Indian J. Microbiol. (September 2008) 48:309–316
DOI: 10.1007/s12088-008-0028-z

REVIEW

Laccase: enzyme revisited and function redefined

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Received: 23 December 2007 / Accepted: 8 February 2008 / Published online: 18 June 2008

Abstract One enzyme, one physiological role, that's how most scientists have traditionally looked at it but there is a growing appreciation that some enzymes "moonlight" i.e. in addition to their "primary" catalytic function, they carry other functions as well. Moonlighting refers to a protein that has multiple functions, which are not because of gene fusion; splice variants or multiple proteolytic fragments. Until recently laccases were reported from eukaryotes, e.g. fungi, plants, insect. However there is some evidence for its existence in prokaryotes, a protein with typical features of multi-copper oxidase enzyme family. The present available knowledge of its structure provides a glimpse of its plasticity, revealing a multitude of binding sites responsible for multifunctional activity. Laccase represents an example of a 'moonlighting' protein that overcomes the one gene-one structure-one function concept to follow the changes of the organism in its physiological and pathological conditions. It is wide spread in plants, where it is involved in biosynthesis of lignin; in fungi it is involved in lignin degradation, development associated pigmentation (melanin synthesis), detoxification and pathogenesis, and in bacteria, laccases are involved in the synthesis of endospore coat protein (cot A).

Keywords Isozyme · Laccase · Moonlight · Oxidoreductase · Lignification

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Introduction

Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is one of the few lignin-degrading enzymes that have been extensively studied since 18th century. Until recently laccases were reported from eukaryotes, e.g., fungi, plants, and insects [1]. There are some evidences, however, for its existence in prokaryotes, a protein with typical features of multi-copper oxidase enzyme family [2]. The first bacterial laccase was detected in the plant root associated bacterium, *Azospirillum lipoferum* [3], were it was shown to be involved in melanin formation [4]. A typical laccase containing six putative copper binding sites was discovered in marine bacterium *Marinomonas mediterranea*, but no functional role was assigned to this enzyme [5–6]. In insects, laccases have been suggested to be active in cuticle sclerotization [7]. Recently, two isoforms of *laccase 2* gene have been found to catalyse larval, pupal, and adult cuticle tanning in *Tribolium castaneum* [8] and a novel laccase has been isolated and characterized from a bovine rumen metagenome library that neither exhibited any sequence similarity to known laccases nor contained hitherto identified functional laccase motifs [9]. They are a diverse group of multi-copper proteins with broad substrate specificity, originally discovered in the exudates of *Rhus vernicifera,* the Japanese lacquer tree [10], and subsequently were demonstrated as a fungal enzyme as well [11–12]. Laccase has been extensively examined

since the mid seventies and a number of reviews have appeared on the subject [13–20]. Broad families of organisms share laccases with diverse biological functions including breakdown of cellulose to provide nutrients to fungi, lignification of plant cell walls and production of melanin in the insect midgut as a primitive immune defense against parasites [1]. Using the diverse functions of this enzyme, *Cryptococcus neoformans* has co-opted laccase into a sinister role as a virulence factor that converts mammalian substrates into reactive intermediates that protect the fungus and allow damage to the mammalian host. Molecular study of this enzyme has yielded insights into the working of laccase and provides reasons for the transformations of the fungal saprophyte into pathogen.

One enzyme with one physiological role is a common phenomenon but there is a growing appreciation that some enzymes exhibit multiple functions, in addition to their primary catalytic function [21]. Moonlighting refers to a single protein that has multiple functions, which is not a consequence of gene fusion, splice variants or multiple proteolytic fragments [21]. Few proteins are known to moonlight, including receptors, channels, enzymes, transcription factors and scaffolds [21, 22]. A recent report of multiple functional proteins has been reported for photosynthesis genes in marine viruses during host infection, [23] which are speculated to be a moonlighting phenomenon. Furthermore, the abundance of cyanobacteria and their phages in the oceans suggests that phage photosynthetic proteins could have a small but significant role in the conversion of light to chemical energy on a global scale [23].

