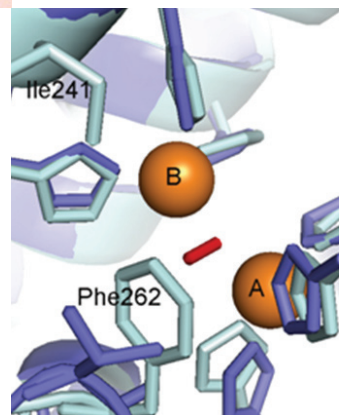# Biochemical and structural characterisation of the copper containing oxidoreductases catechol oxidase, tyrosinase, and laccase from ascomycete fungi

Chiara Gasparetti

V͞T T

Biochemical and structural characterisation of the copper containing oxidoreductases catechol oxidase, tyrosinase, and laccase from ascomycete fungi

Chiara Gasparetti

Biochemical and structural characterisation of the copper containing oxidoreductases catechol oxidase, tyrosinase, and laccase from ascomycete fungi

Abstract

Catechol oxidase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), and laccase (EC 1.10.3.2) are copper-containing metalloenzymes. They oxidise substituted phenols and use molecular oxygen as a terminal electron acceptor. Catechol oxidases and tyrosinases catalyse the oxidation of *p*-substituted *o*-diphenols to the corresponding *o*-quinones. Tyrosinases also catalyse the introduction of a hydroxyl group in the *ortho* position of *p*-substituted monophenols and the subsequent oxidation to the corresponding *o*-quinones. Laccases can oxidise a wide range of compounds by removing single electrons from the reducing group of the substrate and generate free radicals. The reaction products of these oxidases can react further non-enzymatically and lead to formation of polymers and cross-linking of proteins and carbohydrates, in certain conditions.

The work focused on examination of the properties of catechol oxidases, tyrosinases and laccases. A novel catechol oxidase from the ascomycete fungus *Aspergillus oryzae* was characterised from biochemical and structural point of view. Tyrosinases from *Trichoderma reesei* and *Agaricus bisporus* were examined in terms of substrate specificity and inhibition. The oxidation capacity of laccases was elucidated by using a set of laccases with different redox potential and a set of substituted phenolic substrates with different redox potential. Finally, an evaluation of the protein cross-linking ability of catechol oxidase from *A. oryzae*, tyrosinases from *T. reesei* and *A. bisporus* and laccases from *Trametes hirsuta*, *Thielavia arenaria*, and *Melanocarpus albomyces* was performed.

A novel extracellular catechol oxidase from *A. oryzae* (AoCO4; UniProtKB: Q2UNF9; Entrez gene ID: 5990879) was chosen for cloning and expression as representative of the newly discovered family of short tyrosinases sequences. *AoCO4* gene was heterologously expressed in *T. reesei*. The protein produced did not show activity on L-tyrosine and 3,4-dihydroxy-L-phenylalanine (L-DOPA), which are typical substrates for tyrosinases. Consequently, the protein was classified as a catechol oxidase. AoCO4 was produced in a bioreactor and the expression resulted in high yields. The purified AoCO4 was partially processed at a Kex2/furin-type protease site and showed a molecular weight of 39.3 kDa. AoCO4 was able to oxidise a limited range of diphenolic compounds, e.g., catechol, caffeic acid, hydrocaffeic acid, and 4-*tert*-buthylcatechol. AoCO4 oxidised also the monophenolic compounds aminophenol and guaiacol. AoCO4 showed a pH optimum in the acidic range and was observed to be a relatively thermostable enzyme. A crystal structure of AoCO4 was solved at 2.5 Å

resolution. AoCO4 was a monomer and the overall structure of AoCO4 was found to be similar to the known structures of catechol oxidases and tyrosinases.

A detailed characterisation of the substrate-specificity of the extracellular tyrosinase from *T. reesei* (TrT) was accomplished and compared to the commercial tyrosinase from *A. bisporus* (AbT). TrT generally showed lower affinity than AbT on substrates that had a free amino group, such as L-tyrosine, L-DOPA, and YGG tripeptide. The reaction end product of TrT and AbT was studied via mass spectrometry and dopachrome was found to be the only reaction end product of L-DOPA oxidation catalysed by both tyrosinases. Dopachrome produced by AbT-catalysed oxidation of L-DOPA was also shown to inhibit the TrT tyrosinase by an end product inhibition mechanism. Further, when the type of inhibition of potential inhibitors for TrT was analysed with *p*-coumaric acid and caffeic acid as substrates, potassium cyanide and kojic acid were the strongest inhibitors of TrT.

The kinetics of three laccases, with different redox potential ($E°$), for three *p*-substituted dimethoxy phenolic substrates (2,6-dimethoxyphenol, syringic acid, and methyl syringate) with different $E°$ were determined at two pHs. The laccases studied were from *M. albomyces*, *T. arenaria*, and *T. hirsuta*. The enzyme from the ascomycete fungus *T. arenaria* was a novel laccase produced in *T. reesei*. *T. arenaria* laccase was purified, and biochemically and structurally characterised. By comparison of the three laccases it could be shown that both the difference in redox potential ($\Delta E°$) and the pH had an effect on the kinetics. However, the effect of $\Delta E°$ was found to prevail over that of the pH for substrates with a high $E°$, such as methyl syringate.

All oxidative enzymes studied in this work were also tested for their cross-linking ability, with α-caseins utilised as a model substrate with and without a cross-linking agent. TrT was found to be the best enzyme for cross-linking of α-caseins, whereas AbT was found to be the best enzyme for cross-linking α-caseins in the presence of catechol as a cross-linking agent. Interestingly, AoCO4 was also found to cross-link α-caseins in the presence of catechol as a cross-linking agent.

Preface

Academic dissertation

Supervisors   Research Professor Kristiina Kruus
Bio and Process Technology
VTT Technical Research Centre of Finland
Espoo, Finland

Research Professor Johanna Buchert
Bio and Process Technology
VTT Technical Research Centre of Finland
Espoo, Finland

Pre-examiners   Adjunct Professor Taina Lundell
Department of Food and Environmental Sciences
University of Helsinki
Helsinki, Finland

Full Professor Giovanni Sannia
Department of Organic Chemistry and Biochemistry
University of Naples Federico II
Napoli, Italy

Opponent   Professor Lígia O. Martins
Instituto de Tecnologia Química e Biológica
Univesidade Nova de Lisboa
Oeiras, Portugal

Custos   Professor Matti Leisola
Department of Biotechnology and Chemical Technology
Aalto University
Espoo, Finland

List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers.

I   Selinheimo, E., Gasparetti, C., Mattinen, M.-L., Steffensen, C.L., Buchert, J., Kruus, K. Comparison of substrate specificity of tyrosinases from *Trichoderma reesei* and *Agaricus bisporus.* Enzyme and Microbial Technology (2009) 44:1–10.

II   Gasparetti, C., Faccio, G., Arvas, M., Buchert, J., Saloheimo, M., Kruus, K. Discovery of a new tyrosinase-like enzyme family lacking a C-terminally processed domain: production and characterization of an *Aspergillus oryzae* catechol oxidase. Applied Microbiology and Biotechnology (2010) 86:213–226.

III   Kallio, J.P., Gasparetti, C., Andberg, M., Boer, H., Koivula, A., Kruus, K., Rouvinen, J., Hakulinen, N. Crystal structure of an ascomycete fungal laccase from *Thielavia arenaria* – common structural features of asco-laccases. FEBS Journal (2011) 278:2283–2295.

IV   Gasparetti, C., Nordlund, E., Jänis, J., Buchert, J., Kruus, K. Extracellular tyrosinase from the fungus *Trichoderma reesei* shows product inhibition and different inhibition mechanism from the intracellular tyrosinase from *Agaricus bisporus.* BBA – Proteins and Proteomics (2012) 1824:598–607.

Author's contributions

I   The author planned the work in collaboration with the co-authors. The author purified the tyrosinases from *Trichoderma reesei* and *Agaricus bisporus* and determined the kinetic parameters, i.e., K_M and V_{max} for the two tyrosinases. The author interpreted the results and contributed to writing of the publication with Emilia Nordlund (*née* Selinheimo), Sc.D.; Kristiina Kruus, Research Professor; and the other authors.

II   The author planned and conducted the laboratory work related to the purification and the biochemical characterisation of the *Aspergillus oryzae* catechol oxidase. The author interpreted the data and significantly contributed to writing of the publication. The author and the co-author, Greta Faccio, Ph.D., contributed equally to this work.

III   The author planned and conducted the work related to the purification and the determination of the kinetic parameters and pH optima of the laccase from *Thielavia arenaria*. The author interpreted the results and compared the kinetic data of the three laccases, i.e., from *T. arenaria*, *Melanocarpus albomyces* and *Trametes hirsuta*. The author contributed significantly to the writing process.

IV   The author had the main responsibility for designing and carrying out the experiments, for the interpretation of the results, and in writing of the publication. The author conducted the spectroscopy and polarography experiments. The author conducted the experiments using mass spectrometry jointly with Professor Janne Jänis in the Chemistry Department of University of Eastern Finland. The author developed a method to produce dopachrome by utilising the tyrosinase from *A. bisporus* and conducted the dopachrome inhibition experiments of the *T. reesei* tyrosinase. The author determined the K_i parameters of *T. reesei* tyrosinase for several inhibitors.

Contents

Appendices
 Publications I–IV

Publications II is not included in the PDF version.
Please order the printed version to get the complete publication
(http://www.vtt.fi/publications/index.jsp).

List of abbreviations

2,6-DMP	2,6-dimethoxyphenol
AbT	tyrosinase from *Agaricus bisporus*
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AoCO4	catechol oxidase from *Aspergillus oryzae*
BmT	tyrosinase from *Bacillus megaterium*
CD	circular dichroism
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
D-DOPA	3,4-dihydroxy-D-phenylalanine
E^0	redox potential
EPR	electron paramagnetic resonance
GGY	glycine-glycine-tyrosine peptide
GYG	glycine-tyrosine-glycine peptide
IbCO	catechol oxidase from *Ipomea batatas*
L-DOPA	3,4-dihydroxy-L-phenylalanine
LMS	laccase mediator system
MBTH	3-methyl-2-benzothiazolinone hydrazone
MW	molecular weight

PDB	Protein Data Bank
Phe-OH	phenolic substrate
PPO	polyphenol oxidase
*r*MaL	laccase from *Melanocarpus albomyces* (recombinant)
ScT	tyrosinase from *Streptomyces castaneoglobisporus*
T1	type-1 copper site
T2	type-2 copper site
T3	type-3 copper site
TaLcc1	laccase 1 from *Thielavia arenaria*
TBC	4-*tert*-butylcatechol
ThL	laccase from *Trametes hirsuta*
TrT	tyrosinase from *Trichoderma reesei*
VvCO	catechol oxidase from *Vitis vinifera*
Y	L-tyrosine
YGG	tyrosine-glycine-glycine peptide

1.   Introduction

Tyrosinase (EC 1.14.18.1; monophenol monooxygenase), catechol oxidase (EC 1.10.3.1; 1,2-benzenediol:oxygen oxidoreductase) and laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) are copper-containing metalloenzymes that catalyse the oxidation of substituted phenols and use molecular oxygen as a terminal electron acceptor. Molecular oxygen is reduced to water. Tyrosinases oxidise *p*-substituted monophenols and *p*-substituted *o*-diphenols to the corresponding *o*-quinones. Catechol oxidases catalyse the oxidation of *p*-substituted *o*-diphenols to the corresponding *o*-quinones, but do not oxidise *p*-substituted monophenols. Laccases have a wide range of substrate-specificity, e.g., monophenols, diphenols, aryl amines, aminophenols, and inorganic compounds can be oxidised by laccases. While tyrosinases and catechol oxidases remove an electron pair from the hydroxyl groups of a diphenolic substrate and generate quinones, laccases remove single electrons from the reducing group of the substrate and the products generated are usually free radicals (Sanchez-Amat & Solano 1997) (**Figure 1**).

Tyrosinases, catechol oxidases and laccases are essential oxidoreductases in nature, where they are involved, e.g., in defence mechanisms. Tyrosinases, catechol oxidases and laccases, especially those of plant origin, are sometimes also called polyphenol oxidases (PPOs) (Mayer 1987). This classification is misleading for the reason that it does not differentiate these enzymes on the basis of their distinct enzymatic activities (Flurkey & Inlow 2008). Tyrosinase- and catechol oxidase-formed quinones are the building blocks of melanins, which play a physiological role in defence mechanisms, such as sclerotisation, primary immune response, and host defence. The principal role of fungal laccases is degradation of lignin in synergy with other lignin-modifying enzymes, such as lignin peroxidases and manganese peroxidases (for a review, see Lundell et al. 2010). Laccases are also involved in melanogenesis and are important virulence factors (Nagai et al. 2003).

Laccases are used in industrial applications for bleaching of denim in the textile industry (for a review, see Galante & Formantici 2003). Laccases and tyrosinases of fungal origin have been shown to be potent cross-linking agents, i.e., they can be used to introduce specific covalent bonds between proteins, as well as between proteins and carbohydrates (Selinheimo et al. 2007b). Thus far, catechol oxidases have been less studied also in terms of applications.

...
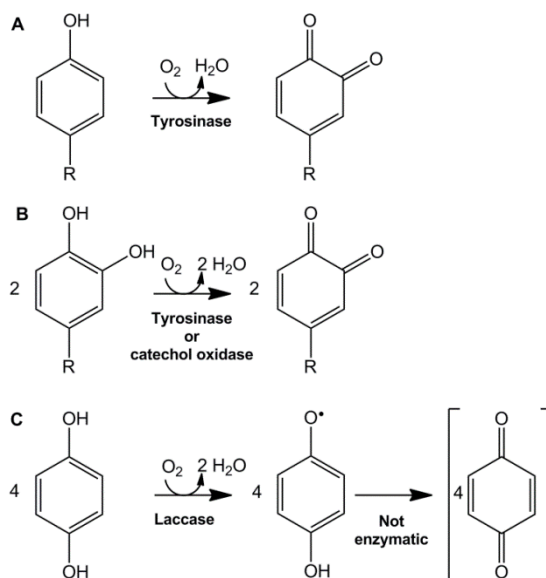
Figure 1. Oxidation of phenolic compounds catalysed by tyrosinase (A and B), catechol oxidase (B), and laccase (C).

1.1 Distribution of tyrosinases, catechol oxidases and laccases

Tyrosinases are scattered among animals, plants, fungi and invertebrates, as well as in prokaryotic and eukaryotic microbes (for a review, see Lerch 1983, Seo et al. 2003, Claus & Decker 2006, Halaouli et al. 2006a, Wang & Hebert 2006). Tyrosinases exist in mammals, including humans (Kwon et al. 1987). While human tyrosinase is a trans-membrane enzyme, tyrosinases of other origins are mainly intracellular enzymes. The first fungal tyrosinase was isolated from the edible mushroom *Agaricus bisporus* (AbT) (Nakamura et al. 1966) and the first bacterial tyrosinase from *Streptomyces glaucescens* (Lerch & Ettinger 1972). Well-characterised intracellular fungal tyrosinases have been reported from the species *Neurospora crassa* (Fling et al. 1963, Lerch 1983, Kupper et al. 1989), *Lentinula edodes* (Kanda et al. 1996), *Aspergillus oryzae* (Nakamura et al. 2000), *Pycnoporus sanguineus* (Halaouli et al. 2005, Halaouli et al. 2006b), and *Thermomicrobium roseum* (Kong et al. 2000). The known extracellular tyrosinases are from the bacteria *Streptomyces michiganensis* (Philipp et al. 1991) and *Streptomyces antibioticus* (Bernan et al. 1985), and from the fungi *Amylomyces rousii* (Montiel et al. 2004) and *Trichoderma reesei* (TrT) (Selinheimo et al. 2006b). Thus far, the tyrosinase from *T. reesei* is the best-characterised extracellular tyrosinase.

