 consumption rate implied

that O_2 was also incorporated in DMPP oxidation products. Oxygen was involved in C_α-C_β cleavage as evidenced by the findings in Table 7.3, showing that the molar VAD yield decreases 2-fold under anaerobic conditions. Together with the observed consumption of O_2 during DMPP conversion, these findings suggest the involvement of intermediate peroxyl radicals in VAD formation. The occurrence of peroxyl radicals in LiP catalyzed reactions is commonly accepted and has been used frequently to explain product formation [10].

TABLE 7.3 **The effect of several incubation conditions on the DMPP conversion and the molar VAD yield (%) after an incubation time of 100 s.**

Condition	Normalized to the air incubation (%)	
	DMPP consumption	Molar VAD yield
Air	100	100
Air + 1 mM Mn^{2+}	56	45
Anaerobic hood	48	54
Anaerobic hood + 1 mM Mn^{2+}	41	22

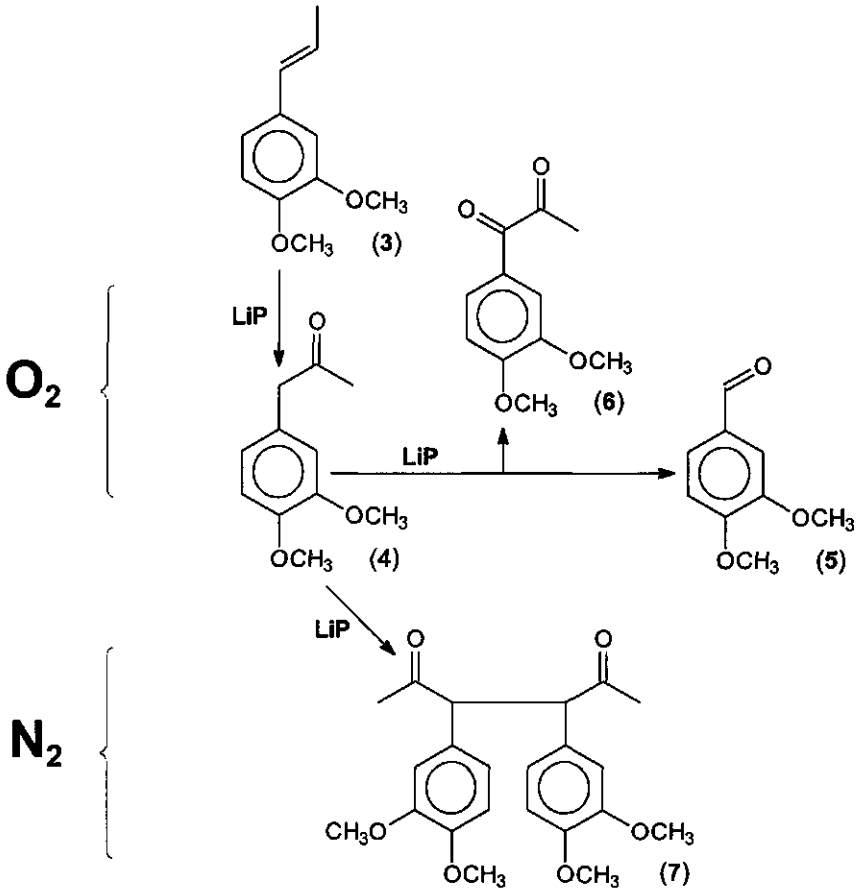
The effect of the well-known peroxyl radical reductant, Mn^{2+} [3], was studied. During the standard LiP-assay in which VA is oxidized to VAD, Mn^{2+} (2 mM) did not affect the VA consumption rate. For that reason it was quite remarkable that Mn^{2+} affected the rate of DMPP consumption of DMPP consumption and the molar yield of VAD.



SCHEME 7.2 **Proposed consumption of DMPP via self-propagating chemical reactions. The consumption is initiated by LiP and is dependent on the presence of O_2. A mechanism that occurs via hydrogen abstraction is depicted.**

Table 7.3 shows that the inhibitory effect of Mn^{2+} on the DMPP consumption was almost completely absent by applying anaerobic

conditions. This finding provided strong evidence for the hypothesis that peroxyl radicals derived from DMPP are also involved in the consumption of fresh DMPP via self-propagating chemical reactions. These peroxyl radicals may add to double bonds as presented in chapter 6, but may alternatively abstract a hydrogen atom as depicted in Scheme 7.2. The Scheme shows that LiP is responsible for the initiation of the chemical DMPP consumption which is followed by a propagation step. The self-propagating chemical DMPP consumption may be terminated by Mn^{2+} or by the coupling of two peroxyl radicals [2].
The inhibitory effect of Mn^{2+} on the molar yield of VAD suggests that at least half of the VAD produced is formed as a result of these self-propagating chemical reactions.
Another VAD producing route was discovered by incubating the DMPP side oxidation product DMPA (4) with LiP (see Scheme 7.3).