Laccase can also be included in the moonlighting list because of its multiple signature functions, depending on cell type and intra- or extra cellular conditions in which its isozymes get expressed (Table 1).

How important laccases are

Based on the mechanism, a majority of the commercial enzymes are hydrolases (including proteases, carbohydrases, and esterases), while oxidoreductases account for a miniscule share [20]. This is in contrast to the high occurrence of oxidoreductases in nature. The gap between a vast natural oxidoreductase repertoire and very limited commercial oxidoreductase products creates the space and potential for developing more oxidoreductase-based biocatalysts.

All the laccases barring those of fungal origin are out of the scope of this discussion, but it is worth noting that one function for laccase is to naturally catalyze the oxidation of phenol-like substrates by molecular oxygen forming water. Because of their high relative nonspecific oxidation capacity, laccases have been found as useful biocatalysts for diverse biotechnological applications. A few oxidoreductases are available at present in the market for textile, food, and other industries and other candidate proteins are being actively developed for future commercialization. The application of laccases can be divided into industrial-technical, speciality chemical synthesis, environmental, food, medicinal, and personal care fields [20]. Being specific, energy saving, and biodegradable, oxidoreductase-based biocatalysts fit well with the development of highly efficient, sustainable, and environment friendly industries. The established or emerging applications having significant economical viability and potential of laccases can be sketched out from a classical review of Thruston and an exhaustive and encouraging review of Xu [18, 20].

Many oxidoreductases have co-substrates that are either chromogenic, fluorogenic, chemiluminescent, or electroactive. They may produce end products that are reactants/substrates for coupled chemical or enzymatic reactions suitable for optic, electric, or other physical measurements. Thus, several oxidoreductases can be applied as biosensors or

Table 1 Examples of few moonlighting enzymes

S. No.	Enzyme	First Function	Second Function	Third Function
1.	Subtilisin	Peptidase	Esterase	-
2.	Aspartate amino transferase (AAT)			
	Transamination	Decarboxylation	-	
3.	Phytase	Phosphomonoesterase	Sulfoxidation	-
4.	Hydroxynitrile lyase (HNL)	Oxynitrilase	Esterase	-
5.	Rubisco	Carboxylase	Oxygenase	-
6.	Decarboxylases (PDC, ADC)	Decarboxylase	Carboligation	-
7.	Chymotrypsin	Amidation	Phosphotriesterase	-
8.	O-Succinyl benzoate (OSBS)	Synthase	Racemization	-
9.	Laccase	Iron oxidase	CotA oxidase	Polyphenol Oxidase (polymerization and depolymerisation)

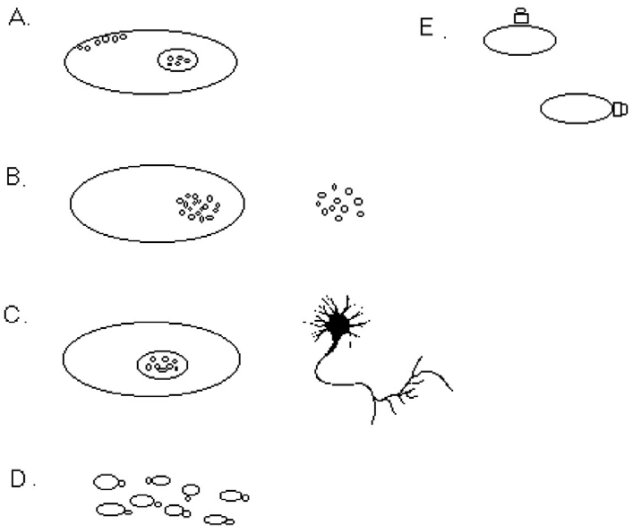

Fig. 1 Different probable mechanisms of switching for multiple functions of laccase.

