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Review

Laccase: new functions for an old enzyme

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Abstract

Laccases occur widely in fungi; they have been characterized less frequently in higher plants. Here we have focused on more recent reports on the occurrence of laccase and its functions in physiological development and industrial utility. The reports of molecular weights, pH optima, and substrate specificity are extremely diverse. Conclusive proof of the occurrence of laccase in a tissue must demonstrate that the enzyme be able to oxidize quinol with concomitant uptake of oxygen. Laccase is involved in the pigmentation process of fungal spores, the regeneration of tobacco protoplasts, as fungal virulence factors, and in lignification of cell walls and delignification during white rot of wood. Commercially, laccases have been used to delignify woody tissues, produce ethanol, and to distinguish between morphine and codeine. A very wide variety of bioremediation processes employ laccase in order to protect the environment from damage caused by industrial effluents. Research in recent years has been intense, much of it elicited by the wide diversity of laccases, their utility and their very interesting enzymology. © 2002 Published by Elsevier Science Ltd.

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Contents

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1. Introduction

Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are part of a larger group of enzymes termed the multicopper enzymes, which includes among others ascorbic acid oxidase and ceruloplasmin. Laccase was first described by Yoshida (1883), and was characterized as a metal containing oxidase by Bertrand (1985). This makes it one of the oldest enzymes ever described. Laccases can be roughly divided into two major groups which show clear differences, i.e. those from higher plants and those from fungi (Harvey and Walker, 1999; Mayer and Harel, 1979; Solomon et al., 1996). The presence of laccase–like enzymes has been reported in bacteria (Claus and Filip, 1997; Givaudan et al., 1993) as well as in insects (Diamantidis et al., 2001; Hopkins and Kramer, 1992; Kramer et al., 2001); however, some of the reports on the presence of laccases in insects must be treated with caution, e.g. the enzyme present in the hemolymph of *Anopheles* is probably not a laccase (Sidjanski et al., 1997).

In this review, we will focus on plant and fungal laccases and stress their possible functions and utility. Laccases have been reviewed a number of times in recent years, generally with emphasis on narrow aspects. The reviews by Messerschmidt (1993, 1997) and by Solomon et al. (1996) provide excellent summaries of the enzymology and electron transfer mechanism of the laccases, and a book edited by Messerschmidt (1997) contains a series of articles dealing with different aspects of laccase kinetics and mechanism of action and the possible roles of the enzymes. The relationship between laccases and other multi-copper enzymes is stressed.

Spectroscopic techniques have provided further insights into the molecular mechanisms of copper proteins (Randall et al., 2001; Solomon et al., 1998). Laccase from *Coprinus cinereus,* expressed in *Aspergillus oryzea* has been crystallysed and its three-dimensional structure determined (Ducros et al., 2001). A cartoon representation of the three-dimensional structure of the *C. cinereus* laccase is shown in Fig. 1.

Fungal laccases have been reviewed by Thurston (1994), and their role in delignification by the white rot fungi has also been discussed (Eggert et al., 1996; Youn et al., 1995). Laccases can degrade lignin in the absence of lignin peroxidase and manganese peroxidase. It is fairly obvious that the laccases are very ancient enzymes from an evolutionary point of view, and that the enzyme activity linked to three different copper sites must have been a very early evolutionary process. At the same time it should be remembered that the properties of different laccases show a great deal of divergence, hence it can be debated whether they should all be lumped together on the basis of their catalytic sites or whether some separation into different groups is indicated. Although the catalytic site seems to have been very conserved, the rest of the molecule appears to show very wide variability We will not refer in this review to the polyphenol oxidases of the tyrosinase type (catecholase and cresolase), but will mention only that although often confused with laccases, these enzymes belong to an entirely different group.

2. Occurrence and characterization of laccases

As already indicated the laccases occur widely in fungi, and reports of their presence in more and more fungal species have been published (Thurston, 1994). In fact, laccases seem to be found in most fungi that have been studied. Their presence has been reported less frequently in higher plants.

2.1. Higher plants

The occurrence of laccases in higher plants appears to be far more limited than in fungi. All laccases described to date have been shown to be glycoproteins. The classical demonstration of laccase in *Rhus vernicifera* is well

documented, and the enzyme has been characterized in great detail (Huttermann et al., 2001). In addition, the entire family of the Anacardiaceae, of which the lacquer tree is a member, appear to contain laccase in the resin ducts and in the secreted resin (Huttermann et al., 2001). Reports on the presence of laccase in other plant species is more limited. Cell cultures of *Acer pseudoplatanus* have been shown to produce and secrete laccase (Bligny and Douce, 1983; Tezuka et al., 1993), and *Pinus taeda* tissue has been shown to contain eight laccases, all expressed predominantly in xylem tissue (Sato et al., 2001). Other reports are those of Wosilait et al. (1954) on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been shown to be present in the xylem tissue of *Populus euramericana* (Ranocha et al., 1999). Other higher plant species also appear to contain laccases, although their characterization is less convincing (Dean and Eriksson, 1994). In contrast to the very many reports on the fungal laccases, surveys of the occurrence of laccase in green plants seems to have been badly neglected. They may be bound to cell walls in some higher plants. It is also difficult to determine their activity in crude extracts against a background of polyphenol oxidase and peroxidase activity. Although it is very likely from present information that laccases are present throughout the plant kingdom, at this stage this is simply surmise.

2.2. Fungi

The presence of laccase has been documented in virtually every fungus examined for it. The genes for numerous laccases have been cloned and the sequences deposited in the appropriate gene register. In many fungal species the presence of both constitutive and inducible laccases have been reported. Usually the enzyme originates in the cytoplasm, but many instances of secretion of laccases have been reported. Relatively little attention has been paid to the sub-cellular location of the enzyme or to the mechanism of secretion. Although the structure of the active site seems to be conserved in all the fungal laccases, there is great diversity in the rest of the protein structure and in the sugar moiety of the enzyme.