The few characterised catechol oxidases are originated from plant and fungi. There are no reports on catechol oxidases of bacterial or mammal origins. All known catechol oxidases are intracellular enzymes. The best-characterised plant catechol oxidases are from *Ipomea batatas* (Eicken et al. 1998), *Populus nigra, Lycopus europaeus* (Rompel et al. 1999a)*,* and *Melissa officinalis* (Rompel et al. 2012). Among fungi, one polyphenol

oxidase from the ascomycete fungus *Alternaria tenuis* has been classified as a catechol oxidase (Motoda 1979a, Motoda 1979b).

Laccases are widely distributed in plants and fungi, and they are also present in some bacteria and insects (for a review, see Hoegger et al. 2006). Unlike tyrosinases, laccases are not found in animals (for a review, see Mayer & Staples 2002). Most of the characterised laccases are of fungal origin; however, the first laccase was found from the tree *Rhus vernicifera* (Yoshida 1883). The majority of the characterised laccases are extracellular enzymes, although intracellular laccases have also been characterised (Froehner & Eriksson 1974, Palmieri et al. 2000, Langfelder et al. 2003, Nagai et al. 2003, McMahon et al. 2007).

1.2 Physiological role of tyrosinases, catechol oxidases and laccases

Tyrosinases and catechol oxidases are the key enzymes in the first reactions of melanogenesis, the formation of melanins. Melanins are involved in many biologically essential functions such as pigmentation, sclerotisation, primary immune response, and host defence. In mammals, melanogenesis is related to the pigmentation of the hair, skin, and eyes. Malfunctions in tyrosinase activity result in hyperpigmentation and albinism in mammals (for a review, see Ito & Wakamatsu 2008). It has been suggested that a tyrosinase activity in bacteria participate in the detoxification of phenolic compounds (Borthakur et al. 1987), and that the formed melanins prevent the dehydration of the cells (Coyne & Al-Harthi 1992).

In plants, the melanins may contribute to the formation of barriers, or be involved in alkylation reactions reducing the bioavailability of proteins, or even in the creation of a toxic environment for the attacker (for a review, see Mayer 1987, Walker & Ferrar 1998). Both tyrosinases and catechol oxidases may contribute to the formation of catechol melanins. Catechol melanins are specific melanins produced in plants, and they are formed by derivatives of catechol quinones. Catechol melanins protect the damaged plant from pathogens and insects (Dervall 1961, Felton et al. 1989, Bi & Felton 1995, Melo et al. 2006). In insects, melanins are involved in the sclerotisation of cuticle, in defence mechanism, and in wound-healing processes (Sugumaran 2002).

In fungi, melanins serve as a mechanism of resistance to stress factors, e.g., UV radiations (Bell & Wheeler 1986), as well as defence against virulence mechanisms (Soler-Rivas et al. 1997, Jacobson 2000). Fungal melanins contribute to cell walls' resistance against hydrolytic enzymes in avoiding cell lysis (Bell & Wheeler 1986). Furthermore, fungal melanins are produced during sexual differentiation (Lerch 1983) and spore formation (Mayer & Harel 1979). It has also been suggested that extracellular tyrosinases play a role in the polymerization and detoxification of plant phenolic compounds in soil environments (Sjoblad & Bollag 1981).

Laccases from plant origin are located in the xylem and play an important role in the early stages of lignin biosynthesis, presumably oxidising monolignols (Bao et al. 1993, O'Malley et al. 1993, Gavnholt & Larsen 2002, Mayer & Staples 2002). Plant laccases have also been shown to be involved in the first steps of healing in wounded leaves (de Marco & Roubelakis-Angelakis 1997). Fungal laccases, in particular from white-rot fungi,

are involved mainly in the degradation of lignin in synergy with other lignin-modifying enzymes, such as lignin peroxidases and manganese peroxidases (for a review, see Hoegger et al. 2006).

More than the two thirds of the known laccase sequences are of fungal origin (for a review, see Valderrama et al. 2003, Hoegger et al. 2006). The characterised laccases from basidiomycete fungi are from *Trametes* species (Coll et al. 1993, Yaver et al. 1996, Yaver et al. 1999, Dedeyan et al. 2000, Bertrand et al. 2002, Galhaup et al. 2002, Klonowska et al. 2002, Xiao et al. 2004), *Pycnoporus cinnabarinus* (Eggert et al. 1996, Sigoillot et al. 1999), *Ceriporiopsis subvermispora* (Karahanian et al. 1998), *Lentinula edodes* (Nagai et al. 2002, Nagai et al. 2003) *Pleurotus ostreatus* (Sannia et al. 1986, Giardina et al. 1999, Garzillo et al. 2001, Palmieri et al. 2003), *Volvariella volvacea* (Chen et al. 2004) *Coprinopsis cinerea* (*Coprinus cinereus*) (Schneider et al. 1999, Yaver et al. 1999)*, Thanatephorus cucumeris* (Wahleithner et al. 1996). The characterised laccases from ascomycete fungi are from *Aspergillus* species (Wood 1980a, Huang et al. 1995)*, Botrytis cinerea* (Marbach et al. 1984, Schouten et al. 2002), *Cryphonectria parasitica* (Rigling & Van Alfen 1993) *Gaeumanomyces graminis* (Litvintseva & Henson 2002) *Melanocarpus albomyces* (Kiiskinen et al. 2002, Kiiskinen et al. 2004), *Chaetomium thermophilum* (Chefetz et al. 1998), *Neurospora crassa* (Froehner & Eriksson 1974, Germann & Lerch 1986) and *Podospora anserina* (Fernandez-Larrea & Stahl 1996). Laccases are produced also in plant-pathogenic fungi, where they play an important role as virulence factors, e.g., the laccase from the grape-vine grey mould *Botrytis cinerea* participates in the infection of a variety of hosts (Bar Nun et al. 1988). Furthermore, a cell wall-localised laccase of *Cryptococcus neoformans* plays a relevant role in the infection of humans (Williamson 1994).

Among bacteria, the laccases from the genus *Streptomyces* are well known. The laccases from *Streptomyces lavendulae* (Suzuki et al. 2003), *Streptomyces cyaneus* (Arias et al. 2003), *Streptomyces coelicolor* (Dubé et al. 2008), and *Streptomyces psammoticus* (Niladevi et al. 2008) have been characterised. Other known bacterial laccases are from *Azospirillum lipoferum* (Faure et al. 1994) and *Bacillus subtilis* (Martins et al. 2002), both involved in pigments production. The role of laccases in insects is related to cuticle sclerotisation (Sugumaran et al. 1992, Dittmer et al. 2004). The distribution and physiological role of tyrosinases, catechol oxidases, and laccases are summarised in **Table 1**.

Table 1. Biochemical and structural properties of tyrosinase, catechol oxidase and laccase.

	Tyrosinase	Catechol oxidase	Laccase	Refer to paragraph
Enzyme classification	EC 1.14.18.1	EC 1.10.3.1	EC 1.10.3.2	
Distribution	Mammals, plants, insects, fungi, bacteria	Plants, insects, fungi, bacteria	Plants, insects, fungi, bacteria	1.1
Physiological role	Pigment formation, would healing, sclerotisation	Pigment formation, would healing, sclerotisation	Lignin degradation, pigment and fruiting body formation, detoxification, plant pathogenesis	1.2
Location in the cell	Mainly intracellular	Mainly intracellular	Mainly extracellular	1.1
Molecular weight	30–50 kDa	30–60 kDa	60–80 kDa (three domain laccases) 30–40 kDa (monomer of two domain laccases)	1.6
Substrates	p-Monophenols, o-diphenols	o-Diphenols	Monophenols, diphenols, aryl amines, amino phenols	1.3 and 1.5
Primary oxidation product	o-Quinone	o-Quinone	Phenoxy radical	1.5
O_2 stoichiometry	1 Monophenol : 1 O_2	2 Diphenol : 1 O_2	4 Phe-OH : 1 O_2 [a]	1.5
Specific inhibitors	Tropolone, salicyl hydroxamic acid, 4-hexyl resorcinol, phenyl hydrazine	Phenylthiourea, N,N-diethylthio-carbamate	Small anions, N-hydroxyl glycine, cetyltrimethylammonium bromide, azide	1.3
Protein family	PF00264	PF00264	PF00394	1.4
Copper centre	T3 [b]	T3 [b]	T1 and trinuclear T2/T3 [b]	1.4
EPR signal	No	No	Yes	1.4
UV/VIS absorption maximum	345 nm (strong); 600 nm (weak) [c]	330 nm (weak)	330 nm (T3 centre); 600 nm (T1 centre)	1.4
Tertiary structure	Monomer, dimer, tetramer	Monomer	Monomer	1.6

[a] Phe-OH is a generic phenolic substrate of laccase.
[b] T1 is a type 1 copper centre; T2 is a type 2 copper centre; T3 is a type 3 copper centre.
[c] UV/VIS absorption maxima of the *oxy*-form of tyrosinase.

1.3 Biochemical properties

1.3.1 Tyrosinases and catechol oxidases

Many tyrosinases have their pH optima in a neutral or acidic range. Only a few tyrosinases have been reported with an optimum in an alkaline pH range: the tyrosinase from *Thermomicrobium roseum* (pH 9.5 on L-DOPA) (Kong et al. 2000), the tyrosinase from *Pinus densiflora* (pH 9–9.5 on L-DOPA) (Kong et al. 1998), and the extracellular tyrosinase from *T. reesei* (pH 9 on L-tyrosine) (Selinheimo et al. 2006b). The known tyrosinases are not very thermostable enzymes and the most commonly known thermostable ones are from *T. roseum* and *P. sanguineus*. *T. roseum* tyrosinase has been shown to retain 100% of its activity on L-DOPA after ten minutes of incubation at 70 °C, but it was rapidly inactivated at temperatures higher than 75 °C (Kong et al. 2000). *P. sanguineus* tyrosinase activity on L-tyrosine was completely inactivated after 20 minutes of incubation at 60 °C (Halaouli et al. 2005). Low temperatures also inactivate tyrosinases, as reported for tyrosinases from *Aspergillus flavipes* (Gukasyan 1999) and *N. crassa* (Fling et al. 1963).

Most of the characterised catechol oxidases are of plant origin and have pH optima in the neutral-basic range. These include catechol oxidases from *I. batatas* (optimum pH 7.8 on catechol) (Eicken et al. 1998), from *P. nigra* (optimum pH 8.0 on catechol) (Rompel et al. 1999a), and from *L. europaeus* (optimum pH 6.5–7.5 on catechol) (Rompel et al. 1999a). In contrast, the characterised catechol oxidase from the ascomycete fungus *A. tenuis* is active in an acidic pH range (4.0–5.8) and has an optimum at pH 4.7 (Motoda 1979a, Motoda 1979b). *A. tenuis* catechol oxidase is not thermostable; it has a half-life of 30 minutes at 44 °C on substrate D-catechin and is inactivated after 30 minutes of incubation at 60 °C (Motoda 1979a, Motoda 1979b).

Tyrosinases and catechol oxidases are not distinguishable from a comparison of their primary sequences, both belong to the same family (PF00264). Substrate-specificity determines the classification of tyrosinase and catechol oxidase activities. While tyrosinases can oxidise a wide range of *p*-substituted mono- and diphenolic compounds, catechol oxidases oxidise various *p*-substituted diphenolic compounds but lack activity on *o*-substituted monophenols (Klabunde et al. 1998, Marusek et al. 2006). L-Tyrosine and L-DOPA, respectively, are the typical monophenolic and diphenolic substrates of tyrosinases and are commonly used to measure tyrosinase activity. In turn, L-DOPA and caffeic acid are the diphenolic substrates mainly used for analysis of the activity of catechol oxidases. It has been reported that *p*-coumaric acid, i.e., the corresponding monophenolic compound of caffeic acid can be oxidised by the tyrosinases from the fungi *P. sanguineus* and *T. reesei*, whereas it cannot be oxidised by the tyrosinase from fungus *A. bisporus* or in general by tyrosinases of plant origin (Halaouli et al. 2005, Selinheimo et al. 2006b, Selinheimo et al. 2007b). Tyrosinases are also capable of oxidising a variety of aromatic amines and *o*-aminophenols (Toussaint & Lerch 1987, Rescigno et al. 1998, Sanjust et al. 2003, Gasowska et al. 2004, Selinheimo et al. 2007b, Muñoz-Muñoz et al. 2011). Tyrosinases can also oxidise larger compounds containing a tyrosyl residue, such as catechins, peptides, and proteins (Selinheimo et al. 2007b, Mattinen et al. 2008a, Mattinen et al. 2008b). Furthermore, some plant catechol oxidases (known also as

polyphenol oxidases) have weak monophenolase activity but cannot oxidise L-tyrosine. It has been suggested that L-tyrosine is a specific substrate for tyrosinase and can be used to distinguish tyrosinases and catechol oxidases (Mayer & Harel 1979, Walker & Ferrar 1998, Gerdemann et al. 2002). The known substrates for tyrosinases and catechol oxidases are summarised in **Table 2**.

Inhibitors of tyrosinases have been extensively studied, because these enzymes are also responsible for undesired reactions, such as browning reactions in fruits and vegetables (for a review, see Seo et al. 2003). The enzymatic browning reactions are started by endogenous tyrosinases, which oxidise the phenolic compounds present in the tissues of fruits, vegetables, and mushrooms. The non-enzymatic reactions consist of Maillard reactions, which are reactions between a nucleophilic amino group of a protein and the carbonyl group of a carbohydrate. Browning reactions cause changes in food products' organoleptic properties and appearance, leading to a short shelf life and a lower market value, so the prevention of browning reactions is highly important (Lertsiri et al. 2003). Antibrowning agents are chemical compounds that decrease the browning reactions. They can be divided into reducing agents and enzyme inhibitors. Reducing agents react with the enzymatic end product, preventing the non-enzymatic reactions that lead to the formation of colourful products. Enzyme inhibitors prevent the primary enzymatic oxidation of a phenolic substrate (for a review, see Seo et al. 2003, Kim & Uyama 2005, Chang 2009). 4-Hexylresorcinol, salicylhydroxamic acid and tropolone are specific inhibitors of tyrosinases, whereas phenylthiourea and *N,N*-diethyldithiocarbamate are specific inhibitors of catechol oxidases (Dawley & Flurkey 1993). Substrate analogues, e.g., cinnamic acids have also been described as having an inhibitory effect on tyrosinases (Lim et al. 1999). Inhibition of tyrosinases has also been reported by small anions, such as azide, cyanide, mercaptoethanol, and carbon dioxide. Small anions bind to the copper ions in the active site (Himmelwright et al. 1980, Beltramini & Lerch 1982).

In humans, the production of anti-tyrosinase antibodies causes vitiligo, an autoimmune disorder that produces a white colour in patches of the skin. Overproduction of tyrosinase in humans, on the other hand, causes melasma and solar lentigines, which represent an aesthetic problem. Skin-whitening agents are chemical substances that reduce the formation of melanins in skin (for a review, see Parvez et al. 2006, Draelos 2007, Chang 2009). Skin-whitening agents react with the tyrosinase-formed quinones reducing them to colourless compounds. Thus far, the skin-whitening agents most often used in cosmetic preparations have been aloesin and arbutin, which are extracted from the plant *Aloe vera* (Yagi et al. 1987). Also, kojic acid, derived from various fungal species, such as those in the *Aspergillus* and *Penicillium* spp. (Parrish et al. 1966), is used in cosmetic and medical preparations as a skin-whitening agent (Masse et al. 2001). Hydroquinone is a strong skin-whitening agent with high melanocyte-specific cytotoxicity. While it is used in depigmenting medication, the use of hydroquinone in cosmetic preparations has been discouraged because of its side effects, including skin irritation (Breathnach 1996).