A. Different function at different location in cell.
B. Protein behaving differentially, intercellularly and intracellularly
C. Proteins with different function when secreted by different cell type
D. Different activity of protein caused by binding of substrate, product, or cofactor.
E. Protein with different binding site for different substrates.

bioreporters [20]. Enzymatic biofuel cells can utilize a diverse and an unlimited supply of fuel sources, which has prompted researchers to focus on miniature, implantable powering devices in the living systems. Unlike conventional fuel cells that need periodic refueling, the implanted micro enzymatic biofuel cells can continue to produce electricity as long as biological host is alive. Reports on laccase catalyzed cathodes for dioxygen reduction exist in the literature [24].

Reactions catalyzed by laccases

Laccase exhibit iron oxidase activity: C. neoformans has emerged as a major fungal pathogen in immuno-compromised individuals such as patients with AIDS, organ transplant recipients and those receiving high doses of corticosteroid treatment [25]. Three of the best-known virulence-associated attributes of *C. neoformans* are: (1) an extensive polysaccharide capsule, (2) the ability to grow at 37°C, and (3) expression of the virulence factor i.e., laccase [25]. Laccase of C. *neoformans* was initially referred to as a phenol oxidase or diphenol oxidase because of its ability to make coloured products from a wide variety of phenolic compounds having two hydroxyl groups, but not tyrosine [26]. However, a number of enzymes including tyrosinase, peroxidase and yeast iron transporter, Fet3, also share these substrate activities, thus suggesting the necessity of additional studies to identify the enzyme as laccase1. Recombinant cryptococcal laccase exhibits iron oxidase activity by converting Fe (II) to Fe (III) [27]. Moreover, laccase from *C. neoformans* is implicated in the virulence of the organism by virtue of its oxidizing action on the brain catecholamines as a defense system against host immune system [2, 29]. Its differential function when secreted by different type of cells adds it to the moonlighting list (Fig. 1). Laccase alone has been demonstrated to confer significant protection against murine alveolar macrophages independent of dopamine, by virtue of its iron oxidase activity, that appears to diminish the host cell oxidative burst by reducing the available Fe (II) stores [27]. Despite considerable work, many fundamental questions about the biological functions of laccase, however, remain unanswered, suggesting several exciting areas of research in a number of disciplines. The development of molecular techniques in the fungus has now allowed the identification of regulators of laccase by methods such as insertional mutagenesis and complementation of mutants.

Laccase shows CotA oxidase activity: The exact function of CotA within the spore coat is still not fully understood, but the assembly of CotA is essential for the full complement of spore resistance properties. Expression of *cotA* gene has been classically implicated in the biosynthesis of a brownish pigment that characterizes sporulating colonies of *Bacillus subtilis,* and which has properties of melanin conferring protection against UV light [30]. *B. subtilis* CotA is significantly similar at the primary structure level with multicopper "blue oxidases", a protein family that includes laccases [31, 2]. Moreover, CotA shows similarity with two members of this family whose structure is known; it has 19.7% sequence identity, and 36.6% similarity with zucchini ascorbate oxidase (ZAO) [32] and 22.4% identity and 39.3% similarity with *Coprinus cinereus* [33]. However, based on sequence comparison, *C. cinereus* laccase and ZAO are more closely related to each other (30.6% identity and 50% similarity) than CotA. Nevertheless, a comparison of the amino acid sequences between CotA and members of the multicopper oxidase family shows that copper ligands are conserved in CotA. The spore-forming bacterium *B. subtilis* synthesizes and deposits a protein coat around the developing endospore during differentiation. The spore coat consists of at least 25 different polypeptides of 5 to 65 kDa, of which some are highly cross-linked [34–36]. These proteins are assembled

