Journal of Korea TAPPI
Vol. 31. No. 5, 1999
Printed in Korea

Review

The Role of Fungal Laccase in Biodegradation of Lignin

Andrzej Leonowicz, Jolanta Luterek, Maria W.-Wasilewska
Anna Matuszewska, M. Hofrichter*1, D. Ziegenhagen*1
Jerzy Rogalski, and Nam-Seok Cho†*2

ABSTRACT

Wood components, cellulose and lignin, are degraded simultaneously and the general outline for the complementary character of carbohydrates and lignin decomposition as well as the existence of enzymatic systems combining these processes is still valid. The degradation of free cellulose or hemicellulose into monosaccharides has long been known to be relatively simple, but the mechanism of lignin degradation was not solved very clearly yet. Anyway the biodegradation of wood constituents is understood at present as an enzymatic process.

Ligninolytic activity has been correlated with lignin and manganese peroxidases. At present the attention is paid to laccase. Laccase oxidizes lignin molecule to phenoxy radicals and quinones. This oxidation can lead to the cleavage of C-C or C-O bonds in the lignin phenylpropane subunits, resulting either in degradation of both side chains and aromatic rings, or in demethylation processes. The role of laccase lies in the "activation" of some low molecular weight mediators and radicals produced by fungal cultures. Such activated factors produced also in cooperation with other enzymes are probably exported to the wood environment where they work in degradation processes as the "enzyme messengers." It is worth mentioning that only fungi possessing laccase show demethylating activity. Thus demethylation, the process important for ligninolysis, is probably caused exclusively by laccase. Under natural conditions laccase seems to work with other fungal enzymes, mediators and mediating radicals. It has shown the possibility of direct Björkman lignin depolymerization by cooperative activity of laccase and glucose oxidase.

1. Introduction

The biodegradation of wood constituents is understood at present as an enzymatic process. The degradation of free cellulose or hemicellulose into monosaccharides has long been known to be relatively simple. Many cellulolytic and some ligninolytic fungi make use of a full range of hydrolases able to produce monosaccharides in large

• Parts of the works cited here and this review were financially supported by the KBN (BS/BiNoZ/4, BW/BiNoZ/4), and International Cooperative Research Project (985-0600-001-2) of KOSEF, Korea.
• Department of Biochemistry, Maria Curie-Sklodowska University, Pl-20031, Lublin, Poland.
*1 Department of Technical Microbiology, Institute of Microbiology, Friedrich Shiller University of Jena, D-07743 Jena, Germany.
*2 School of Forest Resources, Chungbuk National University, Cheongju 361-763, Korea.
† Corresponding author: e-mail: nscho@cbucc.chungbuk.ac.kr

quantities from all polysaccharide components of wood. However, it is mandatory for these components not to occur in a complex with lignin. Thus, lignin with its barrier against hydrolytic enzymes causes the problem. This is why the research on the biodegradation of wood complex has been carried out for years.

During the last decade ligninolytic activity has been correlated with lignin and manganese peroxidases. At present the attention is paid also to laccase (EC 1.10.3.2) as its activity is found in *Phanerochaete chrysosporium*, the fungus mostly investigated for ligninolysis. Among the white rot fungi, especially *Trametes versicolor* and *P. chrysosporium* have been studied in detail because of their high ligninolytic activity and potential for application in bleaching, pulping and wastewater treatment.[1,2] The production of laccase by *T. versicolor* has been known for decades[3] and also relatively early the ligninolytic activity of this enzyme was described.[4] A different situation was in the case of the mostly investigated white rot fungus *P. chrysosporium*, where the enzyme was relatively late found.[5]