2.3. Laccase catalysis

The range of substrates which various laccases can attack is very wide. Basically any substrate with characteristics similar to a *p*-diphenol will be oxidized by laccases. In addition at least some of the fungal laccases can also oxidize monophenols such as cresol and some are able to oxidize ascorbic acid. At the same time it must be remembered that frequently inadequate experimentation has been carried out to differentiate between laccases and peroxidases and laccases and other polyphenoloxidases. An indication of the complexity of this problem is provided by the work of Scherer and Fischer (1998). These authors purified a laccase II from *Aspergillus nidulans*. Sequencing of the enzyme, despite its substrate specificity as a laccase, and the high degree of purification, indicated more similarity to peroxidase than to laccase. It has also been shown in a groundbreaking paper by Xu et al. (1998) that the catalytic properties of a laccase from the fungus *Myceliophthora thermophilica* could be appreciably changed by site-directed mutagenesis. In a triple mutant, catalytic

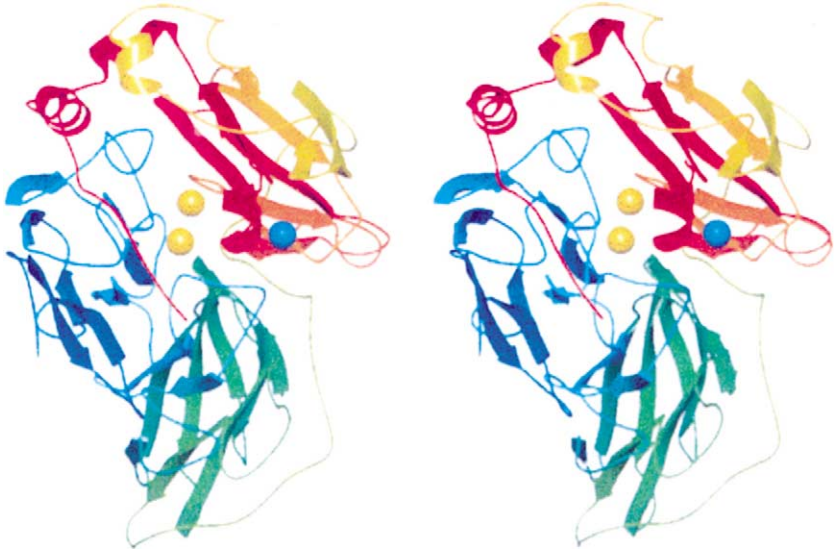

Fig. 1. Cartoon representation of the three-dimensional structure of the *Coprinus cinereus* laccase. The figure is colour-ramped from the N-terminus (blue) to the C-terminus (red). The Cu atoms are shown as shaded spheres, with the T1 site in blue and the T3 pair in yellow. The figure is in divergent (wall-eyed) stereo (after Ducros et al., 2001, with permission).

properties of the enzyme as well as inhibition were markedly altered. Thus it is clear that the properties of laccases can be readily changed, either by changes in their amino acid sequences or in composition of the carbohydrate moiety.

Physical methodology such as circular dichroism (CD) and electron paramagnetic resonance (EPR) have been used to study the metal centers of fungal laccases, showing that they are relatively stable compared to the secondary structure of the protein (Bonomo et al., 2001). The latter can undergo changes thereby altering substrate specificity. Numerous substrates have been used to study laccase activity. The only conclusive proof of laccase activity in our view is that it is able to oxidize quinol as determined by oxygen uptake no matter what other substrates are attacked. The use of syringaldazine as a substrate can be flawed, since this substrate is also oxidized by peroxidase. Special precautions are needed for its use, including the removal of hydrogen peroxide, and this is frequently omitted in work in which the presence of laccase is supposedly shown. Another substrate frequently used is 2,2-azobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). This must also be used with care since it is also oxidized by peroxidase. To differentiate between laccase and peroxidase on the one hand and between laccase and polyphenol oxidase on the other requires painstaking experiments. One of the difficulties is the fact that the immediate product of laccase activity is a semi-quinone i.e. a free radical. These are extremely reactive and are able to interact with numerous compounds which are present particularly in inadequately purified enzyme preparations. The complexity of such reactions has been shown by Ferrari et al. (1997).

In contrast to the rather sparse characterization of the laccases in higher plants, there is an abundance of reports in fungi. Almost every fungus in which a laccase has been reported appears to contain several laccases. Sometimes these are referred to as laccase 1 and laccase 2. Often they differ in molecular weight, and frequently additional forms are present as in *Cryphonectria* (Kim et al., 1995). Four different laccases have been detected in *Rhizoctonia solani* (Wahleithner et al., 1996) and *Fusarium proliferatum* (Kwon and Anderson, 2001), and there appear to be at least three laccases in *Botrytis cinerea* (Marbach et al., 1984; Pezet et al., 1992; Pezet, 1998; Viterbo et al., 1994). While the laccases of *Botrytis cinerea* are predominantly extracellular, laccase activity has been detected in purified extracellular matrix (Doss, 1999; Gil-ad et al., 2001; Schouten et al., 2002), and the fungus contains a constitutive, intracellular laccase (Mayer, unpublished).

New laccases are often discovered when some of the known ones are inactivated using knock out techniques (cf. Kim et al., 1995). The reports on the molecular weight, pH optima, substrate specificity and other properties of the laccases show extreme diversity (Fukushima and Kirk, 1995; Palmieri et al., 1997; Thurston, 1994). To what extent this diversity is the result of isolation and purification procedures is unknown. However, as already mentioned, the sugar moiety is clearly different among the laccases both in amount and composition.