1.3.2 Laccases

Thermotolerant and thermostable laccases have been recently reviewed by Hildén et al. (2009). The most thermostable laccases are of bacterial origin, e.g., the half-life of laccase from *Streptomyces lavendulae* is 100 minutes at 70 °C (activity on catechol)
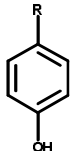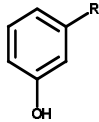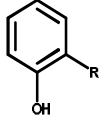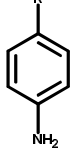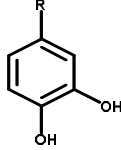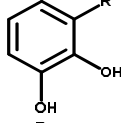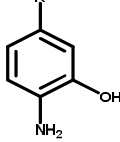
(Suzuki et al. 2003) and that of *Bacillus subtilis* CoTA is 112 minutes at 80 °C (activity on 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS)) (Martins et al. 2002). Fungal laccases are generally stable enzymes in the temperature range 30–50 °C, whereas plant laccases are less thermostable. Among laccases of fungal origin, a laccase from *Agaricus bisporus* has a half-life of ten minutes at 70 °C on *p*-phenylenediamine (Wood 1980a), a laccase from *Trametes sanguinea* is stable for only ten minutes at 70 °C on substrate N,N-dimethyl-*p*-phenylenediamine (Nishizawa et al. 1995), and a laccase from *Coprinopsis cinerea* has a half-life of 4.6 minutes at 70 °C on substrate syringaldazine (Schneider et al. 1999).

Laccases with temperature optima below 35 °C have also been reported: for example, a laccase from *Ganoderma lucidum* has the highest short-time activity at 25 °C on *o*-tolidine (Ko et al. 2001). In general, fungal laccases are more stable at acidic than at alkaline pH (Bollag & Leonowicz 1984), although exceptions exist (Mayer 1987, Baldrian 2006). Laccases generally have a bell-shaped pH activity profiles for phenolic substrates, and optima in the pH range 4–6 (Hoffmann & Esser 1977, Palmieri et al. 1993, Eggert et al. 1996, Xu 1997, Chefetz et al. 1998, Schneider et al. 1999, Garzillo et al. 2001). At alkaline pH values the activity of laccase is negatively affected by the hydroxide ion inhibition, but positively affected by the lower redox potential of the substrates at higher pH values (Xu 1997).
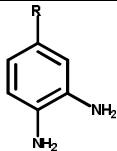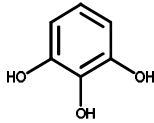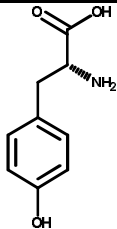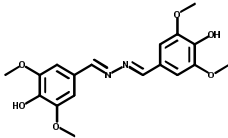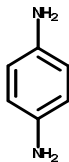
ABTS, guaiacol and 2,6-dimethoxyphenol (2,6-DMP) are substrates generally used to assay a laccase activities. Syringaldazine, tolidine, and *p*-phenylene diamine are considered specific substrates for laccases (Flurkey et al. 1995, Ramirez et al. 2004, Baldrian 2006), whereas L-tyrosine and *p*-cresol are typical substrates of tyrosinases (Käärik 1965). It has, however, been shown also that fungal laccases can oxidise L-tyrosine with a radical mechanism, nevertheless, the affinity and the activity of laccases on L-tyrosine is very low (Selinheimo et al. 2007a). The known substrates for fungal laccases are summarised in **Table 2**.

Small anions, such as azide, cyanide and fluoride ions are the most effective inhibitors of laccases: they bind to the trinuclear copper centre, preventing the binding of molecular oxygen (Solomon et al. 1996, Xu 1996a, Battistuzzi et al. 2003, Johnson et al. 2003). N-hydroxyglycine and cetyltrimethylammonium bromide are more specific inhibitors of laccases (Dawley & Flurkey 1993). EDTA, fatty acids, kojic acid, and coumaric acid are also laccase inhibitors, but they are less effective than small anions (Wood 1980b, Bollag & Leonowicz 1984, Faure et al. 1995, Eggert et al. 1996, Chefetz et al. 1998, Sethuraman et al. 1999, Xu et al. 1999, Jung et al. 2002).

Table 2. Summary of the known substrates of tyrosinase, catechol oxidase and laccase oxidative enzymes. The activity is reported with a plus sign (+) when an activity for the substrate has been reported, with a minus sign (-) when the enzyme is not active on the substrate, or with n.r. for "not reported" when there is no information available from literature. Relevant references are listed in the footnotes [a–c].

Substrate	Structure	Activity		
		Tyrosinase [a]	Catechol oxidase [b]	Laccase [c]
Monophenols				
p-Substituted phenols		+	-	+ (Not tyrosine) [d]
m-Substituted phenols		-	-	+
o-Substituted phenols		-	+	+
Aromatic monoamines		+	-	+
Diphenols				
p-Substituted *o*-diphenols		+	+	+
m-Substituted *o*-diphenols		-	-	+
p-Substituted *o*-aminophenols		+	n.r.	+

23

Substrate	Structure	Activity		
		Tyrosinase [a]	Catechol oxidase [b]	Laccase [c]
p-Substituted o-diamines		+	n.r.	+
Polyphenols				
Pyrogallol		+	+	+
Other polyphenols		+	+	+
Selective substrates				
L-tyrosine		+	-	-[d]
Syringaldazine		-	-	+
p-Phenylene diamine		-	-	+

[a] Espín et al. 1998, Espín et al. 2000, Rodríguez-López et al. 2000, Fenoll et al. 2002, Selinheimo et al. 2006b, Xie et al. 2007.

[b] Mayer & Harel 1979, Motoda 1979a, Motoda 1979b, Eicken et al. 1998, Walker & Ferrar 1998, Rompel et al. 1999b.

[c] Flurkey et al. 1995, Xu 1996a, Xu et al. 1996, Ramirez et al. 2004, Baldrian 2006.

[d] Oxidation of L-tyrosine by *Trametes hirsuta* laccase has been reported by Selinheimo et al. (2007a).

1.4 Active sites of copper-containing enzymes

The copper ions at the active sites of tyrosinases, catechol oxidases, and laccases are co-ordinated by His residues. Definition of the active sites is based on the co-ordination of

the copper ions (for a review, see Solomon et al. 1996, Solomon et al. 2001). Tyrosinase and catechol oxidase belong to the same family (PF00264) and contain a pair of copper ions, i.e., a type 3 (T3) copper site. A T3 copper site contains a pair of two antiferromagnetically coupled copper ions named CuA and CuB, and therefore the site is EPR-silent. Each copper ion is co-ordinated by three His residues. The T3 copper site shows an absorption maximum at a wavelength of 330–345 nm (Jolley et al. 1974). A T3 copper site is also found in haemocyanins, which are oxygen carrier proteins found in molluscs and arthropods (Cuff et al. 1998). Laccases (family PF00394) are multicopper oxidases and they contain four copper ions arranged as a type 1 (T1) copper site and a trinuclear centre formed by one T3 copper site and one type 2 (T2) copper site. The T1 copper site of laccases contains one copper ion co-ordinated by two His residues and one Cys residue. The T1 copper site has a strong absorption at 600 nm due to the $S(Cys)\pi\rightarrow Cu_{dx^2-y^2}$ transition, which also creates the blue colour observed in the oxidised state. The T1 copper site also shows a weak absorption at the wavelengths of 400-485 nm caused by the $S(Cys)\sigma\rightarrow Cu_{dx^2-y^2}$ transition. The T1 copper site in laccases is 13 Å from the trinuclear copper centre. The copper ions in the trinuclear copper centre are co-ordinated by eight His residues, of which six co-ordinate the T3 copper ions and two co-ordinate the copper ion of the T2 copper site in connection with one solvent molecule (e.g., water in *Trametes versicolor* laccase) (Bertrand et al. 2002). Both T1 and T2 copper sites are EPR-detectable.

Proteins containing only a T1 copper site are also known. These proteins, called cupredoxins, are electron transfer proteins. The copper ion in the T1 centre of cupredoxins is co-ordinated by two His residues and one Cys residues and by a weaker axial ligand, usually a Met residue (for a review, see Choi & Davidson 2011). Similarly, also enzymes containing only one T2 copper site are known, i.e., galactose oxidase (EC 1.1.3.9), primary-amine oxidase (EC 1.4.3.21) and diamine oxidase (EC 1.4.3.22). Galactose oxidase catalyses the two-electron oxidation of D-galactose to D-galacto-hexadialose with the concomitant two-electron reduction of oxygen to hydrogen peroxide. In galactose oxidase, the copper ion is co-ordinated by four equatorial ligands (two His residues, one Tyr residue, and one water) and one axial ligand (one Tyr residue). The axial Tyr residue is covalently linked to a Cys residue (Lee et al. 2008, Rokhsana et al. 2012). This Tyr-Cys cross-link participates in the reaction functioning as a second redox centre in the active state of the enzyme (for a review, see Rogers & Dooley 2003). Primary-amine oxidases and diamine oxidases catalyse the deamination of various amines to aldehydes with the concomitant two-electron reduction of oxygen to hydrogen peroxide. The copper ion is co-ordinated by three equatorial His ligands and one modified Tyr residue, i.e., 2,4,5-trihydroxyphenylalanine quinone (TOPA-quinone) (Parsons et al. 1995). Structures of the enzyme copper sites are shown in **Figure 2**.
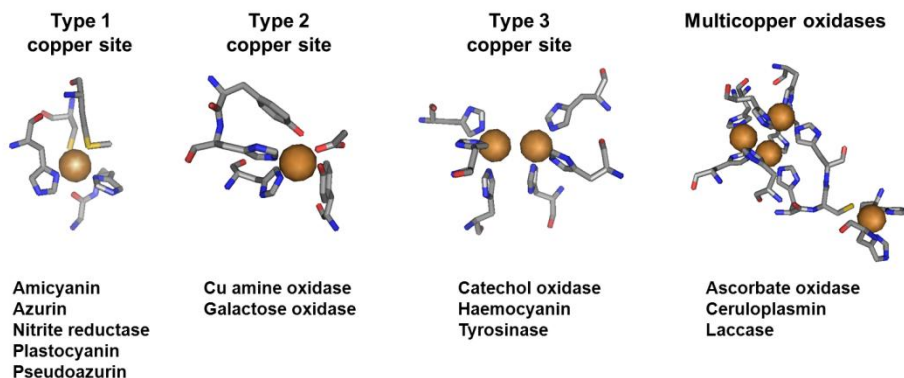
Figure 2. The structure of copper sites and examples of proteins containing these copper sites. Copper ions are illustrated in brown spheres. The figure was made using PyMOL (The PyMOL Molecular Graphics System, Educational Version Schrödinger, LLC) with PDB IDs 1PLC (plastocyanin), 1GOF (galactose oxidase), 1WX2 (tyrosinase), and 1GW0 (laccase).

1.5 Reaction mechanisms

1.5.1 Tyrosinases and catechol oxidases

The active sites of tyrosinases and catechol oxidase exist in different forms during the catalytic cycle: two oxidised forms *oxy*-form ([Cu(II)-O_2^{2}-Cu(II)]) and *met*-form ([Cu(II) - OH^--Cu(II)]), and a reduced unstable *deoxy*-form ([Cu(I) Cu(I)]) (Klabunde et al. 1998, Matoba et al. 2006).

The *met*- and *oxy*-form of tyrosinase and catechol oxidase have different spectroscopic features. While the *met*-form shows a weak absorption maximum at around 330–350 nm, the *oxy*-form shows a strong absorption at 345–350 nm and weaker absorption at 600 nm (Jolley et al. 1974, Himmelwright et al. 1980, Rompel et al. 1999a). The *deoxy*-form is not characterised as well as the *met*- and *oxy*-form, because it is instable and rapidly binds molecular oxygen to generate the *oxy*-form. The *oxy*-form also shows two signals in Raman resonance spectroscopy, but the two signals are not detectable in the *met*-form of the enzyme. Early studies on the tyrosinase from *Agaricus bisporus* showed different circular dichroism (CD) spectra for the *oxy*- and *met*-form (Duckworth & Coleman 1970, Schoot Uiterkamp et al. 1976). All three forms are EPR-silent (Himmelwright et al. 1980, Wilcox et al. 1985). The *met*-form of tyrosinase or catechol oxidase can be transformed into the corresponding *oxy*-form of the enzyme by addition of hydrogen peroxide (Jolley et al. 1974, Rompel et al. 1999a, Rompel et al. 2012). Furthermore, the *met*-form can be partially reduced to *half-met*-tyrosinase [Cu(II) Cu(I)], which is EPR-detectable (Himmelwright et al. 1980, van Gastel et al. 2000, Bubacco et al. 2003). The spectroscopic characteristics of T3 copper enzymes are summarised in **Table 3**.

Table 3. Spectroscopic features of the oxidised and reduced forms of type-3 (T3) copper enzymes.

Property of the active site	oxy-form	met-form	deoxy-form	References
Oxidation state of the copper ions	$[Cu(II)–O_2^{2-}Cu(II)]$	$[Cu(II)-OH^--Cu(II)]$	$[Cu(I)\ Cu(I)]$	Himmelwright et al. 1980
Bridging molecule between the copper ions	H_2O_2	H_2O	H_2O (unstable)	Tepper 2005
Signal in EPR spectroscopy	No	No	No	Himmelwright et al. 1980, Wilcox et al. 1985
Signal in UV/VIS absorption spectroscopy	345 nm (strong) 600 nm (weak)	330–340 nm (weak)	n.d.	Jolley et al. 1974, Hepp et al. 1979
Signal in circular dichroism spectroscopy	260 nm 350 nm	270 nm 290 nm	n.d.	Duckworth & Coleman 1970, Schoot Uiterkamp et al. 1976
Signal in emission fluorescence spectroscopy	330 nm	330 nm	330 nm	Beltramini & Lerch 1982
Signal in Raman spectroscopy	$274\ cm^{-1}$ $755\ cm^{-1}$	n.d.	n.d.	Eickman et al. 1978, Rompel et al. 1999a

n.d. = not detected

Both the *oxy-* and the *met-* form of the tyrosinase oxidise diphenols (diphenolase catalytic cycle). In contrast, the oxidation of monophenols requires the presence of the *oxy-*form of the enzyme (monophenolase or cresolase cycle). One of the atoms of a molecular oxygen molecule that is bound to the *oxy-*form is incorporated in the *o-*position of the monophenolic substrate during the monophenolase cycle (Itoh et al. 2001). The product of tyrosinase-catalysed oxidation of monophenolic or diphenolic substrate is an *o-*quinone. After the *o-*quinone is released, the enzyme is left in the reduced *deoxy-*form. Molecular oxygen has high affinity to the *deoxy-*form and it rapidly binds to the active site, generating the *oxy-*form of tyrosinase (**Figure 3**).

The native form of a tyrosinase typically consists of about 85–90% *met-*form, with the rest being in the *oxy-*form (for a review, see Solomon et al. 1996). When monophenolic substrates are oxidised by tyrosinase, a lag period is usually detected. The lag is related to the state of the active site of the tyrosinase. During the lag period, the enzyme is transformed from the *met-*form to the oxy-form, which can oxidise the monophenolic substrate. The met-form oxidises the diphenolic substrate (e.g., L-DOPA) which is formed because of the non-enzymatic reactions.