Recently laccase has received more attention.[6,7] It has been suggested that two classes of extracellular enzymes, peroxidases and laccases, are involved in ligninolysis owing to their ability to catalyse the cleavage of carbon-carbon or carbon-oxygen bonds in lignin or lignin model compounds.[8-10] Lignin peroxidase (LiP; EC 1.11.1.14) (using veratryl alcohol as the mediating factor) oxidizes the lignin structures by one electron transfer reactions yielding cation radical intermediates that undergo spontaneous ring fission and other bond cleavages.[11-12] Manganese dependent peroxidase (MnP; EC 1.11.1.13) providing hydrogen peroxide through oxalate and glycolate as the radical mediators[13] cooperates with LiP in lignin degradation or directly degrades the polymer using oxalate as the mediator[12,14] although to a smaller extent than LiP. Laccase oxidizes phenolic lignin models to phenoxy radicals and quinones. This spontaneous rearrangement can also lead to the fission of carbon-carbon or carbon-oxygen bonds in the lignin phenylpropane subunits resulting either in degradation of both side chains[15] and aromatic rings,[16] or in demethylation processes.[4,17-19] In this respect, laccase can cooperate with various FAD containing oxidases like glucose oxidase (GOD; EC 1.1.3.4),[20] veratryl alcohol oxidase (VAO; EC 1.1.3.7),[10] cellobiose:quinone oxidoreductase (CBQ; EC 1.1.5.1)[21] and cellobiose dehydrogenase (CDH; EC 1.1.99.18).[22] These reducing quinoids and radicals (through $FADH_2$ oxidation) may prevent polymerization processes.

2. Superoxide Dismutases (SOD_S; EC 1.15.1.1)

This almost universally distributed dismutase[23] was for the first time isolated in a homogeneous form from an oxygen consuming organism by McCord *et al.* in 1971[24] and from a wood rotting basidiomycete by Malarczyk *et al.* in 1995.[25] The enzyme contains iron (FeSOD), manganese (MnSOD), or copper or zinc (CuZnSOD) in the catalytic centre. SOD protects aerobic organisms from the toxic effects of the reactive superoxide radical ($O_2^{\cdot -}$, SOR), which commonly appears during the quinone redox cycle, e.g. in the laccase producing ligninolytic fungus *Pleurotus eryngii*.[26] During the cycle in *P. eryngii*(similarly to the data in our earlier report,[4] the cell-bound divalent reduction of quinones (Q) to hydroquinones (Q^{2-}) is followed by the extracellular laccase-mediated oxidation of hydroquinones into semiquinones ($Q^{\cdot -}$), which are autooxidized to quinones. In both quinone oxidation phases

production of SOR occurs:

$Q^{2-} + O_2 \rightarrow Q^{\cdot-} + O_2^{\cdot-}$

$Q^{\cdot-} + O_2 \rightarrow Q + O_2^{\cdot-}$

subsequently SOR reacts as a reducing or oxidizing agent with other radicals produced by ligninolytic enzymes, contributing to various lignin degradation processes, e.g. aromatic ring fission[16] or demethylation.[27] As an oxidant, SOR produces Mn^{+3} from Mn^{+2}:

$O_2^{\cdot-} + Mn^{+2} + 2H^+ \rightarrow Mn^{+3} + H_2O_2$;

and generates hydrogen peroxide and oxygen:

$O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$;

as a reducing agent SOR reacts with Fe^{3+} reducing it to Fe^{+2} and yielding oxygen:

$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{+2}$;

the reduced iron and hydrogen peroxide react with each other to form OH-radicals via the Fenton reaction:[28-30]

$Fe^{+2} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$;

OH-radicals are also produced from SOR and hydrogen peroxide in the Haber-Weiss reaction:[31]

$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2$.

As the one-electron reduction product of oxygen SOR, is not strong enough for ligninolysis, but produced with the share of oxygen, hydrogen peroxide and hydroxyl radicals that are thought to be important in lignin degradation.[26,32,33]

On the other hand, the highly active hydroxyl radical can react with DNA, proteins, lipids, or other biomolecules which are important for cells and usually kills them. Contrary to this reaction there appears superoxide dismutase (SOD's) which catalyses the dismutation of the superoxide radical $O_2^{\cdot-}$ (SOR) to O_2 and H_2O_2[26,34] as follows:

$2O_2^{\cdot-} + 2H^{\cdot} = H_2O_2 + O_2$.

Hydrogen peroxide yielded here is either transformed to O_2 and H_2O by catalase or included into peroxidative reactions. Consequently, the living cells (also basidiomycetous fungi) commonly possess SOD, catalase and peroxidases, which are able to eliminate $O_2^{\cdot-}$ as H_2O_2.