SCHEME 7.3 **Proposed formation of oxidation products from DMPA (4). Under air DMPA is converted to 1-(3',4'-dimethoxy-phenyl)-1,2-propandione (6) and VAD (5). Under N_2, intermediate radicals couple to yield dimers (7).**

Under air, DMPA was converted to VAD (**5**) and 1-(3',4'-dimethoxyphenyl)-1,2-propandione (**6**). The absence of O_2 completely changed the product spectrum. In this case the side chains of two DMPA radicals coupled forming dimers (**7**). In Scheme 7.3 a simplified mechanism is presented depicting product formation from DMPA and consumption of DMPP.

INDUSTRIAL APPLICABILLITY OF LiP IN VANILLIN PRODUCTION

This research was part of an innovative oriented research programme (IOP, IKA94002) on catalysis in which several industries participated. From that perspective, this research has to meet certain requirements before its applicability is considered seriously.

Right from the start of this project it was clear that the results should at least be as good as those described in the European patent application (nr. 0542348 A2) on preparing phenyl aldehydes. Another requirement is the substrate which should be cheap, natural, and available in bulk amounts. Eugenol, ferulic acid, and e.g. curcumin (see chapter 4) fit this profile and others like isoeugenol, and coniferyl alcohol do not. Unfortunately, only derivatives of the latter two were vanillin precursors. Therefore, the findings from this research are not applicable in the food industry.

LITERATURE

[1] Behar, D., Czapski, G., Rabani, J., Dorfman, L.M., and Schwarz, H.A. (1970) *J. Phys. Chem.* **74**:3209-3213

[2] Chung, N., and Aust, S.D. (1995) *Arch. Biochem. Biophys.* **316**:815-855

[3] Ingold, K.U. (1969) *Acc. chem. res.* **2**:1-9

[4] Kaal, E.E.J., de Jong, E., and Field, J.A. (1993) *Appl. Environ. Microbiol.* **59**:4031-4036

[5] Koduri, R.S., and Tien, M. (1995) *J. Biol. Chem.* **270**:22254-22258

[6] Kersten, P.J., Kalyanaraman, B., Hammel, K.E., Reinhammer, B., and Kirk, T.K. (1990) *Biochem. J.* **268**:475-480

[7] Leonowicz, A., Matuszewska, A. et al. (1999) *Fungal Genetics and Biology* **27**:175-185

[8] Mester, T., De Jong, E. and Field, J.A. (1995) *Appl. Environ. Microbiol.* **61**:188101887

[9] Mester, T., Gimaraes, C., Field, J.A. and Hilhorst, R. (1997) Tappi Proceedings Biological Science Symposium, TAPPI Press, Atlanta, USA, 483-489

[10] Schoemaker, H.E. (1990) *Recl. Trav. Chim. Pays-Bas* **109**:255-272

[11] Swarts, H.J., Verhagen, F.J.M., Field, J.A., and Wijnberg, J.B.P.A. (1996) *Phytochemistry* **42**:1699-1701

List of abbreviations

ε	molar extinction coefficient
$[M]^+$	fragment ion indicating the molecular mass
1,4-DMB	1,4-dimethoxybenzene
AA	*p*-anisyl alcohol
ABTS	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)
BIII-medium	basal III medium
BSA	bovine serum albumin
C_α-C_β	The bond between carbon alpha and beta.
DMPA	1-(3',4'-dimethoxyphenyl)-2-propanone
DMPP	1-(3',4'-dimethoxyphenyl)-propene
DMPPD	1-(3',4'-dimethoxyphenyl)-propane-1,2-dione
EA	eugenyl acetate
EMPP	1-(4'-ethoxy-3'-methoxyphenyl)-propene
FPLC	fast protein liquid chromatography
GC	gas chromatography
H_2O_2	hydrogen peroxide
HOMO	highest occupied molecular orbital
$HOO^\cdot$	hydroperoxyl radical
HPLC	high pressure liquid chromatography
HrP	horseradish peroxidase
IEA	isoeugenyl acetate
IOP	innovative oriented research programme
IP	ionization potential
K_m	Michaelis Menten constant
LiP	lignin peroxidase
LiPII/III	lignin peroxidase compound II/III
MMA	4-methoxymandelic acid
Mn^{2+}	$manganese^{2+}$
Mn^{3+}	$manganese^{3+}$
MnP	manganese
MS	mass spectrometry
M_w	molecular weight
N_2	nitrogen
NMR	nuclear magnetic resonance
N-unregulated	nitrogen unregulated
O_2	oxygen
PAH	polycyclic aromatic hydrocarbon
SEM	standard error of the mean
SOD	superoxide dismutase
TN	turnover number
TNM	tetranitromethane