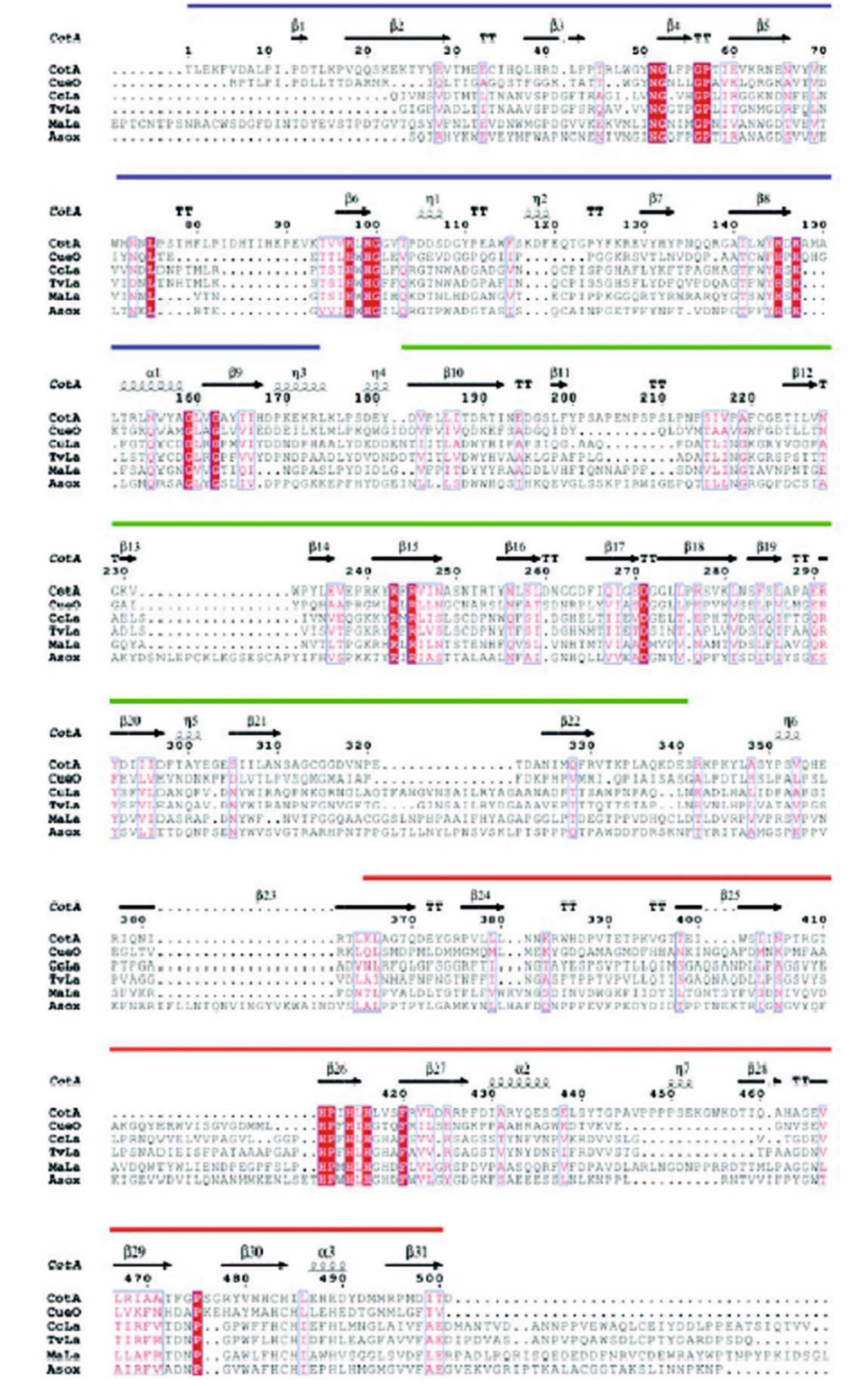

Fig. 2 Sequence alignment of monodomain multicopper oxidases containing four copper atoms and with known three-dimensional structure. Highly conserved regions are *boxed*, within those; invariant residues are represented against a *red* background, whereas conserved residues are *shaded*. CotA, *B. subtilis* Cot A; Cueo, *E. coli* Cueo; CcLa, *Coprinus cinereus* laccase; TvLa, *Trametes versicolor* laccase; MaLa, *Melanocarpus albomyces* laccase; Asox, Zucchini ascorbate oxidase

into a lamella-like inner coat and an electron-dense outer coat, which protects the spore from a diverse range of stress [37]. *B. subtilis* endospore coat protein shows CotA oxidase activity with all the structural features of laccase, including the reactive surface exposed copper center T1 and two buried copper centers (T2 and T3) [29].