The occurrence of laccase-like enzymes which lack the typical absorption around 600 nm has been reported. For example, *Pleurotus* is said to contain a "white laccase" (Palmieri et al., 1997), while "yellow laccases" have also been reported (Leontievsky et al., 1997). Supposedly in this latter enzyme the copper appears in the reduced form, but even if this claim is confirmed the term "yellow" laccase seems entirely unjustified. Such enzymes probably should not be referred to as laccases, despite the similarity in their substrates to the blue laccases.

3. Cell biology

It is assumed, but has never been proven, that laccases in plants are constitutive, and changes in enzyme level during development have never been followed. In the cases when laccase activity has been correlated with lignification, it has been located in or near the cell walls of lignifying cells. In other tissues, such as leaf or stem tissue, neither the cellular nor the sub-cellular location has been determined except in the case of the resin ducts of the Anarcardiaceae. Hence there is a lack of information about some of the basic factors relating to enzyme function.

Laccase activity may contribute to cell-wall reconstitution in regenerating protoplasts of higher plants. De Marco and Roubelakis-Angelakis (1997) measured laccase activity in regenerating and non-regenerating protoplasts isolated from tobacco (*Nicotiana tabacum*) leaves. They found that laccase activity diminished soon after isolation. The activity increased steadily in regenerating protoplasts during a six-day culture period, but was undetectable in non-regenerating protoplasts. Leaf wounding induced an immediate increase of laccase activity, whereas peroxidase activity increased very slowly and peaked only after 4 days. De Marco and Roubelakis-Angelakis (1997) concluded that laccase was the only effective polymerizing enzyme during the first day of protoplast culture and could participate in the first steps of healing in wounded leaves, substituting for peroxidase activity in cell wall reconstitution before hydrogen peroxide became available.

In contrast, information on the role of fungal laccases in development is more abundant. For example, one of the laccases of the edible mushroom *Lentinula edodes* has a role in fungal morphogenesis (Zhao and Kwan, 1999), and a laccase is specifically expressed in the green-spored conidia of *A. nidulans* (Aramayo and Timberlake, 1990; Clutterbuck, 1972). The enzyme has

also been characterized in *A. niger*, but its identity as a laccase is uncertain and its function in sexual development is still not determined (Scherer and Fischer, 1998).

Aspergillus fumigatus, a filamentous fungus producing bluish-green conidia, is an important opportunistic pathogen that primarily affects immunocompromised patients. Conidial pigmentation of *A. fumigatus* significantly influences its virulence. In a recent study, six genes forming a gene cluster spanning 19 kb were identified as involved in conidial pigment biosynthesis. Disruption of each gene in the cluster of six genes confirmed their role in conidial pigment biosynthesis. The gene products of *alb1* (albino 1), *arp1* (aspergillus reddish-pink 1), and *arp2* have high similarity to polyketide synthases, scytalone dehydratases, and hydroxynaphthalene reductases, respectively, found in the dihydroxynaphthalene (DHN)-melanin pathway of brown and black fungi. The *abr1* gene (aspergillus brown 1) encodes a putative protein possessing two signatures of multicopper oxidases. The *abr2* gene product has homology to the laccase encoded by the *yA* gene of *Aspergillus nidulans* (O'Hara and Timberlake, 1989). The function of *ayg1* (aspergillus yellowish-green 1) is unknown. The presence of *arb1, arb2*, and *ayg1* in the gene cluster suggested to Tsai et al. (1999) that conidial pigment biosynthesis in *A. fumigatus* is more complex than the known DHN–melanin pathway.

4. The role of laccases in lignification

Lignin is formed via the oxidative polymerization of monolignols within the plant cell wall matrix (Dean et al., 1998). Peroxidases, which are abundant in virtually all cell walls, have long been held to be the principal catalysts for this reaction. Recent evidence shows, however, that laccases secreted into the secondary walls of vascular tissues are equally capable of polymerizing monolignols in the presence of O_2 (Dean et al., 1998). The possibility that laccases are involved in the lignification process in higher plants was first raised by Freudenberg (1958). This suggestion was based on the observation that fungal laccases could oxidize monomers of lignin to produce dimers similar to those produced when lignin was chemically degraded. After some years, however, this theory was discarded, based on the inability to detect laccases in plant tissues. However, recently the idea has been resurrected because Sterjiades et al. (1992) demonstrated that laccase from *Acer pseudoplantanus* was able to polymerize monolignols, in the complete absence of peroxidase. This group did suggest, however, that laccase was involved only in the early stages of lignification, while peroxidases were involved later. Bao et al. (1993) showed that laccase activity was correlated with lignification of xylem in *P. taeda*. Their results showed that laccase was present in the xylem, is associated with the cell wall, and is present in lignifying cells and could oxidize monoliognols. This concept was further discussed by O'Malley et al. (1993).

The correlation between laccase activity and lignification was also reported by Liu et al. (1994) for stem tissue of *Zinnia elegans*. Similarly, a laccase-like enzyme was shown to be present in the xylem of lignifying tobacco (Richardson and McDougall, 1997). These data show that in at least four different plant species laccase activity is found in tissue which undergoes lignification. The list of species in which laccase is present in cell walls of lignifying tissue has been extended by Richardson et al. (2000) and includes *Arabidopsis*.

In a recent review, Boudet (2000) states that it is impossible to reach a definite conclusion on the role of laccase in the lignfication process, and the involvement of laccase has been challenged by Wallace and Fry (1999). These authors studied the relative rates by which purified laccases and acid and basic peroxidases were able to oxidize several phenolic substrates. Their data showed that the peroxidases had far higher specific activities towards the phenols examined compared to purified laccase, and the role of laccase in lignification remains unresolved. Immunolocalization has been used to demonstrate the involvement of laccase in lignification in Sycamore stem tissue using polyclonal antibodies against the deglycosylated protein (Driouich et al., 1992). The enzyme was found to be located in the cell wall and in the culture medium. It could not be detected elsewhere, although it must have arisen within the cell.