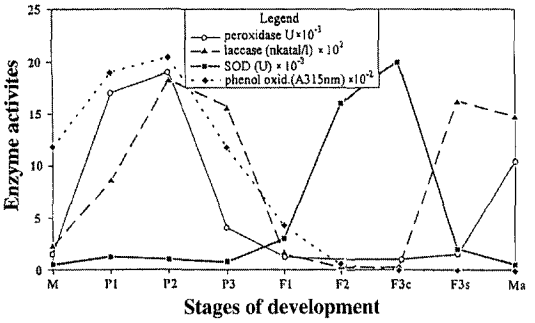

Fig. 1. Activity of SOD and of some ligninolytic enzymes in *Pleurotus sajor-caju* during growth in the stages of primordia and fruiting bodies. P - primordia; 1,2,3 - stages from small to big forms, F - fruit bodies; c - cap; s - stem of fruit body.[25]

The SOD maximum activity in lignin degrading and laccase as well as ligninase producing fungi appear when the activities of the ligninolytic enzymes diminish (Fig. 1). Probably, at the time when ligninolytic enzymes are not working, SOD protects the organisms from the toxic, reactive radicals. On the other hand, during high activity of the enzymes, the radicals might be fully involved in the biodeterioration processes.

3. Low-Molecular Mediators

The molecular sizes of wood rotting enzymes are too large to penetrate in to the wood cell wall. The low molecular mediators of internal or external origin possessing high enough redox potential migrate from the enzymes and oxidize lignin in wood or pulp. Consequently, many possible low-molecular mass compounds have been suggested as candidates for a mobile factor to permeate wood cell walls and initiate decay.

Some of these, such as veratryl alcohol, oxalate, 3-hydroxyanthranilic acid, syringaldehyde and Gt-chelator, are produced as a result of fungal metabolism and their secretion has enabled the fungi to colonize and degrade the wood structure more effectively than other organisms. Synthesis of veratryl alcohol by *P. chrysosporium* was first discovered by Lundquist and Kirk,[35] oxalate was found in *Leucostoma cincta* and *L. personii* by Traquair in 1987,[36] malate and fumarate were recently detected in *Nematoloma frowardii* by Hofrichter *et al.*,[37] 3-hydroxyanthranilic acid was identified in *Pycnoporus cinnabarinus* by Eggert *et al.*,[38] phenolate derivative chelator (Gt-chelator<1 kDa) has been isolated from the brown rot fungus *Gleophyllum trabeum* by Goodell,[39] syringaldehyde was commonly appeared in wood degradation medium.[40] A natural mediator of laccase, 3-hydroxyanthranilic acid (3-HAA) supporting ligninolysis is yielded by *P. cinnabarinus*, a white-rot fungus that does not produce MnP and LiP.[38]

The laccase/3-HAA system mediates the oxidation of non-phenolic lignin model dimers, whereas laccase alone does not affect the compound at all. The dimeric ethers possessing phenolic groups are neither oxidized nor cleaved by the laccase/3-HAA system. The laccase/3-HAA activity on the non-phenolic dimer, besides veratric acid and guaiacol production, results also in a 6-electron oxidation of 3-HAA. It leads to formation of the phenoxazinone ring from anthranilate and finally to the synthesis of cinnabarinic acid which is a common secondary metabolite in *P. cinnabarinus*.[38] It was also shown that, similarly to LiP activity on veratryl alcohol, the laccase/syringaldehyde system can oxidize veratryl alcohol to veratraldehyde through ligninolytic radicals.[41] The presence of syringaldehyde in the wood degrading environment is possible, since syringic acid (readily reduced by fungi) is a lignin degradation metabolite.[40]