Tyrosinases can oxidise a variety of *p-*substituted monophenols and diphenols other than L-tyrosine and L-DOPA. The affinity of a substrate to a tyrosinase strongly depends on the hydrophobic, steric, and electrostatic properties of the group in the *p-*position of the *o-*diphenol. In has been shown that when the diphenolic substrate has an electron-donating group in the *p-*position of the *o-*diphenol, the reaction mechanism will follow the diphenolase pathway. In contrast, when the substrate has a withdrawing-group in the *p-*position of the *o-*diphenol, the reaction mechanism leads the enzyme to a suicide inactivation pathway. In the suicide inactivation pathway, the tyrosinase is irreversibly inactivated by reduction and loss of one of the copper ion from the T3 copper site. Furthermore, certain monophenols can bind to the *met-*form of the enzyme but cannot be oxidised; this leads the enzyme into a dead-end pathway (for a recent review, see Muñoz-Muñoz et al. 2010b) (**Figure 3**).
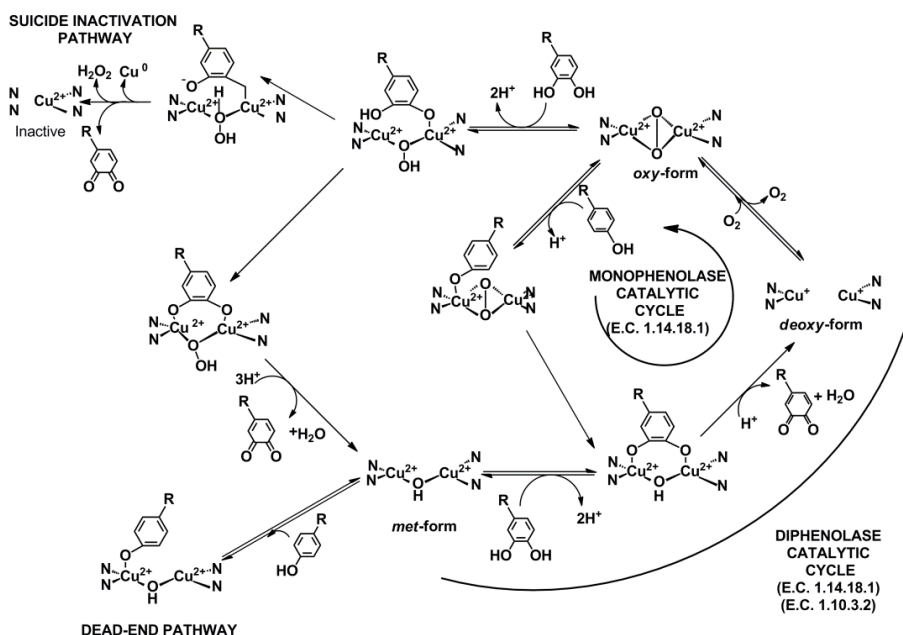
Figure 3. Molecular mechanism for the catalytic cycles of tyrosinase (EC 1.14.18.1) and catechol oxidase (EC 1.10.3.1). The monophenolase catalytic cycle occurs only for tyrosinase. The dead-end and suicide inactivation pathways are also represented (Figure modified from the review articles Kim & Uyama 2005, Muñoz-Muñoz et al. 2010a).

Catechol oxidases catalyse only the oxidation of *p*-substituted diphenols to quinones; but they do not catalyse the hydroxylation of *p*-substituted monophenols, so catechol oxidases cannot complete the monophenolase catalytic cycle (**Figure 3**) (Mayer & Harel 1979, Walker & Ferrar 1998, Gerdemann et al. 2002). Why a catechol oxidase does not show a monophenolase activity is not clearly understood. From the comparison of the three-dimensional structures of tyrosinase and catechol oxidase (see Subsection 1.6.1), it has been shown that the access to the active site of catechol oxidase is partly blocked by a Phe residue (i.e., Phe261 in *I. batatas* catechol oxidase). Furthermore, the corresponding Glu182 residue in *Streptomyces castaneoglobisporus* tyrosinase is smaller; therefore, the active site is more exposed in tyrosinase than in catechol oxidase. The Glu182 residue has been proposed as a putative residue in the orientation of the monophenolic substrate to the active site of tyrosinase (Decker et al. 2006). Thus far, the information on catechol oxidases has been limited, because most of the research has focused on tyrosinases, which appear more attractive also on account of the monophenolase activity.

In view of the three-dimensional structures of tyrosinases (see Subsection 1.6.1), different binding mechanisms of the *oxy*-form of tyrosinase have been proposed for monophenols (Decker et al. 2006, Matoba et al. 2006, Land et al. 2007, Ismaya et al. 2011, Sendovski et al. 2011). Based on the three-dimensional structure of *Streptomyces castaneoglobisporus* tyrosinase in complex with the caddie protein ORF378, Matoba et

al. (2006) proposed that the monophenolic substrate is oriented towards the second copper ion at the active site (CuB), in a similar way to the Tyr98 of the caddie protein. Divergently, Decker et al. (2006) suggested that one His co-ordinating the CuB orients the monophenolic substrate towards the first copper at the active site (CuA) via a hydrophobic interaction and that the hydroxyl group of the substrate binds to the CuA. The binding mechanism proposed by Decker et al. (2006) is supported by Olivares and Solano (2009) and Sendovski et al. (2011). On the basis of a directed-evolution study, Sendovski et al. (2011) suggested that monophenolic substrates bind to the CuA, in agreement with Decker et al. (2006), whereas diphenolic substrates bind to the CuB. In the mutant R209H of the tyrosinase from *Bacillus megaterium* (BmT), the newly introduced His residue blocks the entrance to the active site, interfering with the binding of L-DOPA to CuB. The activity of the mutant R209H of BmT is lower than the activity of the wild-type BmT on L-DOPA 1 mM, by a factor of 1.6 (Shuster Ben-Yosef et al. 2010, Sendovski et al. 2011).

L-tyrosine is the natural substrate of tyrosinase. The *o*-quinones generated by the tyrosinase-catalysed oxidation of L-tyrosine undergo a series of non-enzymatic oxidative and coupling reactions to form hetero-polymers. These reactions are called melanogenesis. Melanogenesis occurs in mammals, fungi and bacteria. In all cases tyrosinase catalyses the first step of this pathway, i.e., the formation of dopaquinone (*o*-quinone of L-DOPA) (Prota 1988). Dopaquinone is the product of a tyrosinase-catalysed oxidation of L-tyrosine and L-DOPA (Cooksey et al. 1997). The tyrosinase-catalysed step is the rate-limiting step in melanogenesis. The reactions following dopaquinone formation can proceed spontaneously at physiological pH (Halaban et al. 2002). The amount of melanins formed is proportional to the production of dopaquinone, and, thereby, to the tyrosinase activity. Melanins can be grouped into eumelanins and pheomelanins. Eumelanins are insoluble brown to black pigments and work as photoprotective antioxidants. Pheomelanins are alkali-soluble yellow to reddish-brown pigments and work as phototoxic pro-oxidants (Chedekel et al. 1980, De Leeuw et al. 2001, Takeuchi et al. 2004, Samokhvalov et al. 2005). Both eumelanins and pheomelanins are produced in melanogenesis and together they form the mixed melanins (**Figure 4**).

The eumelanin pathway occurs in the absence of sulphydryl compounds and can be divided into two phases. The first is a proximal phase in which dopachrome is the end product. The second one is a distal phase, that represents the enzymatic and non-enzymatic reactions occurring from dopachrome to eumelanins (for a review, see Cooksey et al. 1997, Kim & Uyama 2005, Ito & Wakamatsu 2008). In the proximal phase dopaquinone undergoes the intramolecular addition of the amino group to produce leukodopachrome (known also as cyclodopa). A fast redox reaction between leukodopachrome and dopaquinone forms L-DOPA and dopachrome, which is an orange-red unstable compound (Raper 1927, Mason 1948, Canovas et al. 1982, Rodríguez-López et al. 1991). At neutral pH, dopachrome is gradually decarboxylated to 5,6-dihydroxyindole (DHI) (Raper 1927, Palumbo et al. 1987). In mammals, the dopachrome tautomerase activity converts dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Raper 1927, Palumbo et al. 1987, Aroca et al. 1990, Tsukamoto et al. 1992). DHI and DHICA are the main components of eumelanins (Swan & Waggott 1970, Swan 1974). The pheomelanin pathway occurs in the presence of sulphydryl compounds (e.g., Cys), where a nucleophilic attack of the sulphydroxyl group of a Cys on

the benzene ring of dopaquinone occurs and cysteinyldopa isomers are generated. The main isomer formed is 5-*S*-cysteinyldopa (5-*S*-CD), which is considered to be the precursor of the pheomelanins (Prota 1980).
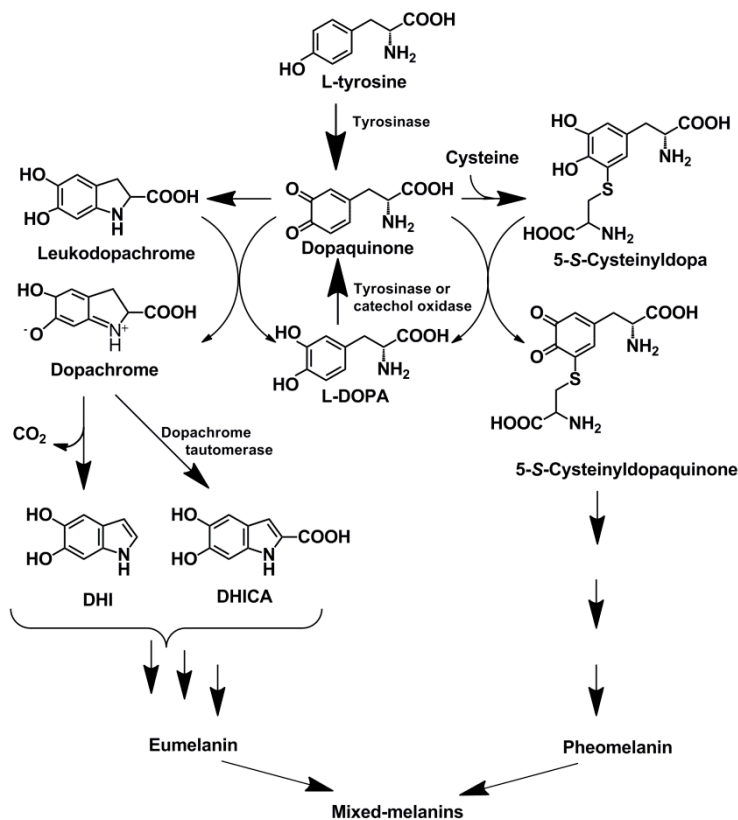
Figure 4. Early stages of the biosynthetic pathways leading to melanin production. 5,6-Dihydroxyindole (DHI) is formed by a spontaneous decarboxylation of dopachrome and 5,6,-dihydroxyindole-2-carboxylic acid (DHICA) by a dopachrome tautomerase activity (Figure modified from the review article Ito & Wakamatsu 2008).

1.5.2 Laccases

Substrates for laccases include various compounds, such as diphenols, substituted phenols, diamines, aromatic amines, benzenethiols, and even inorganic compounds (Xu 1996a). Laccase catalyses the oxidation of these compounds with a radical mechanism. In one catalytic cycle, four electrons in all are removed from the substrate(s) and molecular oxygen is reduced to two molecules of water. In the resting form of laccase, the four copper ions are in an oxidised state. The substrate binds in a pocket close to the T1 copper site of laccase. A single electron is removed from the reducing group of the substrate molecule(s), e.g., the hydroxyl group of a phenolic substrate at the T1 copper

site. The electron is transferred to the trinuclear copper centre through a conserved His-Cys-His tripeptide (Messerschmidt et al. 1992, Bertrand et al. 2002, Piontek et al. 2002). The four-electron reduction of molecular oxygen to water takes place in the trinuclear copper centre (for a review, see Solomon et al. 1996, Solomon et al. 2001, Rodgers et al. 2009, Lundell et al. 2010). A peroxy intermediate is involved in the reduction of molecular oxygen to water. In the absence of reducing substrates (e.g., Phe-OH), the native intermediate slowly transforms into the resting oxidised form of laccase. On the contrary, in the case reducing substrates are available, the native intermediate can be fully reduced in concomitance with the oxidation of the substrates. Radiolabeling studies have shown that at the end of the catalytic cycle one of the oxygen atoms of molecular oxygen is bound as hydroxyl to the T2 copper site (Brändén & Deinum 1977, Brändén et al. 1978, Shin et al. 1996). A schematic representation of the catalytic cycle of laccases is shown in **Figure 5**.

From the three-dimensional structure of the multicopper oxidase ascorbate oxidase, it has been shown that access of the solvents to the trinuclear copper site is provided by a broad and a narrow channel (Messerschmidt et al. 1992). The same channels were found in the laccase from the basidiomycete fungus *Trametes versicolor* (Piontek et al. 2002). Differently, in the laccase from the ascomycete fungus *Melanocarpus albomyces,* only the narrow channel is found and the broad channel is occluded by the four conserved residues of the C-terminus (Asp-Ser-Gly-Leu/Val/Ile known as the C-terminal plug). Accordingly, the entrance of molecular oxygen and the exit of water may not occur via this tunnel in *M. albomyces* laccase. Furthermore, in the laccase from *M. albomyces,* the protons are also transferred from the T1 copper site to the trinuclear copper site via a triangle of amino acids (Ser, Ser and Asp), called a SDS-gate. The SDS-gate is conserved among the sequences of laccases from ascomycete fungi, but is not found in laccases of other origin, such as the laccase from *T. versicolor*, *Rigidoporus lignosus,* and *Bacillus subtilis* (Hakulinen et al. 2008).

Besides the affinity of the substrate to the active site of laccase, the laccase-catalysed reactions also depend on the redox potential of the substrate. The redox potential (E^0) of the substrate must be low enough, since the reaction depends on the difference between the redox potentials of the enzyme and the substrate (ΔE^0). The rate of laccase-catalysed oxidation of a phenolic substrate is expected to increase as the ΔE^0 increases. Furthermore, the E^0 of a phenolic substrate decreases with an increase of a pH value, thus the ΔE^0 increases. Hydroxyl ions inhibit laccases, and at high pH values the dissociation of the hydroxyl group of the phenolic substrate is more favourable; therefore inhibition of the laccase may occur (Xu 1996a, Xu 1997, Xu et al. 2000, Xu et al. 2001). The E^0 of laccases refers to T1 copper. Differently from the phenolic substrate, the E^0 of the T1 copper is not pH-dependent. The redox potential of fungal laccases is in the range of 0.4–0.8 V (Morpurgo et al. 1980, Xu 1996a, Palmer et al. 1999, Xu et al. 1999).
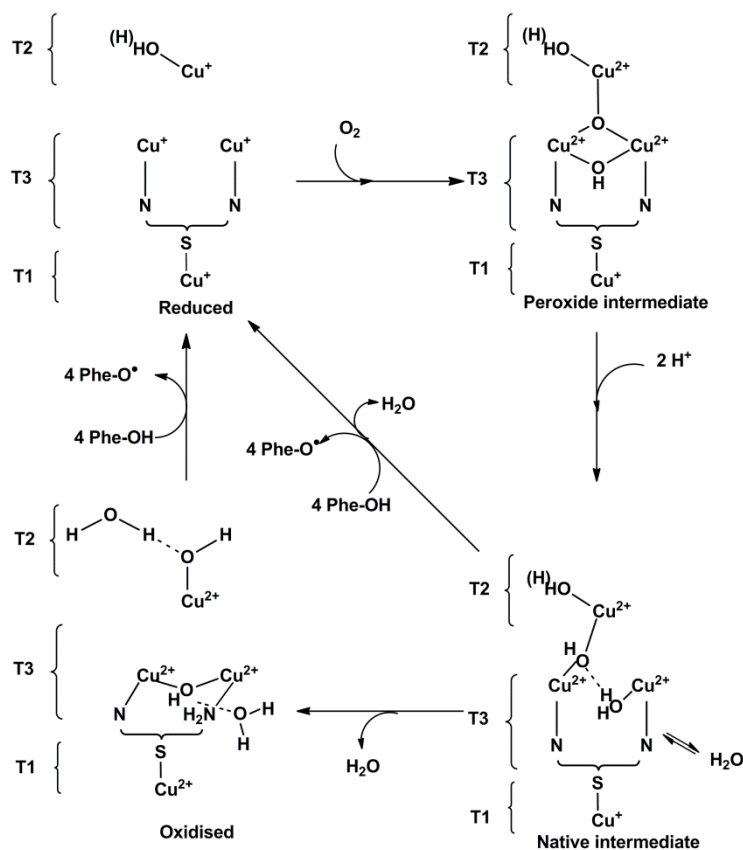
Figure 5. Catalytic cycle of laccase. Phe-OH is the phenolic substrate compound. Phe-O$^\bullet$ is the reactive phenoxy radical. T1 is the type-1 copper centre, T2 is the type-2 copper centre, and T3 is the type-3 copper centre of laccase. (Figure modified from the review articles Solomon et al. 2001, Lee et al. 2002, Yoon et al. 2007)

1.6 Structures

1.6.1 Tyrosinases and catechol oxidases

The primary structures of tyrosinase and catechol oxidase are typically formed by three domains: an N-terminal domain, a central catalytic domain, and a C-terminal domain. The central catalytic domain contains the conserved His residues, which co-ordinate the two copper ions of the T3 copper site. Tyrosinases of animal and plant origins have all three domains, whereas tyrosinases of fungal origin lack the N-terminal domain. Tyrosinases of bacterial origin only have the central catalytic domain, but they are produced in association with a caddie protein that acts as a C-terminal domain. The N-terminal domain directs the protein to the chloroplast thylakoid lumen in plants and is involved in the maturation process of the tyrosinase. The role of the C-terminal domain is to cover

the active site of tyrosinase, keeping the enzyme inactive in the secretory pathway. Tyrosinases are usually activated by cleavage of the N- and C-terminal domains, or in the case of bacterial tyrosinases, by removal of the caddie protein (Marusek et al. 2006, Flurkey & Inlow 2008).