VA	veratryl alcohol
VA$^{\bullet}$	veratryl alcohol radical
VA$^{\bullet+}$	veratryl alcohol radical cation
VAD	veratraldehyde
V_{max}	maximum substrate consumption rate

Summary

This research concentrates on lignin peroxidase (LiP) mediated biotransformations that are useful in producing vanillin.
In order to obtain this extracellular enzyme, the white-rot fungus *Bjerkandera* sp. strain BOS55 was cultivated on nitrogen rich medium. This procedure resulted in a successful LiP production of 600 U/L. Peptone in the culture medium was shown to interfere with the standard LiP assay in which the formation of veratraldehyde (VAD) from veratryl alcohol (VA) is monitored. Removal of peptone by $(NH_4)_2SO_4$ precipitation minimized the interference.
BOS55 excreted at least seven LiP isozymes of which two were purified and characterized. The predominant LiP isozyme (LiP-2) oxidized VA to VAD in the pH range from 2.5 to 6.5. The VA oxidizing activity was optimal at the lowest pH. The K_m for H_2O_2 was strongly depended on the pH. At pH 5.0, a physiological pH, the Km for H_2O_2 was similar to the extracellular H_2O_2 concentration measured in cultures of BOS55.
A model based on calculated ionisation potentials (IP) was developed to predict which potential vanillin precursor would be oxidized by LiP. By testing a series of non-phenolic aromatic compounds, of which the IP was calculated, an IP-threshold value of 9.0 eV was determined. This value was used to select compounds with a lower IP like *O*-acetyl coniferyl alcohol, and *O*-acetyl isoeugenol (isoeugenyl acetate, IEA). Indeed, these compounds were consumed and in part converted into vanillyl acetate, the acetyl ester of vanillin.
IEA was studied to elucidate mechanisms of its oxidation and C_α-C_β cleavage of the propenyl side chain in IEA into vanillyl acetate. It was found that IEA was consumed via redox mediation. IEA was only oxidized in the presence of the redox mediator VA. The latter was first oxidized by LiP to the radical cation ($VA^{\cdot+}$) which in its turn oxidized IEA to its corresponding radical cation $IEA^{\cdot+}$. The following C_α-C_β cleavage reaction was the result of O_2-dependent chemical reactions which resulted in vanillyl acetate and ethanal.
The isoeugenol methyl ether (DMPP) was also used to investigate the consumption and the C_α-C_β cleavage mechanism. DMPP was not only consumed enzymatically, but also by O_2-dependent self-propagating reactions. Mn^{2+} inhibited this chemical consumption. Since Mn^{2+} also inhibited the molar yield of the predominant C_α-C_β cleavage product, VAD, it was concluded that this product was formed also during these chemical reactions. An other VAD producing route was discovered by incubating a DMPP oxidation side product, 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA) with LiP. Interestingly, VAD was only formed in the presence of O_2. Without O_2, solely DMPA dimers were detected by GC-MS.