The laccase/mediator systems may work also in delignification of kraft pulp. It was found that this process can be accelerated by laccase and some external (i.e. non-produced by fungi and absent in pulp) low molecular dyes or other aromatic hydrogen donors like 2,2' azinobis-(3-ethylbenzen thiazoline-6-sulfonic acid) (ABTS)[42-44] or 1-hydroxybenzotriazole (HBT).[45] An R-NO· radical is the active form of this last mediator and selective for lignin oxidation. The laccase/HBT couple causes up to 50% of pulp delignification.[46] HBT is more effective in the bleaching processes than ABTS is. The discovery of HBT introduced a new class of mediators with N-OH as the functional group yielding the NO· radical, which is stabilized by the mediator structure, and selective for lignin oxidation. Such mediators as 4-hydroxy-3-nitroso-1-naphtalenesulfonic acid (HNNS), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS) and Remazol brilliant blue (RBB) promoted delignification only to a smaller extentthan HBT and ABTS. Promazine (PZ) and chlorpromazine (CPZ) did not act in this system.[47] However, as ABTS and other proposed mediating compounds do not appear in pulp, it is necessary to search for novel natural mediators originating from the mycelium or the substrate. 3-hydroxyanthranilate (3-HAA) and syringaldehyde would perform this role. The first one is produced as a natural redox mediator by *P. cinnabarinus*, and the second is aproduct of lignin degradation. 3-HAA has been reported to be capable of mediating the oxidation of veratryl alcohol and ^{14}C-ring labelled synthetic lignin.[38]

It was also shown that similarly to LiP activity on veratryl alcohol the laccase:syringaldehyde system can oxidize veratryl alcohol to veratraldehyde through ligninolytic radicals.[41] The presence of syringaldehyde in the wood degrading environment is possible, since

syringic acid is one of lignin degradation metabolites.[40] Thus syringaldehyde can be considered as a natural mediator, equivalent to the laccase/ABTS in delignification of wood and pulp. On the other hand, the commercialization of this system for pulp bleaching needs cheeper and more effective mediator.

The ligninolytic enzymes - in particular laccase - often carry out their ligninolytic activity by cooperating with low molecular weight compounds acting as redox mediators. The ideal mediator should "form a high redox potential oxidation product in a highly reversible reaction."[47] Cheaper bleaching of pulp needs optimization of both factors.47) The hypothetical relationship between veratryl alcohol oxidase, laccase and other enzymes and radicals in the process of wood degradation is illustrated in Fig. 2.

In the proposed system laccase, LiP and MnP are secreted from fungal hyphae close to the hyphae environment where they cooperate each other and with VAO producing mediating factors. The yielded chelators and mediating radicals are exported further to the wood tissue where they work as enzyme "messengers" in wood degradation. Therefore it is the consecutive proposition of the feedback type enzymatic system, that works in wood degradation with a meaningful participation of different ligninolytic enzymes. Ligninolytic radicals are produced by SOD from quinones and semi-quinones yielded by laccase. During fungal growth on medium reach in lignin derivatives the maximum of SOD activity in lignin-degrading environment appeared when the activities of the ligninolytic enzymes decreased.[25] This may be caused by the fact that when the ligninolytic enzymes are not active, SOD protects the organisms from the toxic, reactive radicals. On the other hand, during the high activity of the enzymes, the radicals might be fully involved in biodeterioration processes. As was already mentioned, SOD

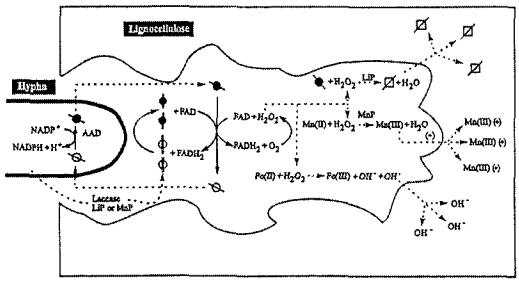

Fig. 2. Hypothetical relationship among fungal hyphae enzymes and veratryl alcohol dehydrogenase with mediators and mediating radicals during degradation of wood. From left to right: AAD - aryl alcohol dehydrogenase with NADP as the prosthetic group; aryl alcohol; aryl aldehyde; LiP; MnP; lignin derived radicals or quinones and their reduced forms, VAO with FAD as the prosthetic group; LiP with Fe; MnP with Mn; metal chelating agents, e.g. oxalic acid.[48]

is most likely involved in the production of these factors from the intermediates of quinone redox cycling caused by laccase (see Fig. 3).