Until now, there have been three crystal structures of tyrosinases available, from *Streptomyces castaneoglobisporus* (ScT) (Matoba et al. 2006), from *Bacillus megaterium* (BmT) (Sendovski et al. 2011), and from *A. bisporus* (AbT) (Ismaya et al. 2011) (**Figure 6**), and two crystal structures of plant catechol oxidases, from *I. batatas* (IbCO) (Klabunde et al. 1998), and from *Vitis vinifera* (VvCO) (Virador et al. 2009) (**Figure 7**). The PDB IDs of the structures of tyrosinases and catechol oxidases are shown in **Table 4**.

The three-dimensional structure of tyrosinase is mainly α-helical. Two β-strands located in the N- and C-terminus of the central catalytic domain form a short parallel-sheet structure. The residues forming this short parallel-sheet structure are highly conserved and mark the beginning and the end of the central catalytic domain. The core of the enzyme is formed by a four-helix bundle where the catalytic dinuclear copper centre is located. The active site is lodged in the bottom of a large concavity, where the substrate is bound (Matoba et al. 2006, Ismaya et al. 2011, Sendovski et al. 2011).

The first three-dimensional structure presented for a tyrosinase is from *S. castaneoglobisporus* (ScT) (Matoba et al. 2006). The ScT three-dimensional structure was solved as a complex with a caddie protein, the dimensions of the heterodimer are 40 x 55 x 60 Å. The caddie protein is involved in the copper assembly, and in the secretion and activation of the enzyme. A Tyr residue (i.e., Tyr98) from the caddie protein occupies the putative substrate-binding site of ScT, but it is at a 3.0 Å distance from the active site, so too far away to be oxidised. The structure of the bacterial tyrosinase from BmT was solved with a resolution of 2.0–2.3 Å (Sendovski et al. 2011). BmT is a homodimer, the dimensions of each unit are 45 x 25 x 80 Å. Each unit contains an active site, with the two substrate-binding sites facing alternate directions. The structure of AbT was also very recently solved (Ismaya et al. 2011). This is the first structure revealed for a full-length tyrosinase of fungal origin. The enzyme is a tetramer composed of two heavy (H) chains and two light (L) chains, the dimensions of the tetramer are 139 x 97 x 59 Å. The active site is located in the H subunit, where it is lodged in the four-helix bundle as in ScT and BmT. The substrate-binding site is found at the bottom of a spacious cavity in the surface of the H subunit. The L chain has a β-trefoil fold, consisting of 12 antiparallel β-strands. The L chain is about 25 Å away from the active site and it does not hinder access to the active site. The L chain does not show any sequence or structure similarity to the caddie protein of ScT. The functional role of the L chain is not yet clear; it is possible that it plays a role in the folding, copper-binding, and stability of AbT *in vivo* (**Figure 6**) (Ismaya et al. 2011).
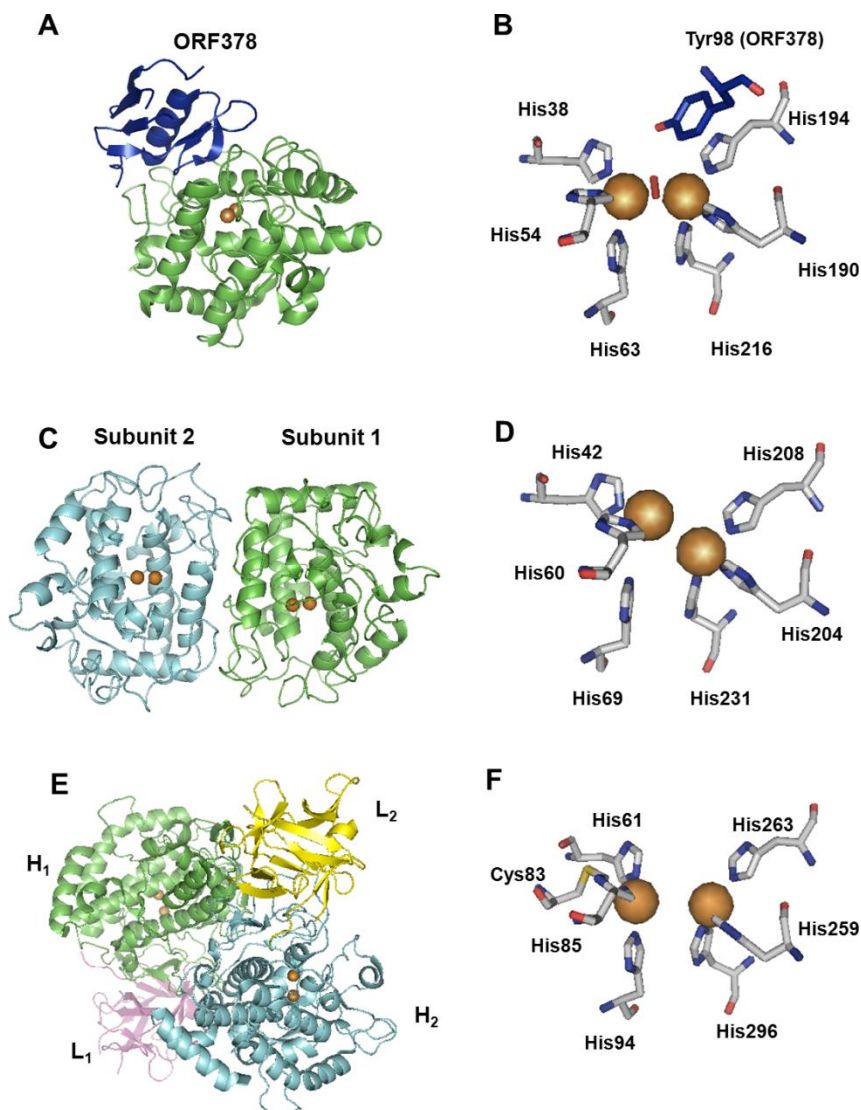
Figure 6. (A) Three-dimensional structure of *Streptomyces castaneoglobisporus* tyrosinase (PDB ID 1WX2) in complex with the caddie protein ORF378. The tyrosinase is in green and the caddie protein ORF378 in blue. (B) The active centre of *S. castaneoglobisporus* tyrosinase. (C) Homodimeric structure of *Bacillus megaterium* tyrosinase (PDB ID 3NM8). Subunit 1 and subunit 2 are shown in green and light blue, respectively. (D) Active centre of *B. megaterium* tyrosinase. (E) Tetrameric structure of *Agaricus bisporus* tyrosinase (PDB ID 2Y9W). Chains are shown as H (heavy chain) and L (light chain). (F) Active centre of *A. bisporus* tyrosinase. The copper ions in the active centre are shown with brown spheres, the bridging molecule in red and the thioether bond in yellow. The figure was created with PyMOL (The PyMOL Molecular Graphics System, Educational Version Schrödinger, LLC).

The first solving of a three-dimensional structure of a catechol oxidase from *I. batatas* (IbCO) was in 1998 (Klabunde et al. 1998) (**Figure 7**). IbCO is a monomer with dimensions 55 x 45 x 45 Å. The overall structure of IbCO is similar to that of tyrosinase, i.e., IbCO is mainly α-helical, and the short-parallel sheet structure formed by two β-strands located in the N-terminus and C-terminus of the central catalytic domain is also present. The active site of IbCO is found in the four-helix bundle as in ScT, but, differently from ScT, it is partially covered by a gatekeeper residue (i.e., Phe261 in IbCO), which shields the CuA and prevents the docking of a monophenolic substrate to the active site of catechol oxidase. IbCO contains a thioether bond between the Cε carbon of His109 (i.e., one copper ligand) and the sulphur of a Cys92 (Klabunde et al. 1998). The thioether bond was not found in ScT, and it has been suggested that the His54 of ScT co-ordinating CuB was flexible during catalysis, whereas in IbCO the flexibility of the corresponding residue His109 is restricted by the thioether bond. More recently, the thioether bond was also found in the structures of AbT (Ismaya et al. 2011) and VvCO (Virador et al. 2009). The Cys residue forming the thioether bond is also found in the sequence of the fungal tyrosinase from *N. crassa* (Lerch 1982). Hence, the presence or absence of the thioether bond and the flexibility of the His co-ordinating the CuB are not enough for elucidation of the different substrate-specificity of tyrosinases and catechol oxidases.
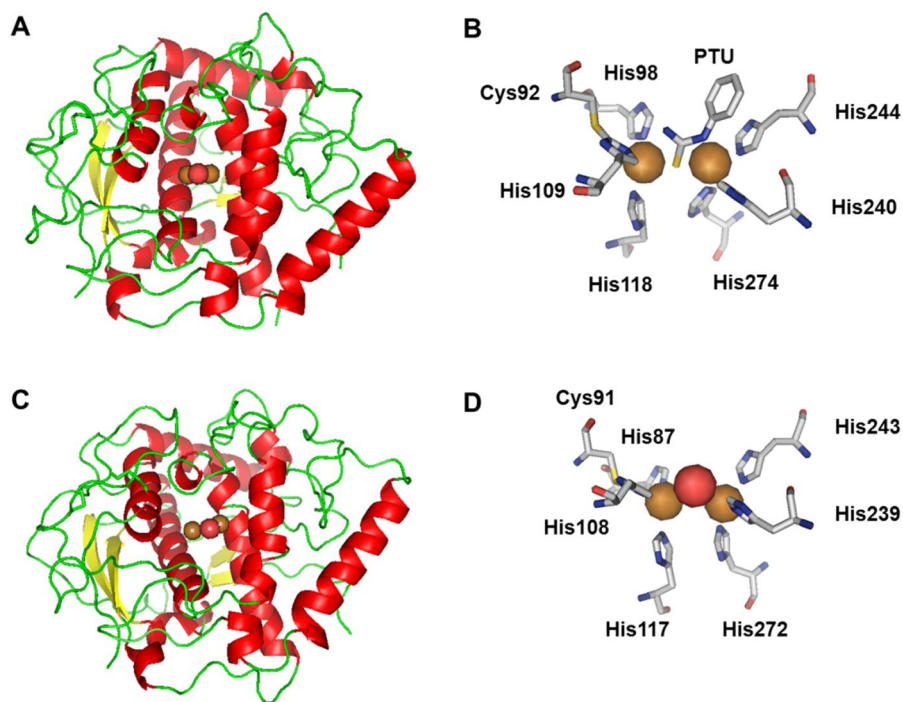
Figure 7. (A) Three-dimensional structure of *Ipomea batatas* catechol oxidase (PDB ID 1BT1). (B) Active centre of the phenylthiourea (PTU) inhibitor-bound *I. batatas* catechol oxidase. (C) Three-dimensional structure of *Vitis vinifera* catechol oxidase (PDB ID 2P3X). (D) Active centre of the *Vitis vinifera* catechol oxidase. The secondary structures are shown in red (α helix), yellow (β sheet), and green (loop). The copper ions are shown with brown spheres, the bridging molecule in red and the thioether bond in yellow. The figure was created with PyMOL (The PyMOL Molecular Graphics System, Educational Version Schrödinger, LLC).

1.6.2 Laccases

The three-dimensional structures of laccases published so far are of laccases of fungal and bacterial origins (**Table 4**). The three-dimensional structure of a plant laccase has not been solved yet. The first three-dimensional structure published was of the laccase from the basidiomycete fungus *Coprinopsis cinerea*, but it was T2 copper depleted (Ducros et al. 1998). The first complete three-dimensional structures of laccases became available in 2002, when the structures of the laccase both from the basidiomycete fungus *Trametes versicolor* (Bertrand et al. 2002, Piontek et al. 2002) and from the ascomycete fungus *M. albomyces* (Hakulinen et al. 2002) were published. Thus far, most of the three-dimensional structures of fungal laccases reported have been from basidiomycete fungi, such as *T. versicolor* (Bertrand et al. 2002, Piontek et al. 2002), *Rigidoporus lignosus* (Garavaglia et al. 2004), *Coriolus zonatus* (Zhukova et al. 2006, Lyashenko et al. 2007),

Lentinus tigrinus (Ferraroni et al. 2007), *Trametes trogii* (Matera et al. 2008), *Coriolopsis gallica* (De La Mora et al. 2008), *Cerrena maxima* (Lyashenko et al. 2007), and *Trametes hirsuta* (Polyakov et al. 2009). The only three-dimensional structure of an ascomycete fungus laccase published so far is from *M. albomyces* (Hakulinen et al. 2002, Hakulinen et al. 2006, Hakulinen et al. 2008). Among bacterial laccases, the structures published are from *Bacillus subtilis* (Enguita et al. 2003), *Escherichia coli* (Li et al. 2007), *Nitrosomonas europaea* (Lawton et al. 2009), and *Streptomyces coelicolor* (Skálová et al. 2011). Furthermore, a model of the plant laccase from *Populus trichocarpa* has been predicted through homology modelling approach using the ascorbate oxidase from zucchini (PDB ID 1AOZ) as a template, and it was compared to the bacterial laccase from *B. subtilis* and the fungal laccase from *T. versicolor* (for a review, see Dwivedi et al. 2011). Two examples of three-dimensional structures of laccases, from the ascomycete fungus *M. albomyces* and from the basidiomycete fungus *T. versicolor,* are presented in **Figure 8**.

Laccases of different origin share similar structural features. The overall structure of laccases consists of three cupredoxin-like domains (A, B, and C), with each domain having a "Greek key motif" β-barrel structure. The mononuclear T1 copper site is located in domain C. The putative substrate-binding site is in a cleft between domains B and C. The trinuclear T2/T3 copper cluster is located at the interface between domains A and C, with both domains providing residues for the co-ordination of the coppers ions. The electrons are donated from the substrate to the copper ion of the T1 copper site, and subsequently they are transferred to the trinuclear site where the reduction of molecular oxygen to water occurs. Electron transfer from the T1 copper site to the T2/T3 copper cluster occurs via a His-Cys-His pathway. The substrate-binding sites of laccases are rather dissimilar, i.e., the bacterial laccase from *B. subtilis* has a larger putative substrate-binding site cavity as compared to the fungal laccases from *T. versicolor* (Dwivedi et al. 2011) and *M. albomyces* (Hakulinen et al. 2008). Messerschmidt et al. (1992) reported the presence of two solvent channels in the ascorbate oxidase from zucchini. A broad channel leads to the pair of copper ions at the T3 site, and a narrow channel leads to the T2 copper. Similar channels are found in laccases of fungal origin; however, in the laccase from *M. albomyces,* the broad channel is plugged by the C-terminal residues Asp-Ser-Gly-Leu (Hakulinen et al. 2002, Hakulinen et al. 2008). The four residues of the C-terminus are conserved also in other ascomycete laccases, so it is possible that the C-terminal plug is present also in these ascomycete laccases. The C-terminal plug is not found in the basidiomycete laccases. One characteristic feature of fungal laccases is that they are glycosylated proteins, typically with 3–10 glycosylation sites per monomer (Solomon et al. 1996, Rodgers et al. 2009). The functional role of the glycans in laccases is not yet clear.