The laccases especially involved have not always been adequately characterized. One of the laccases used by Wallace and Fry (1999) was from *Acer* cell cultures, which do not normally lignify, and another of the laccases was from the xylem of *Populus*. It is not clear whether specific activity alone is an indicator of the role of the enzymes in lignification. In view of previous discussions on the differences between fungal and higher plant laccases, it is not to be expected that the fungal laccases would be involved in lignification. It also remains to be shown whether the oxidation products of the monolignols, resulting from either laccase or peroxidase activity bind and react with the dirigent protein suggested by Lewis and coworkers (Davin et al., 1997; Lewis et al., 1998) to be involved in lignification. The linkage specificity has been discussed by Hatfield and Vermerris (2001). This problem can probably be finally resolved only by using mutants or antisense RNA experiments, although even then the option always exists that if one enzyme system is removed or inhibited something else can take its place. An interesting suggestion by De Marco and Roubelakis-Angelakis (1997) assigns a function to laccase in cross-linking tyrosine containing proteins in regenerating tobacco protoplasts. This still implicates laccase in cell-wall formation, but not necessarily in lignification.

5. Pathogen virulence

Laccase has been shown to be an important virulence factor in many diseases caused by fungi. Among other roles, laccase can protect the fungal pathogen from the toxic phytoalexins and tannins in the host environment (Pezet et al., 1992). For example, in the root pathogen, the aggressiveness of *Heterobasidion annosum* is related to the presence of laccase (Johansson et al., 1999).

Dutch elm disease has also been associated with laccase secretion by the causative organisms (Binz and Canevascini, 1996), but no clear role was ascribed to laccase. Extracellular laccase production of *Ophiostoma ulmi* and *Ophiostoma novo-ulmi*, the two sub-populations of dutch elm disease, previously known as the Eurasian and North American races (Brasier and Kirk, 2001), respectively, was monitored during growth in vitro. *O. novo-ulmi* secreted appreciable amounts of laccase in parallel with growth whereas laccase activity in *O. ulmi* was barely detectable. The differential production of laccase provides an example of the extensive physiological differences between *O. ulmi* and *O. novo-ulmi*. The recently described Himalayan subpopulation, *O. himal-ulmi* (Pipe et al., 2000) apparently has not been studied yet.

5.1. Human pathogens

Cryptococcus neoformans is an encapsulated fungus that has emerged as a life-threatening infection in immunocompromised patients, especially those infected with human immunodeficiency virus. First identified by Williamson (1994), laccase is present as a tightly associated cell wall enzyme that is readily accessible for interactions with host immune cells (Zhu et al., 2001). In a review, Williamson (1997) describes laccase and its product melanin as an important virulence factor of *C. neoformans*. Direct chemical evidence has been provided to show that eumelanin polymers are formed by laccase-assisted oxidation of catecholamine followed by oxidative coupling of dihydroxyindole (Williamson et al., 1998). In fact, melanin synthesis was shown to be dependent on a single copper-dependent laccase, a role confirmed by studies using knock-out strains of the fungus. Laccase is not produced by the mammalian host, and thus is a potential drug target. Williamson (1997) speculated that in human patients, melanin may protect *C. neoformans* by acting as an anti-oxidant or by interacting with the cell wall surface, thereby offering protection against numerous effectors of cellular immunity. In fact, studies with CNLAC1, the laccase structural gene of *C. neoformans* has been shown to be a fungal virulence factor (Salas et al., 1996). In mice, however, Liu et al. (1999) suggested from their studies on infected mouse brain, which do not contain melanin, that the iron oxidase activity of laccase may protect *C. neoformans* from alveolar macrophages by oxidation of phagosomal iron to Fe(III) with a resultant decrease in hydroxyl radical formation. The ability of a multinuclear copper enzyme to oxidize divalent iron has been reported previously (Hassett et al., 1998).

Among species of the heterobasidiomycetous yeasts, *Filobasidiella neoformans* is the only serious pathogen that causes fatal infections in both immunocompromised as well as immunocompetent patients. Three phenotypic characteristics are known to play major roles in the pathogenicity of the fungus, i.e. growth at 37 °C, an extracellular capsule composed of polysaccharide, and a gene, CNLAC1, that encodes a laccase (Petter et al., 2001). CNLAC1 was present in several closely related species of the genus *Filobasidiella*, however, CAP59, involved in polysaccharide capsule formation, was unique to *F. neoformans*. It may be that the two genes, CNLAC1 and CAP59, acting together, compose the pathogenic phenotype.

5.2. Phytoanticipins

Phytoanticipins are antibiotic compounds present in plant tissue prior to microbial infection and those that are produced from preformed constituents during infection (VanEtten et al., 1994). They provide a protective barrier against infection by microorganisms. Laccase is a polyphenoloxidase, and while the full range of its substrates is not known, the enzyme attacks polyphenols, methoxy-substituted phenols, but not tyrosine. In 1988, Bar-Nun et al. (1988) suggested that laccase secreted by *B. cinerea* acted as a detoxifying enzyme to protect the fungus from toxic metabolites, and to reduce lignification activities by the host.

Cucumber fruits, *Cucumis prophetarium*, and the common weed, *Ecballium elaterium,* contain a family of cyclic triterpenoids, cucurbitacins, that protect against attack by the fungus, *B. cinerea* (Bar-Nun et al., 1988; Bar-Nun and Mayer, 1989). Resistance to *B. cinerea* is correlated with the ability of extracts from the fruit to repress laccase secretion. As reviewed by Bar-Nun and Mayer (1989), cucurbitacins appear to protect *E. elaterium* against *B. cinerea* by inhibiting the synthesis of laccase protein (Viterbo et al., 1994). The repression of laccase formation by the cucurbitacins with the result that *B. cinerea* is then only a weak or disabled parasite, confirms the importance of laccase to pathogen virulence.