Samenvatting

Dit onderzoek heeft zich geconcentreerd op lignine peroxidase (LiP) gemedieerde biotransformaties die nuttig zijn bij de productie van vanilline.
Om dit extracellulaire enzym te verkrijgen is de wit-rot schimmel *Bjerkandera* sp. stam BOS55 gekweekt op stikstofrijk medium. Deze procedure resulteerde in een succesvolle LiP productie van 600 U/L. Pepton in de cultuurvloeistof bleek te storen tijdens de standaard LiP assay waarbij de vorming van veratraldehyde (VAD) uit veratryl alcohol (VA) spectrofotometrisch wordt gevolgd. De interferentie werd geminimaliseerd door pepton te verwijderen middels $(NH_4)_2SO_4$ precipitatie.
BOS55 scheidt tenminste zeven LiP isozymen uit. Twee ervan werden gezuiverd en gekarakteriseerd. Het voornaamste isozym (LiP-2) oxideerde VA tot VAD in het pH-gebied van 2.5 tot 6.5. De hoogste oxidatiesnelheid werd gevonden bij de laagste pH-waarde. Bij pH 5.0, een fysiologische pH, was de K_m voor H_2O_2 ongeveer gelijk aan de normale extracellulaire H_2O_2 concentraties zoals die gemeten zijn in de cultuurvloeistof van BOS55.
Om te kunnen voorspellen of een potentiële vanilline precursor wordt geoxideerd door LiP is een model ontwikkeld dat gebaseerd is op berekende ionisatie potentialen. Er is een IP-drempelwaarde van 9.0 eV vastgesteld door een serie non-fenolische aromaten te testen waarvan de IP berekend was. Deze waarde werd gebruikt om verbindingen als *O*-acetyl coniferyl alcohol en *O*-acetyl isoeugenol (isoeugenyl acetate, IEA) te selecteren. Deze verbindingen werden geconsumeerd en bovendien gedeeltelijk omgezet in vanillyl acetate, de acetyl ester van vanilline.
IEA is gebruikt voor verdere studies m.b.t. de oxidatie en de C_α-C_β splitsing van de propenylzijketen. IEA werd alleen geconsumeerd in de aanwezigheid van de redox mediator VA. LiP oxideerde VA tot het radicaal cation ($VA^{\cdot+}$). $VA^{\cdot+}$ oxideerde vervolgens IEA tot $IEA^{\cdot+}$. De daarop volgende C_α-C_β splitsingsreaktie was het resultaat van O_2-afhankelijke chemische reakties die resulteerde in vanillyl acetaat en ethanal.
Ook werd de consumptie en de C_α-C_β splitsingsreaktie van de isoeugenol methyl ether (DMPP) bestudeerd. Het bleek dat DMPP niet alleen enzymatisch maar ook via zelf propagerende chemische reakties werd geconsumeerd. Mn^{2+} remde deze chemische consumptie. Eveneens werd de molaire opbrengst aan VAD, het voornaamste splitsingsproduct, geremd door Mn^{2+}. Daarom werd geconcludeerd dat VAD ook tijdens deze chemische reakties werd gevormd. Een andere VAD producerende route werd ontdekt door 1-(3',4'-dimethoxyphenyl)-2-propanone (DMPA), een bijproduct van de DMPP oxidatie, te incuberen met LiP. Interessant genoeg werd VAD alleen gevormd in de aanwezigheid van O_2. Zonder O_2 werden alleen DMPA dimeren gedetecteerd met GC-MS.

Curriculum vitae

Rimko ten Have werd op 25 september 1971 te Lienden geboren. In 1988 behaalde hij zijn HAVO-diploma aan het Linge College te Tiel. Vervolgens startte hij met een opleiding tot chemisch analist aan de Internationale Agrarische Hogeschool Larenstein te Wageningen. Deze studie werd afgerond met een stage en een afstudeeropdracht bij de werkgroep Fytochemie (Organische Chemie, LUW). In 1992 studeerde hij verder aan de Landbouwuniversiteit en volgde een doorstroomprogramma Moleculaire Wetenschappen, fysisch chemische oriëntatie. Tijdens deze opleiding werden afstudeervakken bij Organische chemie (LUW) en bij Moleculaire Fysica (LUW) gedaan.
In 1995 startte Rimko met zijn promotie-onderzoek bij de vakgroep Levensmiddelentechnologie en Voedingswetenschappen, leerstoelgroep Industriële Microbiologie van de Landbouwuniversiteit Wageningen.

List of publications

ten Have, R. Hartmans, S., and Field, J.A. (1997) Interference of peptone and tyrosine with the lignin peroxidase assay. *Appl. Environ. Microbiol.* **63**:3301-3303.

ten Have, R., Hartmans, S., Teunissen, P.J.M., and Field, J.A. (1998) Purification and characterization of two lignin peroxidase isozymes produced by Bjerkandera sp. strain BOS55. *FEBS Lett.* **422**:391-394.

ten Have, R., Rietjens, I.M.C.M., Hartmans, S., Swarts, H.J., and Field, J.A. (1998) Calculated ionisation potentials determine the oxidation of vanillin precursors by lignin peroxidase. *FEBS Lett.* **430**:390-392.

ten Have, R., de Thouars, R.G., Swarts, H.J., and Field, J.A. (1999) Veratryl alcohol-mediated oxidation of isoeugenyl acetate by lignin peroxidase. *Eur. J. Biochem.* **265**:1008-1014.

ten Have, R., Franssen, M.C.R., and Field, J.A. (2000) C_{α}-C_{β} cleavage of 1-(3',4'-dimethoxyphenyl)-propene by lignin peroxidase from *Bjerkandera* sp. strain BOS55. Accepted for publication in the *Biochemical Journal.*

Additional pages for notes

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