Recently, the three-dimensional structures of two-domain laccases have been established. Two-domain laccases are smaller than the three-domain laccases, but they are surprisingly temperature stable and have high activity on typical substrates of laccases, such as 2,6-dimethoxy phenol (2,6-DMP). The two-domain laccase from *Nitrosomonas europaea* was characterised already in 1985 (DiSpirito et al. 1985), but the three-dimensional structure was published only recently (Lawton et al. 2009). The three-dimensional structure of a two-domain laccase from *Streptomyces coelicolor* (Skálová et al. 2009) is also known. The three-dimensional structure of two-domain laccases is a

homotrimer, with each monomer is comprised of two cupredoxin-like domains (domain 1 and domain 2). The T1 copper site is in domain 1, and the trinuclear site is embedded in the inter-molecular interface between the subunits (Komori et al. 2009, Lawton et al. 2009, Skálová et al. 2011).
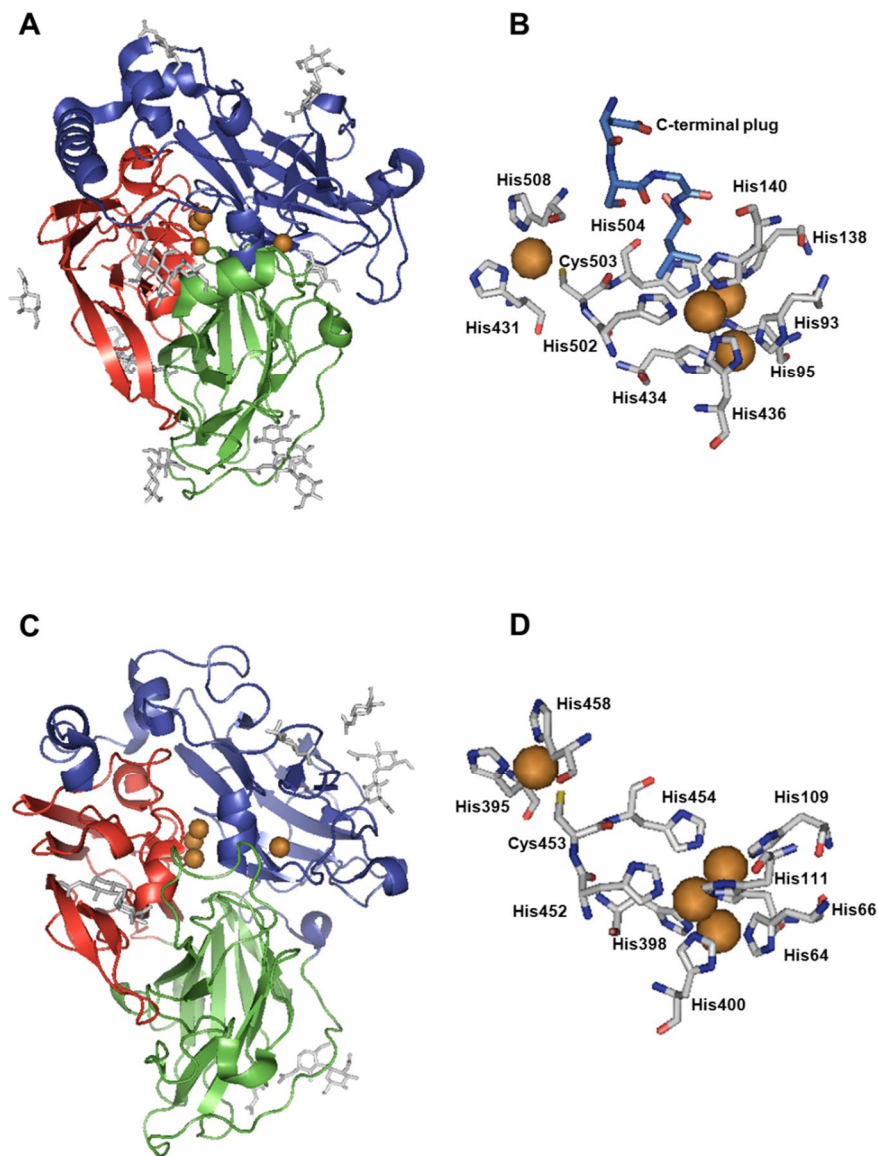
Figure 8. (A) Three-dimensional structure of the laccase from ascomycete fungus *Melanocarpus albomyces* (PDB ID 1GWO). (B) Active centre of *M. albomyces* laccase. The C-terminal plug (Asp556-Ser557-Gly558-Leu559) is also shown. (C) Three-dimensional structure of the laccase from basidiomycete fungus *Trametes versicolor* (PDB ID 1GYC). (D) Active centre of *T. versicolor* laccase. Domain A is represented in red, domain B in green, and domain C in blue. The copper ions are represented with brown spheres. The figure was created with PyMOL (The PyMOL Molecular Graphics System, Educational Version Schrödinger, LLC).

Table 4. Summary of the crystal structures of tyrosinase, catechol oxidase and laccase available from RCSB PDB (www.pdb.org).

Enzyme	Kingdom	Species	PDB ID	Best resolution (Å)	References
Tyrosinase	Bacteria	*Bacillus megaterium*	3NM8, 3NPY, 3NQ0, 3NQ1, 3NQ5, 3NTM	2.00 Å	Sendovski et al. 2011
Tyrosinase	Bacteria	*Streptomyces castaneoglobisporus*	1WXC, 1WX2, 1WX4, 1WX5, 2AHK, 2AHL, 2ZMX, 2ZMY, 2ZMZ, 2ZWD, 2ZWE, 2ZWF, 2ZWG, 3AWS, 3AWT, 3AWU, 3AWV, 3AWW, 3AWX, 3AWY, 3AWZ, 3AX0	1.16 Å	Matoba et al. 2006, Matoba et al. 2011
Tyrosinase	Fungi	*Agaricus bisporus*	2Y9W, 2Y9X	2.30 Å	Ismaya et al. 2011
Catechol oxidase	Plantae	*Ipomea batatas*	1BT1, 1BT2, 1BT3, 1BUG	2.50 Å	Klabunde et al. 1998
Catechol oxidase	Plantae	*Vitis vinifera*	2P3X	2.20 Å	Virador et al. 2009
Laccase	Bacteria	*Bacillus subtilis*	1GSK, 1OF0, 1UVW, 1W6L, 1W6W, 1W8E, 2BHF, 2WSD, 2X87, 2X88	1.60 Å	Enguita et al. 2003
Laccase	Bacteria	*Escherichia coli*	2FQD, 2FQE, 2FQF, 2FQG, 2ZWN, 1Z9T	1.54 Å	Li et al. 2007
Laccase [a]	Bacteria	*Nitrosomonas europaea*	3G5W	1.90 Å	Lawton et al. 2009
Laccase [a]	Bacteria	*Streptomyces coelicolor*	3CG8, 3KW8	2.29 Å	Skálová et al. 2009, Skálová et al. 2011

Enzyme	Kingdom	Species	PDB ID	Best resolution (Å)	References
Laccase	Fungi	*Cerrena maxima*	2H5U, 3DIU	1.76 Å	Lyashenko et al. 2007
Laccase	Fungi	*Coprinopsis cinerea* (*Coprinus cinereus*)	1A65, 1HFU	1.68 Å	Ducros et al. 1998
Laccase	Fungi	*Coriolus zonatus*	2HZH	2.60 Å	Lyashenko et al. 2007
Laccase	Fungi	*Coriolopsis gallica*	4A2H, 2VDZ, 2VE0	1.70 Å	De La Mora et al. 2008
Laccase	Fungi	*Melanocarpus albomyces*	1GW0, 2IH8, 2IH9, 2Q9O, 3DKH, 3FU7, 3FU8, 3FU9, 3QPK	1.30 Å	Hakulinen et al. 2002, Hakulinen et al. 2006, Hakulinen et al. 2008, Andberg et al. 2009, Kallio et al. 2009
Laccase	Fungi	*Lentinus tigrinus*	2QT6	1.50 Å	Ferraroni et al. 2007
Laccase	Fungi	*Trametes hirsuta*	3FPX, 3PXL	1.20 Å	Polyakov et al. 2009
Laccase	Fungi	*Trametes sp. AH28-2*	3KW7	3.44 Å	Ge et al. 2010
Laccase	Fungi	*Trametes trogii*	2HRG, 2HRH	1.58 Å	Matera et al. 2008
Laccase	Fungi	*Trametes versicolor*	1GYC, 1KYA	1.90 Å	Bertrand et al. 2002, Piontek et al. 2002
Laccase	Fungi	*Rigidoporus lignosus*	1V10	1.70 Å	Garavaglia et al. 2004

[a] Two-domain laccase

1.7 Applications of tyrosinases and laccases

Among tyrosinases, catechol oxidases, and laccases, laccases are the only commercially available enzymes for industrial purposes and they are already used on a large scale in the textile industry. The tyrosinase from the edible mushroom *Agaricus bisporus* is commercially available for research purposes, whereas catechol oxidase preparations are not available. Several potential applications of tyrosinases and laccases in food and non-food processes have been reported, whilst only a few promising applications for catechol oxidases have been described. Moreover, several potential applications that use catechol oxidase, and monophenolase activity have been reported without a clear indication of whether the enzyme applied is a tyrosinase or a catechol oxidase. Indeed, catechol oxidases could be utilised as an alternative to tyrosinases in the oxidation of diphenolic substrates. It is worth noticing that most of the research on tyrosinases and catechol oxidases focuses on their inhibition mechanism, because of the interest in the related reactions of browning and hyperpigmentation (see Subsection 1.5.1). However, more recently interest in the utilisation of tyrosinases has rapidly increased. Food- and non-food-related applications of tyrosinases, catechol oxidases, and laccases are discussed below and summarised in **Table 5** and **Table 6**.

1.7.1 Food-related applications

1.7.1.1 Development in the organoleptic properties of food products

The enzymatic activity of tyrosinases, catechol oxidases and laccases present in fruits and seeds and in the microbes associated with fruits and seeds are exploited in the fermentation processes of black tea, coffee, cocoa, and black raisins. They confer organoleptic properties and colour on the final food products (for a review, see Yoruk & Marshall 2003). In the fermentation process of tea leaves, tyrosinase can oxidise the catechins to theaflavins. Theaflavins, antioxidant compounds with an orangish colour, impart colour and brightness to the black tea and reduce the astringency, i.e., the dry feeling in the mouth of black tea liquors (Subramanian et al. 1999).

Tyrosinases and laccases have been shown to have a positive effect in the fermentation of cocoa beans. Tyrosinase from *A. bisporus* can oxidise the polyphenols that contribute to astringency, thus improving the taste of cocoa (Selamat et al. 2002). Also laccase from *Pleurotus osteratus* has been shown to reduce the tannin content of cocoa. Tannins have astringency properties and negatively affect the digestion process by binding to various digestive enzymes (Mensah et al. 2012).

1.7.1.2 Cross-linking reactions related to food applications

A cross-linking reaction introduces a covalent bond between proteins (homo-cross-link) or between a protein and a carbohydrate (hetero-cross-link). Cross-linking reactions can be used for food structure modification and can affect the sensory properties of the final product. Laccases and tyrosinases can be used to cross-link proteins. The reactive residues in proteins are Gln, Lys, Tyr, and Cys.

Tyrosinase can oxidise protein-bound Tyr residues to the corresponding quinone, which can further react with protein-bound Lys, Tyr, and Cys residues (Ito et al. 1984, Bittner 2006, Mattinen et al. 2008a, Mattinen et al. 2008b, Selinheimo et al. 2008). As compared to other fungal and plant tyrosinases, the extracellular tyrosinase from the fungus *T. reesei* has shown extremely good cross-linking abilities for random coil proteins such as α-caseins (Selinheimo et al. 2007a, Selinheimo et al. 2007b, Selinheimo et al. 2008, Monogioudi et al. 2009, Ercili Cura et al. 2010) as well as short tyrosine-containing peptides (Mattinen et al. 2008b). However, application of *T. reesei* tyrosinase in the food industry is so far limited by the formation of undesired colour (Monogioudi et al. 2009).

Differently from that with tyrosinases, laccase-catalysed cross-linking is based on the formation of free radicals, which, in turn, can further react with other phenolic radicals or free aromatic and amino groups present in proteins. Laccases act on the protein-bound Tyr, Trp, and Cys residues. Laccases can act also on ferulic acid and *p*-coumaric acid residues, which are present in arabinoxylans and pectins (for a review, see Harjinder 1991). Among laccases, the effects of the fungal laccase from *T. hirsuta* have been extensively investigated in the baking applications (Selinheimo et al. 2006a, Selinheimo et al. 2007a, Selinheimo et al. 2008).

Other than laccases and tyrosinases several enzymes are known to be able to cross-link proteins. For example, peroxidase (EC 1.11.1.7), and sulphydryl oxidase (EC 1.8.3.3) have also been extensively studied (for a review see Buchert et al. 2007, Buchert et al. 2010). Transglutaminase (EC 2.3.2.13) reacts with protein-bound Gln and Lys residues, resulting in the formation of an isopeptide linkage. During the reaction, one molecule of ammonia is generated per a cross-link (Folk & Finlayson 1977, Griffin et al. 2002). A transglutaminase from *Streptoverticillium mobaraense* was the first cross-linking enzyme to see a market launch (Ando et al. 1989). At present, microbial transglutaminases are the only commercially available food-grade proteins directly cross-linking enzymes.

Table 5. Food related applications of tyrosinases and laccases. Industrial applications are indicated with *** when the enzyme is commercially available, the commercial name and the distributor are also indicated in brackets. Pilot-scale developed applications are indicated with **, and applications tested at research-level are indicated with *.

Food related applications	Tyrosinase	Laccase	References
Development of organoleptic properties [a]	Removal of polyphenols during fermentation of cocoa beans* Oxidation of catechins to theaflavins during fermentation of black tea leaves* Better flavour in coffee*	Removal of tannins during fermentation of cocoa beans*	Subramanian et al. 1999, Selamat et al. 2002, Mensah et al. 2012
Baking	Increased softness of bread crumbs and added volume for bread*	Improved loaf volume, dough-handling, and crumb structure of bread*	Si 1994, Labat et al. 2000, Labat et al. 2001, Selinheimo et al. 2007a
Beverages		Cork stopper treatment (Suberase®, Novozymes)*** Wine and beer stabilisation* Clarification of juices*	Conrad et al. 2000, Minussi et al. 2002, Minussi et al. 2007
Dairy	Cross-linking of dairy proteins*	Cross-linking of dairy proteins*	Færgemand et al. 1998, Thalmann & Lötzbeyer 2002, Halaouli et al. 2005, Selinheimo et al. 2006a, Selinheimo et al. 2007a, Selinheimo et al. 2008
Emulsions	Improved emulsifying properties via conjugation of different types of proteins and polysaccharides*	Stabilisation of beet pectin emulsions*	Kato et al. 1991, Kato et al. 1993, Littoz & McClements 2008
Meat	Increased firmness of myofibrillar gels*	Improved forming of myofibrillar gels at high salt concentrations* Decreased formation of myofibrillar gels with high laccase dosage*	Lantto et al. 2005b, Lantto et al. 2007

[a] Endogenous enzymes are utilised in these applications.

1.7.2 Non-food-related applications

1.7.2.1 Large-scale applications of laccases

A laccase from ascomycete fungus *Myceliophthora thermophila* distributed by Novozymes with the commercial name of Suberase® is used in the preparation of cork stoppers for wine bottles. Cork is a desirable stopper material; however, frequently a cork taint (i.e., an off-flavour) is conveyed to the wine in contact with the cork. The enzymatic treatment polymerises phenolic compounds in the cork and thus reduces the astringency, bitterness, and tannic flavour imparted to the wine by the cork (Conrad et al. 2000).