5.3. Phytoalexins

Phytoalexins are toxic compounds synthesized by plants in response to microbial infection. Some fungal pathogens can tolerate a host-produced phytoalexin, but the ability to metabolize a phytoalexin does not always confer tolerance to the fungus (Van Etten et al., 1989).

Some newer research on phytoalexin destruction deals with laccases from the necrotrophic fungal plant pathogen, *B. cinerea*, which infects the fruits, flowers, or green tissues of at least 235 plant species (Jarvis, 1977). The fungus is able to withstand the toxic effects of plant defense compounds with varying structures such as stilbenes, isoflavonoids, coumarins, and sesquiterpenes. For example, resveratrol is a phenolic phytoalexin that arises in grapevine in response to attack by *B. cinerea* (Heale, 1992; Jeandet et al., 1995; Pezet et al., 1992). A 32-kDa laccase (Pezet, 1998) has been shown to oxidize and detoxify these phytoalexins (Adrian et al., 1998; Bar-Nun et al., 1988; Breuil et al., 1999; Pezet et al., 1991, 1992). The oxidation of resveratrol may be deleterious to human health, since it acts as an antioxidant; thus laccase activity could be harmful (Espin, 2000).

Recently, Schouten et al. (2002) suggested that resveratrol could act as a pro-fungicide that is converted by the laccase from *B. cinerea* into a compound inhibitory to the fungus. Thus the function of the laccases in *B. cinerea*, whose formation and level is regulated in a complex fashion (Gil-ad et al., 2000, 2001), seems to be much more complicated than first thought, and the task of each of the three laccases, known to be present, will have to be studied separately.

More general mechanisms that also provide protection against a broad range of toxicants such as compartmentalization and reduction of accumulation allows pathogens to cope with various defence compounds that occur in many different host species. For example, reduction of accumulation of resveratrol, can be achieved with active efflux by ATP-binding cassette (ABC) transporters (VanEtten et al., 2001), and Schoonbeek et al. (2001) have shown that an ABC transporter functioning in *B. cinerea*, coded for by the gene *BcatrB*, can reduce the toxicity of resveratrol to the fungus.

5.4. Effect of mycoviruses on laccase gene expression

Sometimes the expression of laccase is regulated by the presence of mycoviruses that infect the fungal pathogen, and which can both up-regulate and down-regulate fungal virulence.

5.4.1. Down-regulation

Probably the best documented evidence for the significance of laccase as a virulence factor comes from the work on *Cryphonectria parasitica*, the fungus that causes chestnut blight. The fungus, *C. parasitica,* causes a severe blight of chestnut, *Castanea dentata*, and chestnut blight has been so severe in North America, that mature chestnut trees are rarely seen. Strains of *C. parasitica* occur in nature that have reduced levels of virulence (hypovirulence) that have been used to control disease progress (Anagnostakis, 1988; Anagnostakis and Day, 1979; Rigling, 1994). The strains have been shown to be infected with dsRNA mycoviruses; in addition to virulence, these strains are distinguished by associated traits, e.g. reduced levels of sporulation, orange pigmentation (Anagnostakis, 1982, 1984; Elliston, 1978; Hillman et al., 1990), reduced levels of cellulase activity (Hillman et al., 1990), and oxalate accumulation (Havir and Anagnostakis, 1983). Hypovirulent strains can convert virulent strains to hypovirulence by transfer of dsRNA via hyphal anastamosis (Anagnostakis and Day, 1979), and in 1992, Choi and Nuss (1992a,b) demonstrated that hypovirulence of the chestnut blight fungus was conferred by a viral dsRNA genetic element.

Choi and Nuss (1992a) transformed a strain of *C. parasitica* that was free of viral dsRNA with a cDNA copy of ORF A of 622 codons, one of two contiguous coding domains in the mycovirus associated with reduced virulence (hypovirulence). Both open reading frames encode polyproteins that undergo autoproteolytic processing mediated by two papain-like protease activities (Choi et al., 1992; Heath and Steinberg, 1999; Senbongi et al., 1999; Wildt and Deuschle, 1999). After transformation, the transformed strain of *C. parasitica* acquired the traits of hypovirulence including reduced pigmentation, reduced laccase accumulation and suppressed conidiation, thereby demonstrating a direct cause and effect relationship between a viral dsRNA genetic element present in a hypovirulent *C. parasitica* strain and specific phenotypic traits. They further demonstrated that these traits were not the result of a general reaction of the fungus to the presence of the replicating viral RNA, but are caused by a specific viral coding domain.

Symptoms of virus infection appear to be the result of reduced expression of a small number of specific fungal genes in viral infected strains (Kazmierczak et al., 1996; Powell and Van Alfen, 1987a,b). Specific deletion of *Vir2*, one of these viral suppressed genes, resulted in a phenotype that mimicked a portion of the viral-induced, sporulation-related symptoms (Zhang et al., 1993). Other fungal genes cloned from *C. parasitica* that have been shown to be down-regulated by the virus are *Vir1*, a gene that, like *Vir2*, also appears to be sporulation-related and encodes a fungal sex pheromone (Powell and Van Alfen, 1987b), *Crp,* a gene encoding an abundant tissue-specific cell-surface hydrophobin (Zhang et al., 1994) and *Lac1*, an extracellular laccase (Choi et al., 1992).