It has been unequivocally stated that lignindegradation is accelerated in the presence of cellulose or its oligomers.[50] The idea of a feedback type interdependence of delignification and cellulose degradation processes was postulated for the first time by Westermark and Eriksson.[21,49,51] Depolymerization of cellulose and lignin are interrelated in certain point and accelerate each other. They discovered the enzyme CBQ which cooperates with laccase and cellulose in the depolymerization process of both components of the ligninocellulose complex in a feedback fashion. CBQ, accelerating degradation of both elements of the complex in a feedback system, thus removes decomposition products of cellulose. The authors suggested that in the system laccase

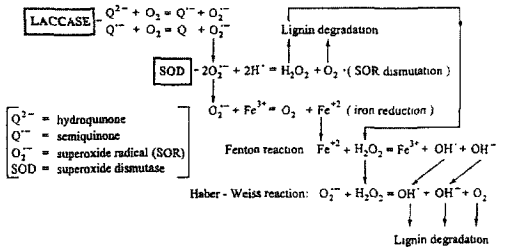

Fig. 3. The possible cooperation of laccase and SOD in production of ligninolytic factors and radicals (own unpublished results).

might function as a link in an extracellular "electron transport chain."

Ander and Marzullo in 1997[48] modified the Westermark and Eriksson's scheme including the recently discovered enzymes and radicals which are active in degradation of the ligninocellulose complex as follows (Fig. 4). The CDH/CBQ system (Fig. 4) functions at the presence of oxygen and hydrogen peroxide; oxygen can be supplied with air, but hydrogen peroxide (needed *e.g.* by LiP) is only produced at a low rate, by CDH and CBQ as suggested by the authors. The crucial H_2O_2 is probably produced by superoxide radical ($O_2^{\cdot-}$) yielded in quinone cycling (see SODs), and superoxide radical ($O_2^{\cdot-}$) oxidizes Mn^{+2} to Mn^{+3}:

$$Q^{2-} + O_2 = Q^{\cdot-} + O_2^{\cdot-}$$
$$Q^{\cdot-} + O_2 = Q + O_2^{\cdot-}$$

$$O_2^{\cdot-} + Mn^{+2} + 2H^+ = Mn^{+3} + H_2O_2$$

H_2O_2 produced here supports further production of Mn^{+3} from Mn^{+2} and finally both are used by MnP for oxidation (but not depolymerization) of lignin or its phenolic derivatives.[52]

Therefore it seems that one should look for another natural system generating enough quantity of hydrogen peroxide for biodeterioration of the ligninocellulose complex. Various possibilities have been considered.

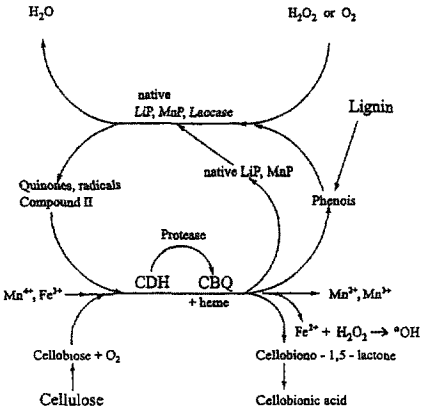

Fig. 4. Interaction of the CDH/CBQ system with other enzymes, quinones and phenoxy radicals produced by these enzymes.[48]

First of all, the function of some cytosolic fungal enzymes were analyzed, *e.g.* glyoxal oxidase (GLO; EC 1.2.3.5), VAO, MnP or GOD. These enzymes can function both in the cytoplasm and extracellularly. Other enzymes generating hydrogen peroxide like pyranose-2-oxidase (P_2O; EC 1.1.3.10), methanol oxidase (MEO; EC 1.1.3.13), and fatty-acyl-CoA oxidoreductase (FAR; EC 1.2.1.42) work only intracellularly.[53] Therefore, it is hard to understand that wood degradation occurs extracellularly. The first listed enzymes are relatively stable and resistant to outside factors. They are known as rather defensive in ligninolysis, but cooperating with other enzymes in degradation of lignin. Among all these enzymes only GOD can join both lignin and carbohydrate metabolic processe in wood. The enzyme possesses two necessary functions for acceleration of wood metabolism, namely oxidation of glucose for the hydrogen peroxide production, and reduction of quinoids and phenoxy radicals produced by laccase during lignin oxidation. Other above listed enzymes, although reduce quinones and produce hydrogen peroxide (like