Cot A is naturally associated with the coat structure in active form, which suggests *B. subtilis* endospore coat structure as a surface display system for biocatalyst applications involving the stable CotA laccase [38]. Comparison of *B. subtilis* CotA with other multicopper oxidases shows that copper binding motives are conserved in all sequences. Further, similarities are more significant in N- and C-terminal regions, corresponding to domain 1 and 3 in the CotA structure (Fig. 2).

Laccase as polyphenol oxidase in lignin degradation

Laccase is small group of blue oxidases that can utilize the full oxidizing capacity of oxygen to form two molecules of water. It is one of the best-understood and widely studied oxidases, in terms of its catalytic mechanism. Most laccases reported so far are extracellular enzymes and differ markedly in their redox potentials, carbohydrate contents, thermal stabilities and substrate specificities [39–40]. Due to their high non-specific oxidation capacity, laccases are useful biocatalysts for diverse biotechnological applications [18, 41].

Fungal laccase is responsible for demethylation of lignin and lignin related model compounds, which is thought to be an initial step in lignin biodegradation. Side chain elimination is also responsible for lignin breakdown [42–43]. All the enzymes putatively involved in lignin cleavage, produce highly reactive and toxic compounds, which are ultimately scavenged via polymerization before their entry into the fungal hypha [18].

The discovery of low molecular weight organic compounds acting as mediators [43] has significantly expanded the role of laccase in lignin degradation [44]. Bourbonnais and Paice (1990) [44] discovered that laccase/ABTS {2,2'-azino-bis-(3 ethylbenzothiazoline-6-sulfonate)} did not only degrade non-phenolic lignin model compounds such as veratryl alcohol but also decreases pulp kappa number, resulting in the release of methanol from lignin methoxy groups during bleaching [45]. A naturally occurring laccase-mediator, 3-hydroxyanthranilic acid (3-HAA) has been found in the white-rot fungus *Pycnoporus cinnabarinus* [45]. Call and Mucke (1997) [46] discovered another effective laccase mediator, 1-hydroxybenzotriazole (1-HBT) which was suitable and applicable for bleaching of pulps at pilot plant scale. A detailed understanding of these processes will allow the future design of optimized enzymes by protein engineering and, of novel and more efficient mediators by molecular modeling techniques.

Laccase gene family

Laccase gene family can be used as a potential tool to define its moonlighting functions. The copy numbers of laccase genes vary among fungi. A laccase gene family in which the genes encoding two of five laccases were located on the same chromosome of *Trametes villosa* [47–48], and three laccase genes were found to be clustered within approximately 11 kb of each another in the plant pathogenic fungus, *Rhizoctonia solani* [49] Giardiana et. al., (1996) [50] isolated two-phenol oxidase genes (*pox1* and *pox2*) that showed 84% homology with each other and thus demonstrated the existence of a multigene family that encoded for isoforms of laccase in *Pleurotus ostreatus. C. cinereus* also contains a laccase gene family consisting of at least three genes [51]. The presence of multiple gene families for the secreted laccases requires systematic genetic analysis to elucidate their functions. Gene families probably produce closely related proteins that are subtly different in their activities, allowing transformation of a wider range of substrates or showing differential regulation [52]. Moreover, until transcripts for all the laccase genes are not detected, the possibility that some of the non-expressed laccase genes are pseudogenes or are expressed under different physiological conditions cannot be ruled out.