The effect of the virus on laccase activity was first studied by Rigling et al. (1989), research that has included studies on *Lac1* gene expression (Rigling, 1995; Rigling and Van Alfen, 1991). *C. parasitica* produces two extracellular laccases, LAC1 and LAC3, and an intracellular laccase, LAC2, and the virus reduces the activity of the enzymes (Kim et al., 1995). The function of the laccases is unknown, but Kim et al. (1995)

speculate that they somehow enable the fungus to live in the tannic acid-rich environment of the chestnut bark where the pathogen causes the well-known stem cankers. The virus interferes with induction of *Lac1* by reducing transcription in response to amino acid deficiency (Larson and Nuss, 1994), including transcriptional activation by cycloheximide (Wang and Nuss, 1998), apparently by disrupting G-protein signal transduction pathways (Gao and Nuss, 1996; Kasahara et al., 2000; Kasahara and Nuss, 1997; Nuss, 1996) primarily through modification of the CPG-1 signaling pathway, a negative modulator of adenylyl cyclase (Chen et al., 1996).

Diaporthe ambigua is a fungal pathogen of apple, pear, and plum rootstocks in South Africa. Strains of the fungus infected with a virus-like dsRNA have reduced laccase activity, reduced gallic acid oxidation, diminished oxalic acid accumulation, and suppressed sporulation (Smit et al., 1996). The fungus is spread to healthy strains of the same vegetative compatibility group by hyphal anastomosis.

5.4.2. Up-regulation

Not all mycoviruses reduce the virulence of a fungal host. A dsRNA was shown to up-regulate the fungal virulence of *Nectria radicicola*, the causal fungus of ginseng root rot (Ahn and Lee, 2001). Curing tests showed that the presence of a 6.0-kbp dsRNA was associated with high levels of virulence, sporulation, laccase activity, and pigmentation in the fungus. Fungal strains cured of the 6.0-kbp dsRNA completely lost the virulence-related phenotypes, and virulence was restored when the dsRNA was reintroduced into the cured strain by hyphal anastomosis. Biochemical analyses suggested that the dsRNA may regulate fungal virulence through signal-transduction pathways involving cAMP-dependent protein kinase and protein kinase C.

5.5. Defense functions of higher plant laccases

The presence of laccases in resin ducts of the Anarcardiaceae suggests a rather obvious function, i.e. defense against predators, herbivores and invasion by bacteria and fungi. The excretion of an enzyme which will cause oxidative reactions at the wound surface certainly suggests a role as a deterrent or in wound healing; however, there is no direct proof of such a function. The physiology of secretion, the amount of enzyme present at different developmental stages and the relation between laccase activity, its secretion and exposure to attack remains to be investigated. The number of higher plant laccsses, distributed among very diverse species and a variety of lignified and non-lignified plant tissues, certainly should stimulate further and much more detailed research.

6. Some recent applications

The practical applications of the use of laccase, has lead to a search for sources of the enzyme from white rot fungi, and the use of mediators, which promote or facilitate enzyme action. This work has contributed much knowledge to laccase biology, but it is not the main focus of this review. The potential use of laccase for bleaching has been investigated and this has even led to the esoteric suggestion of using laccases in the presence of hydroxy stilbenes as hair dyes (bleaches) (Onuki et al., 2000; Pruche et al., 2000). Laccases can be absorbed onto solid surfaces and this has resulted in an electrode for detecting azide (Leech and Daigle, 1998). Such electrodes showed electrocatalytic reduction of oxygen in the presence of a mediator (Hyung and Shin, 1999). Basically all these varied uses of laccase, and many more we have not mentioned, can be ascribed to one property of the enzyme, its ability to produce a free radical from a suitable substrate. The ensuing secondary reactions are responsible for the versatility of laccases in producing so many varied products. The use of laccases in bioremediation has also been proposed, again presumably due to this single basic reaction.

6.1. Laccases and delignification

The removal of lignin from woody tissues is a process that has attracted a very great deal of research, especially due to its importance in the pulp and paper industry. One of the approaches to delignification of wood fibers for preparation of pulp has been the use of laccases for this purpose. We will not cover this facet of laccase action because a very recent publication (Argyropoulos, 2001) covers many aspects of this problem, particularly the development of mediators, i.e. chemicals which enable the large enzyme molecule to react with fiber lignin via the small mediator molecule (Poppius-Levlin et al., 2001).

In decay of wood by white rot fungi, there can be little doubt that laccase is one of the main enzymes involved in delignification. The presence of laccase, alone or together with lignin peroxidase and manganese peroxidase, has been demonstrated in a wide variety of white rot fungi. The list of these organisms is long, and we will not specify them here. The structure of many of these laccases has been described and surprisingly, despite the fact that lignocellulose is a common substrate, they show a considerable diversity in molecular weight, pH optimum and other properties. The stability of some of these laccases is enhanced by the presence of phenolic substances (Mai et al., 2000). It has also been shown that the ability of laccases to break down ligno-cellulose is increased by certain phenolic compounds acting as mediators (Bourbonnais et al., 1995; Eggert et al., 1996).

Surprisingly, laccase only attaches to the substrate cell wall after decay has already begun (Evans and Gallagher, 1991). An additional interesting feature is that laccase, for example from *Trametes*, can be expressed efficiently in a yeast *Saccharomyces cervisiae*. The transformed yeast showed considerable resistance to phenolic compounds (Larsson et al., 2001). These results suggest that laccase from white rot fungi utilize the products of lignin breakdown and are resistant to the phenolic compounds produced, although these might be thought to be inhibitory compounds. It must be assumed that the free radicals, produced as a result of laccase action, are rapidly removed by secondary reactions.

A puzzling observation was made that laccase was quite stable in leaf litter for example of oak, perhaps due to absorption to humic acid components in the litter (Criquet et al., 2000). An obvious question arises whether this persistence of laccase has any ecological significance in the continued decomposition of the litter. The white-rot fungus, *Marasmius quercophilus,* apparently secretes a laccase when degrading leaf litter from oak (Dedeyan et al., 2000). One of the laccases formed by *Pleurotus* shows activity inside the cell or in the cell wall (Palmieri et al., 2000). This is a little difficult to reconcile with a role in delignification of a substrate. Thus, although the function of laccase in delignification is not in doubt, many aspects remain unclear despite the advances made in this area. The interaction of different wood-decaying basidiomycetes has shown a highly variable pattern of laccase formation, and this subject requires more detailed and reliable experiments (Iakoviev and Stenlid, 2000).