AAO/VAO), have been taken into consideration, rather to a smaller extent, because hydrogen peroxide production does not proceed from glucose oxidation, which is a key metabolite in carbohydrates degradation. It means that probably only GOD cooperates with the system of cellulases oxidizing glucose, generated by these enzymes during cellulose hydrolysis.

The GOD enzyme is produced by all white-rot fungi[54] yielding laccase[55] and LiP. *P. chrysosporium*, known as one of the best lignin degrader deprived GOD by mutagenesis does not decompose lignin at all.[56] This fungus produces not only LiP,[9] but also laccase.[5] Thus, GOD seems to be a feedback type enzyme that might be important in wood degradation. When oxygen is indispensable in the second stage of the GOD reaction (Fig. 7), it can be supplied by radicals or quinones generated when phenolic or methoxyphenolic compounds are exposed to laccase.[20] On the other hand, since excess quinones produced by laccase inhibits the enzyme,[20] it can be concluded that glucose oxidase counteracts the poisonous level of quinones in the medium, and enables laccase to continue its function. Taking all these reasons into consideration we have postulated, over ten years ago, a hypothetical mechanism of ligninocellulose complex degradation (Fig. 5).[55]

According to the mechanism GOD cooperates with lignin and manganese peroxidase providing hydrogen peroxide and with laccase reducing yielded by this enzyme quinones to adequate phenols. For this reason, GOD operates as a feedback system where lignin and manganese peroxidase function as the first lignin decomposing agent and laccase as the demethylating factor. Glucose which is produced as a result of cellulose hydrolysis by the cellulase complex becomes the substrate for GOD. Oxygen needed by GOD can be replaced by

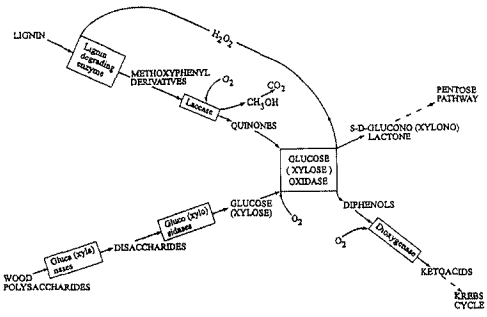

Fig. 5. Hypothetical mechanism of wood degradation by enzymes of whiterot fungi. The initial products of partial wood hydrolysis distinctly induce the enzymatic system accelerating the degradation processes.[55,57]

quinones produced by laccase from lignin oligomers. As a result of glucose oxidation, γ-D-gluconolactone is formed which after certain degradation, reinforces the metabolism of the fungus in the pentose phosphate cycle or in glycolysis. Hydrogen peroxide produced in the reaction catalyzed by GOD, in turn, activates lignin and manganese peroxidase. Lignin exposed to peroxidases undergoes decomposition into lower molecular weight fragments containing methoxyl groups. Laccase demethylates oligomers yielded by LiP and degrade them to even lower fragments. Generation of excess quinones and possible secondary polymerization are counterbalanced by GOD which reduces them to the respective phenols. The phenols, in turn, become the substrate for dioxygenase, present in fungal cultures, which catalyze cleavage reaction of the aromatic rings, the products obtained in the form of ketoacids easily find their ways to the Krebs cycle.