In some applications, laccases are used in combination with mediators in so-called laccase mediator systems (LMS). Mediators are small molecules with a redox potential ($E°$) between the $E°$ of the laccase and the $E°$ of the substrate. Mediators are used to oxidise bulky substrates that could not be oxidised by laccase alone. Thus the use of a mediator enlarges the substrate-specificity of the laccase. In LMS, the laccase first oxidises the mediator, and subsequently a redox reaction occurs between the oxidised mediator and the substrate. The reaction products are the oxidised substrate and the reduced mediator, which can often be recycled. The first LMS to bleach denim garments (i.e., to decolourise indigo) was developed with a laccase from *M. thermophila* and the mediator methyl ester of syringic acid (Berka et al. 1997). This LMS oxidises indigo, and the reaction products are two molecules of isatin, which is a colourless chemical more biodegradable than indigo. Currently there are various commercial laccase preparations available for bleaching of denim (for a review, see Galante & Formantici 2003, Rodriguez Couto & Toca Herrera 2006). For instance DeniLite (Novozymes) utilises a laccase from *Aspergillus niger* (Niladevi 2009).

Laccases have also been used in the enzymatic removal of lignin. The LignoZym® system (GmbH) utilises a laccase from *T. versicolor* and a group of mediators containing N-OH-, N-oxide-, oxime- or hydroxamic acid-functional groups to remove lignin from pulp. The Lignozym® system has been tested on pilot-scale, and it has been shown that 50–70% of lignin can be removed within 1–4 hours (for a review, see Call & Mücke 1997). Moreover, the combination of a laccase from *T. hirsuta* with cellulases has been shown to increase the sugar yield in the enzymatic hydrolysis of steam-pre-treated softwood by 13% (Palonen & Viikari 2004). Generally, the industrial applicability of the LMS has suffered on account of high costs, recycling, and toxicity of the mediators used (Bourbonnais & Paice 1992).

1.7.2.2 Other potential non-food applications of tyrosinases and laccases

Tyrosinases and laccases may be used in non-food applications, but these enzymes are not yet utilised in large-scale applications. Tyrosinase- and laccase-catalysed cross-linking may be used in tailoring biopolymers: e.g., the softness of wool fibres can be increased via tyrosinase or laccase-cross-linking of the fibres (Lantto et al. 2005a, Freddi et al. 2006, Jus et al. 2008, Mattinen et al. 2008b). Tyrosinase-catalysed cross-linking may be utilised in grafting of carboxyl groups to chitosan with potential application in cosmetics (Chao et al. 2004), as well as to immobilise antibodies on membranes with potential for applications in microarray technology (Ahmed et al. 2006).

The oxidation of phenols catalysed by tyrosinases, catechol oxidases and laccases has a potential application in biosensors (e.g., quantification of phenols) and in bioremediation (i.e., removal of phenols). Phenols are commonly found in industrial waste waters and can have toxic effects. Some phenols are endocrine disrupters: i.e., they are able to mimic or antagonise the effects of endogenous hormones. Therefore, the detection of low concentrations of phenols is of fundamental importance (Mita et al. 2007). Several tyrosinase-based biosensors have been proposed (for a review, see Dong & Chen 2002). A biosensor based on *A. bisporus* tyrosinase was tested for the determination of bisphenol A, which can be found in food and drink packaging (Mita et al. 2007). A catechol oxidase electrode made with a catechol oxidase from *Solanum melangena* L. (eggplant) immobilised in gelatine was found to be a specific biosensor for catechol and to be stable for 70 days (Dinckaya et al. 1998).

Laccases have potential in decolourisation of brewers and olive-mill waste waters. One laccase from *Coriolopsis gallica* has been suggested as an efficient tool to remove the colourful tannins seen in brewery waste waters (Yagüe et al. 2000). The efficacy of an immobilised laccase from *Lentinula edodes* has been tested for removal of toxic phenols from oil-mill effluents. This system was most effective on *o*-diphenols (D'Annibale et al. 2000).

Biofuel cells are devices that convert chemical energy into electrical energy via electrochemical reactions catalysed by oxidoreductases. The electrons for production of electricity are produced via the enzymatic oxidation of a fuel (sugar or alcohol) at the bioanode of the fuel cell. The electrons are transferred to the biocathode and gained by the electron acceptor, typically molecular oxygen or peroxide, through an enzymatic reaction. Laccases have been investigated in biofuel applications (Palmore & Kim 1999, Lim & Palmore 2007, Smolander et al. 2008).

Tyrosinases, catechol oxidases, and laccases may be used in medical applications, such as drug development and diagnostics. Thus far, of the three enzymes, tyrosinases have been the most studied in this field. Human tyrosinase is overexpressed in melanosome malignant cells. A vaccine against melanosome that uses specific antibodies for tyrosinase has been developed and successfully passed phase I clinical trials (Chen et al. 1995, Bergman et al. 2003, Liao et al. 2006). Also, a melanocyte-directed enzyme prodrug therapy (MDEPT) has been developed. The prodrug is a tyrosinase substrate (dopamine) linked to a drug (phenyl mustard) by a carbamate linkage. The tyrosinase-catalysed oxidation of dopamine releases the drug *in situ* (Morrison et al. 1985, Jordan et al. 1999, Jordan et al. 2001). L-DOPA, used as a drug for treatment of Parkinson's disease, is currently chemically produced (Knowles 2003). A cost-effective means of enzymatic production of L-DOPA has been proposed, with immobilised tyrosinase used to oxidise L-tyrosine to dopaquinone and ascorbic acid used to reduce dopaquinone to L-DOPA (Carvalho et al. 2000).

The catechol oxidase from *I. batatas* has potential application in the medical field in the quantitative determination of L-DOPA and carbidopa in pharmaceutical preparations. L-DOPA is used as a neurotransmitter against Parkinson's disease, and carbidopa is often present in the same preparation with L-DOPA as an inhibitor of decarboxylase activity. The catechol oxidase oxidation products of L-DOPA and carbidopa absorb at different wavelengths so can be detected and quantified (Fatibello-Filho & da Cruz Vieira 1997).

A laccase system has been proposed as an alternative to a peroxidase system to convert iodide to iodine, which is used as a disinfectant. This laccase system allows *in situ* production of iodine and, differently from the peroxidase system, does not require the presence of hydrogen peroxide, which is a harmful chemical. The laccase used in this system is from *Myceliophthora thermophila* (Xu 1996b). Laccases from *Trametes* sp. have potential application in the synthesis of novel antibiotics: i.e., laccases catalyse the amination of catechols with amino-β-lactams to produce antibiotics, such as cephalosporins, penicillins, and carbacephems (Mikolasch et al. 2006, Mikolasch et al. 2008).

Table 6. Non-food related applications of tyrosinases and laccases. Industrial applications are indicated with * when the enzyme is commercially available, the commercial name and the distributing company are also indicated in brackets. Pilot-scale level applications are indicated with **, and applications tested at research-level are indicated with *.

Non-food-related applications	Tyrosinase	Laccase	References
Biosensors and biofuel cells	Electrodes in pure organic phase* Quantification of polar organic solvents* Biosensor of phenol* Phenols and amines degradation* Catechol oxidation* Phenolic compound polymerisation* Pesticides detection (based on inhibition)*	Detection of phenolic compounds* Detection of oxygen* Detection of azides* Immobilised in the biocathode of biofuel cells*	Yaropolov et al. 1995, Dinckaya et al. 1998, Palmore & Kim 1999, Streffer et al. 2001, Minussi et al. 2002, Chao et al. 2004, Busch et al. 2006, Gill et al. 2006, Lim & Palmore 2007, Liu & Dong 2007, Mita et al. 2007, Smolander et al. 2008, Akyilmaz et al. 2010
Medical field	Melanosome vaccine (clinical trials)** Treatment of malignant melanoma in the form of a melanocyte-directed enzyme prodrug therapy (MDEPT)* Production of L-DOPA as a drug for Parkinson's treatment * Grafting of phenolic moieties or proteins onto chitosan to create hydrogels for skin substitutes* Immobilisation of antibody via tyrosinase-catalysed reaction*	Skin lightening* In-situ generation of iodine (disinfectant)* Production of antibiotics* Glucose determination* Detection of morphine* Hair dyes*	Morrison et al. 1985, Roure et al. 1992, Chen et al. 1995, Aaslyng et al. 1996, Fatibello-Filho & da Cruz Vieira 1997, Lang & Cotteret 1998, Jordan et al. 1999, Carvalho et al. 2000, Jordan et al. 2001, Takada et al. 2003, Ahmed et al. 2006, Liao et al. 2006, Littoz & McClements 2008
Textile industry		Denim finishing (DeniLite® and DeniLite IIs®, Novozymes; Zylite®, ZyTex)*** Synthesis of dyes* Decolourisation of dyes-house	Reyes et al. 1999, Setti et al. 1999, Wong & Yu 1999, Abadulla et al. 2000, Galante & Formantici 2003, Peralta-Zamora et al. 2003, Ossola & Galante 2004, Pazarlioğlu et al. 2005, Zille et al.

Non-food-related applications	Tyrosinase	Laccase	References
		effluents* Bleaching of cotton* Rove scouring* Dyeing of wool* Anti-shrinkage treatment for wool*	2005, Rodriguez Couto & Toca Herrera 2006
Lignocellulose processing		Lignin modification (Lignozym®, GmbH and Novozym® 51003, Novozymes)** Pulp-bleaching*	Bourbonnais & Paice 1992, Bourbonnais et al. 1997, Poppius-Levlin et al. 1999, Palonen & Viikari 2004, Camarero et al. 2007, Gutiérrez et al. 2007
Tailoring of biopolymers by means of cross-linking reactions	Grafting of chitosan to silk proteins* Grafting of peptides or fluorescent proteins to chitosan* Modification of wool fibres* Protein cross-linked chitosans with adhesive properties* Protein cross-linked chitosans for use as matrices for drug delivery*	Modification of wool fibres*	Lantto et al. 2005a, Freddi et al. 2006, Anghileri et al. 2007
Nanotechnology	Modification of surfaces of nano-scale structures*		Basnar et al. 2007
Bioremediation and decontamination of feed	Xenobiotic compounds' removal*	Oxidation of polycyclic aromatic hydrocarbons and chlorophenols* Removal of dioxins and polychlorinated biphenyls from fishmeal*	Torres et al. 2003, Zille et al. 2005, Baron et al. 2007, Martin et al. 2007, Canfora et al. 2008

2. Aims of the work

The overall aim of the work was to characterise oxidative cross-linking enzymes that can be used for cross-linking of proteins, as well as proteins with carbohydrates in industrial food production. The work focused on the characterisation of three oxidoreductases: catechol oxidase, tyrosinase, and laccase.

The aims of the work at more detailed level were:

1. Biochemical and structural characterisation of a novel catechol oxidase from the fungus *Aspergillus oryzae*. The gene encoding for the *A. oryzae* catechol oxidase belongs to the newly discovered short-tyrosinases sequence group (Publication II and unpublished).

2. Detailed characterisation of the extracellular tyrosinase from the fungus *Trichoderma reesei* in terms of substrate-specificity and inhibition, and comparison with the commercially available tyrosinase from *Agaricus bisporus* (Publications I and IV).

3. Elucidation of the oxidation capacity of fungal laccases using a set of laccases with different redox potential and a set of substituted phenolic substrates with different redox potential. The laccases used in this work were the basidiomycete laccase from *Trametes hirsuta* and the ascomycete laccases from *Thielavia arenaria* and *Melanocarpus albomyces* (Publication III).

4. Evaluation of the cross-linking ability of catechol oxidase from *A. oryzae*, tyrosinases from *T. reesei* and *A. bisporus* and laccases from *T. hirsuta, T. arenaria,* and *M. albomyces* through the use of α-casein proteins as a model substrate (unpublished).

3. Material and methods

A summary of the materials and methods used in the work is presented in this Chapter, detailed descriptions can be found in the original articles (I–IV).

3.1 Substrates and inhibitors

The substrates and inhibitors used in this work are listed in **Table 7**. Various low-molecular-weight monophenolic and diphenolic compounds were used for determination of the substrate-specificity of tyrosinases and catechol oxidase. Tripeptides were used as a simplified model of protein bound tyrosine for tyrosinases. A range of small chemical compounds were tested for analysis of the inhibition of *Trichoderma reesei* tyrosinase (TrT). 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxy phenol (2,6-DMP), syringic acid, and methyl syringate were used specifically for the laccase work (Publication III), and α-casein proteins were used as model proteins for assaying of the cross-linking ability of the enzymes.

3.2 Enzymes

The enzymes studied in this work are listed in **Table 8**. TrT was produced and purified as described by Selinheimo et al. (2006b). The intracellular tyrosinase from *Agaricus bisporus* (AbT) was purchased from Sigma-Aldrich and purified as described in Publication I. The extracellular catechol oxidase from *Aspergillus oryzae* (AoCO4) was produced in *T. reesei* under the *cbhI* promoter and purified as described in Publication II. Laccase 1 from *Thielavia arenaria* (TaLcc1) was produced and purified as described in Publication III. Laccase from *Trametes hirsuta* (ThL) was produced and purified as described by Rittstieg et al. (2002), and laccase from *Melanocarpus albomyces* (*r*MaL) was produced and purified as described in Kiiskinen et al. (2004).

Table 7. Substrates and inhibitors used in this work.

Common name	IUPAC name	Supplier	Studied in publication
2,6-DMP	2,6-Dimethoxy phenol	Aldrich	III
4-Aminophenol	4-Aminophenol	Aldrich	II
4-*tert*-Butylcatechol	4-*tert*-Butylbenzene-1,2-diol	Fluka	I–II, Unpublished
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)	Roche	III
Aniline	Phenylamine	Sigma	I
Benzaldehyde	Benzaldehyde	Fluka	IV
Benzoic acid	Benzoic acid	Merck	I, IV
Caffeic acid	3,4-Dihydroxycinnamic acid	Fluka	I–II, IV
α-Caseins		Calbiochem	Unpublished
(+)-Catechin hydrate	*trans*-3,3',4',5,7-Pentahydroxyflavane	Sigma	II
Catechol	1,2-Dihydroxybenzene	Fluka	I–II, Unpublished
p-Coumaric acid	*trans*-4-Hydroxycinnamic acid	Sigma	I, IV
L-DOPA	3,4-Dihydroxy-L-phenylalanine	Fluka	I–II, IV
L-Dopamine	4-(2-Aminoethyl)benzene-1,2-diol	Sigma	I
(-)-Epicatechin	(-)-*cis*-3,3',4',5,7-Pentahydroxyflavane	Sigma	II
GGY	Glycine-glycine-tyrosine	Bachem	I
Guaiacol	2-Methoxyphenol	Sigma	II
GYG	Glycine-tyrosine-glycine	Bachem	I
Hydrogen peroxide	Dihydrogen dioxide	Merck	Unpublished
Hydroxycaffeic acid	3-(3,4-Dihydroxyphenyl) propanoic acid	Aldrich	I–II
Kojic acid	5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one	Merck	IV
MBTH	3-Methyl-2-benzothiazolinone hydrazone	Sigma	I
Methyl syringate	Methyl 3,5-dimethoxy-4-hydroxybenzoate	Aldrich	III
Phenol	Hydroxybenzene	Merck	I–II
Phloretic acid	3-(4-Hydroxyphenyl) propanoic acid	Aldrich	I
Potassium cyanide	Potassium cyanide	Merck	IV
L-Tyramine	2-(4-Hydroxyphenyl) ethylamine	Sigma	I
L-Tyrosine	3-(4-Hydroxyphenyl)-L-alanine	Sigma	I–II, IV
p-Tyrosol	2-(4-Hydroxyphenyl) ethanol	Aldrich	I–II
Sodium azide	Sodium azide	Merck	IV
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	Fluka	III
YGG	Tyrosine-glycine-glycine	Bachem	I

Table 8. Enzymes studied in this work.