6.2. Ethanol production

To improve the production of fuel ethanol from renewable raw materials, laccase from the white rot fungus, *Trametes versicolor*, was expressed under control of the PGK1 promoter in *S. cerevisiae* to increase its resistance to phenolic inhibitors in lignocellulose hydrolysates (Larsson et al., 2001). The results from a detailed study of the system showed that phenolic compounds were important as fermentation inhibitors, and that there was a definite advantage of using laccase-expressing yeast strains for producing ethanol from lignocellulose.

6.3. Drug analysis

A new enzymatic method based on laccase has been developed to distinguish morphine from codeine simultaneously in drug samples injected into a flow detection system (Bauer et al., 1999). An enzyme sensor was constructed based on laccase and glucose dehydrogenase immobilized at a Clark oxygen electrode. Morphine is oxidized by laccase with consumption of oxygen and regenerated by glucose dehydrogenase. Laccase cannot oxidize codeine, so the sensor is selective for morphine. Morphine is detected between 32 nM and 100 μM. The rapid and technically simple method allows discrimination between morphine and codeine in less than 1 min after injection at a sampling rate for quantitative measurements of 20 per hour.

6.4. Wine clarification

Laccase immobilized on a copper-chelate carrier that can be regenerated was used successfully to remove phenols from white grape must (Servili et al., 2000).The phenols were partially removed by the enzymatic treatment, especially (−)-epicatechin, ferulic and *o*-coumaric acids. Laccase was shown to form two complex compounds from ferulic acid, i.e. trans-5-((*E*)-2-carboxyvinyl)-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid and (*Z*)-β-(4-((*E*)-2-carboxyvinyl)-2-methoxyphenoxy)-4-hydroxy-3-methoxy cinnamic acid (Carunchio et al., 2001). Copper (II) had an important role in the reaction mechanism.

6.5. Bioremediation

Enzymes from fungi have been shown to be useful for the degradation of a variety of persistent environmental pollutants. Many of the enzymes responsible for pollutant degradation are extracellular, and in nature probably are involved in the degradation of wood. Some white rot-fungi such as *Pycnoporus cinnabarinus* produce high levels of laccase, however, the potential for laccase in bioremediation has not been well characterized (Wong and Yu, 1999). A laccase produced in the yeast, *Pichia pastoris*, was engineered by site-directed mutagenesis to improve the rate of electron transfer between the copper-containing active site of laccase and an electrode (Gelo et al., 1999). Thus laccases may be usefully engineered to improve the efficiency of particular bioremediation processes.

6.5.1. Trichlorophenol

The toxicity of the isomers of 2,4,6-trichlorophenols was studied in liquid cultures of the white rot fungi, *Panus tigrinus* and *Coriolus versicolo*r. In both cases the ligninolytic enzyme systems of both fungi were found to be responsible for the transformation of trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone by both intact fungal cultures and the purified ligninolytic enzymes, Mn-peroxidases and laccases of both fungi (Leontievsky et al., 2000). However, the primary attack on 2,4,6-TCP in cultures of *P. tigrinus* was catalyzed predominately by Mn-peroxidase, while in *C. versicolor* it was catalyzed predominantly by laccase, suggesting that the mode of regulation of these enzymes was different in these two fungi.

6.5.2. Alkenes

Laccase from the white-rot fungus, *Trametes hirsuta*, has been used to oxidize alkenes (Niku and Viikari, 2000). The oxidation is the effect of a two-step process in which the enzyme first catalyzed the oxidation of primary substrate, a mediator added to the reaction, and then the oxidized mediator oxidizes the secondary substrate, the alkene, to the corresponding ketone or aldehyde. The best results were obtained by using hydroxybenzotriazole as mediator, and aliphatic polyunsaturated and aromatic allyl alcohols were completely oxidized within 2 h at 20 °C. Aliphatic allyl alcohols were oxidized up to 70% at 45 °C for 20 h. By contrast, the oxidation of other alkenes, such as allyl ether, *cis*-2-heptene and cyclohexene, did not exceed 25%.

6.5.3. Other compounds

Laccase purified from a strain of *Coriolopsis gallica* oxidized carbazole, *N*-ethylcarbazole, fluorene, and dibenzothiophene in the presence of 1-hydroxybenzotriazole and 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid as free radical mediators (Bressler et al., 2000). Although carbazole and *N*-ethylcarbazole were both completely removed within 18 h, no oxidation or condensation metabolites were detected.

Crude laccase from *Pleurotus ostreatus* immobilized to Eupergit was found to be useful to remove phenolic pollutants (Hublik and Schinner, 2000). Continuous elimination of 2,6-dimethoxyphenol by immobilized laccase was carried out in a packed-bed reactor followed by filtration of the formed precipitate.

6.5.4. Industrial wastes

An isolate of the fungus, *Flavodon flavus*, was shown to be able to decolourize the effluent from a Kraft paper mill bleach plant (Raghukumar, 2000). The important enzymes, produced by the fungus in the presence of the effluent, were laccase, manganese-dependent peroxidase, and lignin peroxidase. The culture appeared to be a potential candidate for bioremediation of coloured industrial effluents.

Laccase purified from *Trametes villosa* degrades bisphenol A, an endocrine-disrupting chemical (Fukuda et al., 2001). Not all reaction products were identified, but one was 4-isopropenylphenol. The removal of this estrogenic activity from bisphenol and also nonylphenol was shown to be most effective when laccase was supplemented with 1-hydroxybenzotriazole (Tsutsumi et al., 2001). Polyoxometalates have also been observed to be an effective promoter of laccase-assisted reactions (Carneiro et al., 2000).