The mechanism could occur in those fungi which possess all the above mentioned enzymes, *e.g. P. chrysosporium*, *Phlebia radiata*, and *T. versicolor*. For those species in which the presence of LiP has not

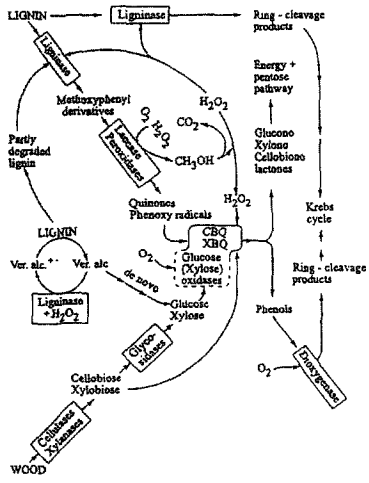

Fig. 6. Hypothetical scheme for degradation of lignin, cellulose and xylan in wood.[58,59]

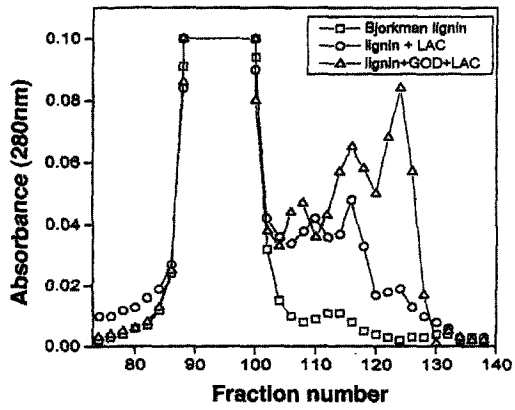

Fig. 7. Elution pattern of Björkman lignin on Sepharose Cl-6B column after incubation with both laccase and GLO.[63]

been confirmed although they transform wood, the mechanism suggested by Westermark and Eriksson where CBQ is the crucial, feedback type enzyme seems to function very well. It is not out of question that both systems function in concert as it was shown in the Eriksson's Laboratory and published twice in a monograph on microbia lwood degradation[58] and in a mini-review article (Fig. 6).[59]

The mechanism of wood degradation was further developed by Eriksson's team by supplementation of CBQ and mediating factors. It enriched greatly its areas of function (not one, but two enzymes with feedback activity, veratryl alcohol, phenoxy radicals). We are in full agreement with this proposition, all the more, since it takes also into consideration the contribution of fungal laccase and peroxidases in the demethylation of lignin, and methoxyphenyl derivatives as it has been postulated by our group many years ago.

The contemporary discoveries concerning the role of VAO, CDH, AAO (aryl alcohol oxidase; EC 1.1.3.7) and especially low molecular mediators and radicals in wood biodeterioration[48] support the proposed route by explaining how the high molecular weight enzymes can function in the wood environment that is normally resistant to the microbial attack. However, the existence of other, still to be discovered, mechanisms as well as other enzymes also operating as feedback systems in the process of wood degradation are not ruled out. Detailed investigations into these processes would definitely help, *e.g.* to solve the problem: how to utilize the supply of waste lignocellulose which is accumulated as a result of human activity or how to protect our forests from microbial attack. It is worthwile to say that recently we have shown the possibility of direct Björkman lignin depolymerization by cooperative activity of laccase and GOD (see Fig. 7). However, the yield of the process is rather low. Better would be probably with using mediators and carbohydrates.

4. Conclusions

Considering the earlier and present results we can conclude that cellulose and lignin are degraded simultaneously and the general outline for the complementary character of carbohydrates and lignin decomposition as well as the existence of enzymatic systems combining these processes is still valid. Demethylation caused by laccase after the LiP and MnP have acted on lignin seems to be important as the depolymerizing activity of LiP is inhibited by the phenolic groups of native lignin.

The role of laccase (inducing toxic lignin degradation products or stress conditions) lies probably in the "activation" of some low molecular weight mediators and radicals produced by fungal cultures. Such activated factors produced also in cooperation with other enzymes are probably exported to the wood environment where they work in degradation processes as the "enzyme messengers." It is worth mentioning that only fungi possessing laccase show demethylating activity. Thus demethylation, the process important for ligninolysis, is probably caused exclusively by laccase. The depolymerising activity of laccase is unusual, because its routine enzyme activity consists to polymerization or coupling reactions. But, this routine activity is employed usuallly in laboratory. Under natural conditions laccase works in the wood environment in concert with other fungal enzymes, mediators and mediating radicals. In these processes laccase found the proper position among other ligninolytic enzymes. It has shown the possibility of direct Björkman lignin depolymerization by cooperation of laccase and GOD.

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