Phylogenetic data of the moonlighting protein i.e., Fet3, CotA and polyphenol oxidase from various sources proves that diverse paralogus laccase genes may have descended from progenitor gene or master gene, which has duplicated and diverged prior to speciation. These paralogus genes can give information on the early ancestors of families of proteins now residing in many contemporary organisms and showing moonlighting behavior (Fig. 3).

Conclusion

It is possible that several other proteins might possess additional functions that remain to be elucidated. Characterization of a novel protein generally involves finding a function for a protein, but does not necessarily include a search for all possible additional functions of the protein. There is currently no general straightforward method to identify which proteins encoded by a genome sequence that have multiple functions or which determines whether a protein of interest is a moonlighting protein. Moonlighting may be a common mechanism of communication and cooperation

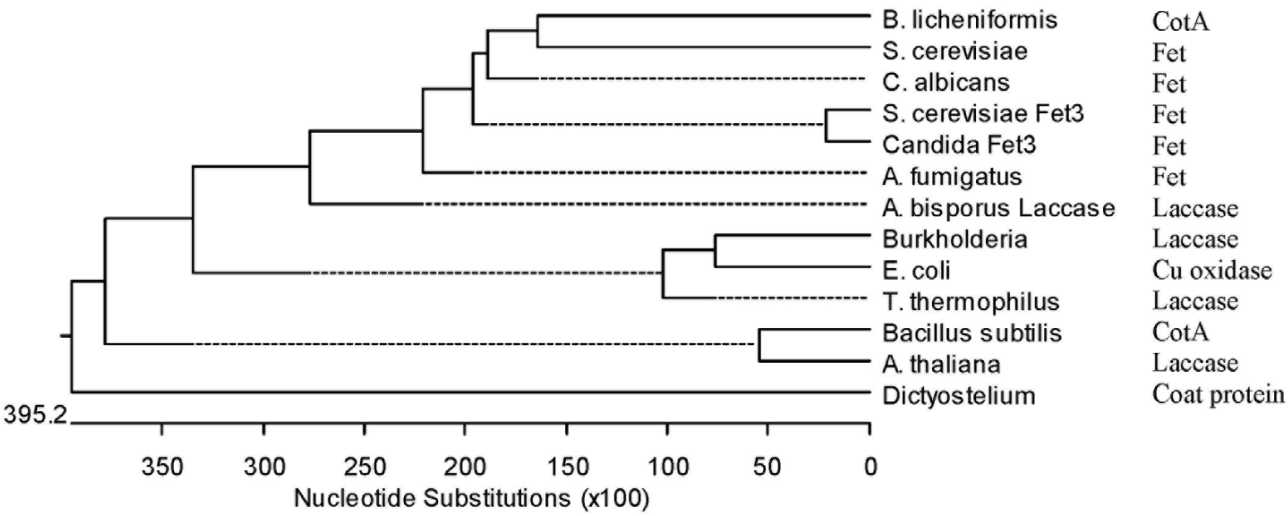

Fig. 3 Dendrogram constructed of different form of laccases having moonlighting functions i.e. Fet3, CotA and Polyphenol oxidase, from diverse sources

between different functions and pathways within a complex modern cell or between different cell types within an organism [21].

As natures own catalysts, laccase possess very diverse specificity, reactivity, and other physicochemical, catalytic, and biological properties that are desirable for various industrial and medical applications. The moonlighting concept will spark our brain to design a biocatalyst having desirable, diverse and multiple functionality, which could cater to the need of future biotechnology. The ability of laccase to moonlight can complicate the future use of proteomics, which results in understanding diseases and developing new therapeutics. Moonlighting phenomenon of laccases can be better understood under the light of evolutionary pathways. Some proteins might have been partially evolved or still at their transitional phase, which can be used as connecting links to solve some of the lineage problem.

Acknowledgement The authors are grateful to Prof. B. N. Johri, Emeritus-Scientist, C.S.I.R. (Gov. of India), presently with Department of Biotechnology, Barkatuallah University, Bhopal, for his critical editing of the manuscript.

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