6.5.5. Decolorisation of dyes

F. flavus decolourized several synthetic dyes like Azure B, Brilliant green, Congo red, Crystal violet, and Remazol Brilliant Blue R in low nitrogen medium (Raghukumar, 2000). Alternatively, laccase, along with stabilizers, may be suitable for treatment of wastewater (Cameron et al., 2000; Soares et al., 2001). In contrast, addition of several laccase activity activators, such as ethanol, veratryl alcohol, and aeration level, can be used to increase laccase production by *Trametes versicolor* (Maceiras et al., 2001). Grown on Nylon sponge functioning as a physical support on which the mycelium was bound, these cultures of *T. versicolor* were shown to decolourize the polymeric dye, Poly R-478, and about 90% decolourization was achieved with the veratryl alcohol-supplemented cultures. Laccases from *T. versicolor* may also be used to decolor humic acids derived from lignite (brown coal) (Fakoussa and Frost, 1999), and to convert 4-methyl-3-hydroxyanthranilic acid to 2-amino-4,6-dimethyl-3-phenoxazinone-1,9-carboxylic acid, a phenoxazinone chromophore occurring in actinomycins (Osiadacz et al., 1999).

Partial decolorization of two azo dyes (orange G and amaranth) and complete decolorization of two triphenylmethane dyes (bromophenol blue and malachite green) was achieved by cultures of *Pycnoporus sanguineus* producing laccase as the sole phenoloxidase (Pointing and Vrijmoed, 2000). The fungus was grown in submerged liquid cultures. Enzyme production was correlated with dye decolorization, and sorption of dye to mycelia accounted for less than 3% of dye removal.

Trametes hirsuta, and a laccase purified from the fungus, were able to degrade triarylmethane, indigoid, azo, and athraquinonic dyes used in dyeing textiles (Abadulla et al., 2000) as well as 23 industrial dyes (Rodriguez et al., 1999). Immobilization of the *T. hirsute* on alumina (Abadulla et al., 2000) enhanced the thermal stabilities of the enzyme and its tolerance against inhibitors such as halides, copper chelators, and dyeing additives (Abadulla et al., 2000). Treatment of the dyes with immobilized laccase reduced their toxicity by up to 80% based on oxygen consumption rate of *Pseudomonas putida*. Textile effluents decolorized with immobilized laccase could be used for dyeing, and acceptable colour differences were measured for most dyes.

6.5.6. Herbicide degradation

Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide (Mougin et al., 2000). The diketonitrile derivative undergoes cleavage to the inactive benzoic acid analogue. Laccase enzymes in two fungi, *Phanerochaete chrysosporium* and *T. versicolor*, are able to convert the diketonitrile to the acid, as will purified laccase in the presence of 2 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) acting as a redox mediator at pH 3 (Mougin et al., 2000).

7. Perspective

Laccases are exceptionally versatile enzymes, catalyzing one basic reaction from which all its activities spring. In higher plants the function of laccases are only now being evaluated, and more investigations on the occurrence, development and function of higher plant laccases are needed. Fungal laccases have been studied far more extensively. Their function in fungi is quite varied and includes regulation of morphology, control of virulence and nutrition, and their ability to delignify woody tissues. In addition to the strictly biological functions, laccases are increasingly being investigated for a variety of practical applications ranging from use in the pulp and paper industry to their possible use in bioremediation and analytical uses.

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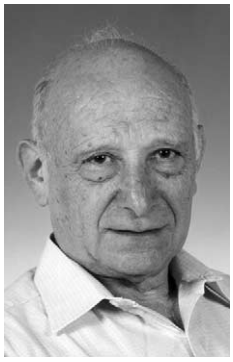

Alfred M. Mayer, born in Germany, because of the rise of the Nazi regime, emigrated to Holland in 1933 and to England in 1939. His BSc degree in Chemistry was followed by a PhD in Plant Physiology, in the University of London, under the supervision of W.H. Pearsall. Since 1950, has lived in Israel, and worked in the Department of Botany of the Hebrew University of Jerusalem, as Full Professor since 1969 and as Emeritus since 1995. His interest in Phytochemistry was stimulated during a stay in 1959 in Cambridge, UK, working with L.W. Mapson, T. Swain and J. Friend, while also learning from the wisdom and charm of Robin Hill. Throughout the years he has pursued his interests in plant metabolism, in addition to teaching plant sciences and filling a variety of administrative tasks in the University. His publications include a book: ''The Germination of Seeds'', a textbook of plant physiology in Hebrew and an autobiography. The collaboration with R.C. Staples started in 1987 during a sabbatical leave at Cornell University. He has received an honorary doctorate from the University of Bordeaux II for his work on laccase in relation to wine.

Richard C. Staples joined the Boyce Thompson Institute in 1952, and earned his PhD degree in Plant Biochemistry at Columbia University in 1957 under the supervision of Professor Ray F. Dawson. He was appointed the G.L. McNew Scientist in 1988, and the G.L. McNew Scientist Emeritus in 1992. In 1980, he was given the Senior US Scientist award by the Alexander von Humboldt Foundation, and from 1982 to 1983 he went to Aachen, Germany, to work with Professor Dr. Hans J. Reisener on the germination of urediospores of the rust fungi. In 1994, Staples was presented with the Ruth Allen Award by the American Phytopathological Society for his work on stomatal recognition and differentiation of appressoria by urediospore germlings, especially that done in collaboration with Prof. Harvey C. Hoch at Cornell. Staples' interest in laccase arose from his research on the virulence factors expressed by the fungal pathogen, *Botrytis cinerea*, an interest that led to a fruitful collaboration with Professor Mayer.