Enzyme	Origin	Abbreviation	Reference	Studied in publication
Tyrosinase (EC 1.14.18.1)	*Trichoderma reesei*	TrT	Selinheimo et al. 2006b	I, IV
	Agaricus bisporus	AbT	Purchased from Sigma-Aldrich	I, IV
Catechol oxidase (EC 1.10.3.1)	*Aspergillus oryzae*	AoCO4	Produced and purified as described in Publication II	II, Unpublished
Laccase (EC 1.10.3.2)	*Thielavia arenaria*	TaLcc1	Produced and purified as described in Publication III	III
	Trametes hirsuta	ThL	Rittstieg et al. 2002	III
	Melanocarpus albomyces (recombinant)	*r*MaL	Kiiskinen et al. 2004	III

3.3 Enzyme activity assays

Tyrosinase activity was measured with 15 mM L-DOPA or 2 mM L-tyrosine as the substrate. Activity assays were performed in 0.1 M phosphate buffer pH 7.0 at 25 ºC, either by monitoring of dopachrome formation at 475 nm ($\varepsilon_{dopachrome}$=3400 M^{-1} cm^{-1}) as described by Robb (1984) or by monitoring of the consumption of the co-substrate molecular oxygen, with a single-channel fibre-optic oxygen meter for mini-sensors (Precision sensing GmbH, Regensburg, Germany) as described by Selinheimo et al. (2006b).

Catechol oxidase activity was measured with 15 mM catechol and 15 mM 4-*tert*-buthylcatechol (TBC) as substrates. Activity assays were performed at 25 ºC in 0.1 M phosphate buffer pH 7.0, with monitoring of the quinone formation at 400 nm ($\varepsilon_{quinone}$=1450 M^{-1} cm^{-1}; $\varepsilon_{o\text{-}tert\text{-butylquinone}}$=1200 M^{-1} cm^{-1}) as described by Robb (1984).

Laccase activity was measured with ABTS as a substrate. Activity assays were performed at 25 ºC in 25 mM succinate buffer pH 4.5, with the product formation monitored at 436 nm ($\varepsilon_{product}$=29300 M^{-1} cm^{-1}) as described by Niku-Paavola et al. (1988).

3.4 Production and purification of enzymes

3.4.1 Production and purification of catechol oxidase from *Aspergillus oryzae* (Publication II)

The putative tyrosinase protein sequences were retrieved via the VTT in-house database (Arvas et al. 2007). The putative tyrosinase *AoCO4* gene (Entrez gene ID 5990879) was amplified by PCR from the genomic DNA of the *A. oryzae* strain VTT-D-88348. The *AoCO4* gene was ligated into the *T. reesei* expression construct pMS186 to create the plasmid pGF007. The construct pGF007 was transformed into *T. reesei* strain VTT-D-00775 (Penttila et al. 1987). Transformants were selected as described by Selinheimo et al. (2006b). Based on shake flask cultures, the best overexpressing transformant of *AoCO4* was grown in a bioreactor of a volume of 10 L.

For purification of AoCO4, a concentrated culture supernatant was first desalted on a PD-10 desalting column (Sephadex G-25 medium, GE Healthcare) in 20 mM sodium acetate buffer, pH 4.8. The desalted sample was applied to a HiPrep™ 16/10 CM Sepharose Fast Flow column (20 mL volume, Amersham Biosciences, Uppsala, Sweden), in 20 mM sodium acetate buffer, pH 4.8. Bound proteins were eluted with a linear NaCl gradient (0–150 mM in 20 column volumes) in the equilibration buffer. Catechol-positive fractions were pooled, concentrated with a Vivaspin concentrator (20 mL, 5000-Da MWCO; Vivascience, Hannover, Germany), and subjected to anion-exchange chromatography in a ResourceQ column (6 mL volume, Amersham Biosciences, Uppsala, Sweden) in 10 mM Tris-HCl buffer, pH 7.2. Bound proteins were eluted with a linear NaCl gradient (0–150 mM in 20 column volumes) in the equilibration buffer. Active fractions were pooled, concentrated, and stored at −20 °C in aliquots. SDS-PAGE (12% Tris-HCl Ready Gel, Bio-Rad) was performed according to Laemmli (1970). Coomassie Brilliant Blue (R350; Pharmacia Biotech, St Albans, UK) was used for staining the proteins, and prestained SDS-PAGE standards (Broad Range, Cat no. 161-0318) were used for the determination of molecular weight.

3.4.2 Production and purification of laccase from *Thielavia arenaria* (Publication III)

Thielavia arenaria laccase 1 (TaLcc1) was produced at Roal Oy (Rajamäki, Finland) with *T. reesei* used as a host organism, as described by Paloheimo et al. (2006).

For purification of TaLcc1, a concentrated culture supernatant was first desalted on a Sephadex G-25 M column (70 mL volume, Pharmacia Biotech, Uppsala, Sweden) in 5 mM Tris-HCl buffer pH 8.5. The desalted sample was applied to a DEAE Sepharose Fast Flow column (70 mL volume, Amersham Biosciences, Uppsala, Sweden), in 5 mM Tris-HCl buffer, pH 8.5. Bound proteins were eluted with a linear sodium sulphate gradient (0–100 mM in 6 column volumes) in the equilibration buffer. ABTS-active fractions were pooled, concentrated with a Vivaspin concentrator (20 mL, 10000-Da MWCO; Vivascience, Hannover, Germany), and subjected to anion-exchange chromatography in a ResourceQ column (6 column volume, Amersham Biosciences, Uppsala, Sweden) pre-equilibrated in 5 mM Tris-HCl buffer pH 8.5. Bound proteins were eluted with a linear sodium sulphate gradient (0–100 mM in 20 column volumes) in the equilibration buffer.

ABTS-active fractions were pooled, concentrated with a Vivaspin concentrator (20 mL, 10000-Da MWCO; Vivascience, Hannover, Germany). Subsequently, the buffer was changed to 20 mM Tris-HCl pH 7.2 on a PD-10 desalting column (Sephadex G-25 medium, GE Healthcare).

3.5 Biochemical characterisation of enzymes

3.5.1 Biochemical and structural characterisation of catechol oxidase from *Aspergillus oryzae* (Publication II and unpublished results)

The isoelectric point of AoCO4 was determined by isoelectric focusing on an LKB 2117 Multiphor II Electrophoresis System. Catecholase activity was visualised by staining of the gel with 15 mM 4-*tert*-butylcatechol (TBC) in 0.1 M phosphate buffer, pH 7.0, and proteins were visualised via Coomassie Blue staining. The molecular weight of AoCO4 was determined by MALDI-TOF MS with an Ultraflex TOF/TOF instrument (Bruker Daltonik, Bremen, Germany). Tryptic peptides were analysed by MALDI-TOF MS. The N-terminus of AoCO4 was sequenced with a PE Biosystems Procise Sequencer (PE Biosystems, Foster City, CA, USA) on the basis of Edman degradation as described by Kiiskinen et al. (2002).

Determination of the pH optimum of AoCO4 was carried out at 25 ºC with 15 mM TBC as a substrate in 50 mM sodium acetate buffer in the pH range 4.0–5.6, in 50 mM phosphate buffer in the pH range 5.8–8.0, and in 50 mM Tris-HCl buffer in the pH range 7.5–8.6. The stability of AoCO4 at different pH-values was determined through incubation of the enzyme at different pHs at 25 ºC for one, two, and three days in 50 mM McIlvaine universal buffer, and with measurement of the residual activity on TBC as described in Section 3.3. Temperature stability of AoCO4 was determined with the enzyme placed at 30 ºC, 40 ºC, 50 ºC, and 60 ºC in Tris-HCl buffer, pH 7.2, with measurement of the residual activity on TBC as described in Section 3.3.

The substrate-specificity of AoCO4 was qualitatively evaluated on the basis of following the colour change of the substrate visually after 30 minutes of incubation of the enzyme with different substrates: L-tyrosine, *p*-coumaric acid, tyramine, phloretic acid, phenol, 4-aminophenol, tyrosol, 4-mercaptoethanol, guaiacol, creasol, aniline, caffeic acid, hydrocaffeic acid, dopamine, L-DOPA, 3,4-dihydroxy-D-phenylalanine (D-DOPA), catechol, TBC, ABTS, (−)-epicatechin, and (+)-catechin at 25 °C. All the substrates tested were dissolved in 0.1 M phosphate buffer, pH 7.0, to a final concentration of 15 mM, except L-tyrosine, which was used in 2 mM final concentration. Kinetic constants K_M, V_{max}, and k_{cat} of AoCO4 on catechol, caffeic acid and hydrocaffeic acid were determined by means of spectrophotometric measurements at 25 ºC in 0.1 M phosphate buffer, pH 7.0, monitoring the quinone formation at 400 nm for catechol and TBC ($\varepsilon_{quinone}$=1450 M^{-1} cm^{-1}; $\varepsilon_{o\text{-}tert\text{-}butylquinone}$=1200 M^{-1} cm^{-1}), and at 460 nm for hydrocaffeic acid ($\varepsilon_{quinone}$=1120 M^{-1} cm^{-1}) (Waite 1976). The enzyme concentration was 6.4 mM (0.25 mg mL^{-1}) with all the three substrates. The Michaelis–Menten curves for determination of K_M, V_{max}, and k_{cat} were obtained by curve-fitting analysis with the GRAPHPAD PRISM 4.01 program (GraphPad Software Inc., San Diego, CA, USA).

For the characterisation of the secondary structure of AoCO4, intrinsic fluorescence intensity measurements were carried out by means of a Varian Cary Eclipse Fluorescence Spectrophotometer according to Gheibi et al. (2005), and far UV (250–190 nm) circular dichroism (CD) spectra were recorded on a Jasco model J-720 CD spectrophotometer as described by Boer and Koivula (2003). The UV/VIS absorption spectra were obtained with a Varian Cary 100 Bio spectrophotometer at 25 ºC using a quartz cuvette (1 cm path length). Titration of AoCO4 with hydrogen peroxide (H_2O_2) was performed in 10 mM Tris-HCl buffer, pH 7.2. The spectrum was recorded immediately after an addition of equivalents (0–10 equivalents) of hydrogen peroxide to a solution containing AoCO4 (0.1 mM). Difference spectra of AoCO4 were obtained by subtracting a spectrum of the native AoCO4 from that of H_2O_2 treated AoCO4. The percentage of *oxy*-form present in the native form of AoCO4 was calculated as relative to the 100% *oxy*-form of AoCO4 generated after addition of 10 equivalents of H_2O_2 using the following equation:

$$oxy - \textbf{form in native AoCO4 (\%)} = \frac{\left(\frac{A350}{A280}\right) \textbf{ of native AoCO4}}{\left(\frac{A350}{A280}\right) \textbf{ of 10 eq.} H_2O_2 \textbf{ treated AoCO4}} * \textbf{100}$$

Crystallisation experiments were performed via the hanging-drop vapour-diffusion method in Linbro 24-well plates and the commercial screens Crystal Screen (Hampton Research) and Clear Strategy Screen (Molecular Dimensions) as described by Kaljunen et al. (2011). Several optimisation screens were done and the best measurable crystals grew in a solution containing 12% PEG 8000, 10% ethanol, 0.2 M magnesium chloride, and 0.1 M acetate buffer, pH 4.5. The X-ray data from crystals of a preparation containing both processed and non-processed forms of AoCO4 were collected at DESY bean line X12, whereas the X-ray data from crystals of a preparation containing only the processed form of AoCO4 were collected at ESRF beam line ID14-1. X-ray data analysis of these crystals is still in progress.

3.5.2 Biochemical characterisation of tyrosinase from *Trichoderma reesei* (Publications I and IV)

The substrate specificity of TrT and AbT was analysed on small mono- and diphenolic compounds and tyrosine-containing tripeptides. Kinetic constants K_M and V_{max} of TrT and AbT with the small mono- and diphenolic compounds were determined in line with the enzymatic reaction by spectrophotometer as described in Section 3.3. The enzyme concentrations of TrT and AbT were 3.8 µg mL^{-1} and 3.1 µg mL^{-1}, respectively for the monophenols and 0.9 µg mL^{-1} and 0.8 µg mL^{-1}, respectively for the diphenols. Kinetic constants K_M, V_{max} and k_{cat} for TrT and AbT with the tyrosine-containing peptides were determined on the basis of the consumption of the co-substrate oxygen during the enzymatic reaction. The enzyme concentrations of TrT and AbT were 22 µg mL^{-1} and 17 µg mL^{-1}, respectively.

The oxidation of L-DOPA by TrT and AbT was also followed by mass spectrophotometry, using a 4.7-T Bruker APEX-Qe Fourier-transform ion cyclotron resonance (FT–ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in the negative mode. Ions of interest were further isolated by mass-selective quadrupole, and

subsequently irradiated with IR-photons for infrared multiphoton dissociation (IRMPD). Enzymatic oxidation of L-DOPA to dopachrome by tyrosinases was carried out at 25 ºC in 0.1 M ammonium acetate solution pH 7.0. The reaction was started by an addition of the tyrosinase (15 µg mL^{-1}) to L-DOPA (1 mM) solution, and the formation of dopachrome was followed by mass spectrophotometry at predetermined time intervals.

To evaluate the product inhibition of TrT, dopachrome was prepared via 30 minutes' enzymatic oxidation of 5 mM L-DOPA with AbT (0.7 mg mL^{-1}) at 25 ºC in 0.1 M sodium acetate buffer, pH 5.6. The enzyme was removed by means of ultracentrifugation in a Vivaspin concentrator (20 mL, 5000-Da MWCO; Vivascience, Hannover, Germany). The concentration of the dopachrome formed in the solution was calculated from the intensity of absorbance at 475 nm, with the extinction coefficient $\varepsilon_{dopachrome}=3400$ M^{-1} cm^{-1}. Dopachrome product inhibition of TrT was analysed by oxygen consumption measurements at 25 ºC in 0.1 M sodium acetate buffer, pH 5.6. The reaction mixture typically contained 8 mM L-DOPA and had a varying concentration of dopachrome (from 0 to 1.5 mM). The enzyme concentration of TrT was 7 µg mL^{-1}.

The inhibition of TrT by potassium cyanide, sodium azide, arbutin, kojic acid, benzaldehyde, and benzoic acid was analysed with caffeic acid and *p*-coumaric acid used as substrates. The reactions were carried out in 0.1–15 mM substrate and 0–60 mM inhibitor concentrations at 25 ºC in 0.1 M phosphate buffer, pH 7.0. The enzyme concentrations were 3 µg mL^{-1} for caffeic acid and 6 µg mL^{-1} for *p*-coumaric acid oxidation. The initial rate of product formation was determined as the increase of absorbance at wavelength 480 nm per minute for caffeic acid and at 360 nm per minute for *p*-coumaric acid. The type of inhibition and the K_i constant for each inhibitor were determined, with the data plotted as Lineweaver-Burk and Dixon plots. The Cornish plot was used for determination of the K_i' constant.

3.5.3 Determination of kinetic parameters of laccases from *Thielavia arenaria, Melanocarpus albomyces,* and *Trametes hirsuta* (Publication III)

Kinetic parameters K_M and V_{max}, for the laccases *r*MaL, TaLcc1, and ThL were determined at 25°C with 2,6-DMP, syringic acid, and methyl syringate both in 25 mM succinate buffer, pH 4.5 and in 40 mM MES buffer, pH 6.0. Kinetic measurements were performed by spectrophotometric assay, following the product formation at specific wavelengths as described in Section 3.3. The kinetic parameters were calculated via curve-fitting analysis by means of the GRAPHPAD PRISM 4.01 program (GraphPad Software Inc., San Diego, CA, USA). The pH optima of the three laccases were measured at 25 °C with a substrate concentration of 1.7 mM in McIlvaine's universal buffer at pHs from 2 to 8.

3.5.4 Enzymatic cross-linking of α-caseins by catechol oxidase, tyrosinases, and laccases (Unpublished results)

The ability of catechol oxidase (AoCO4), tyrosinases (TrT and AbT), and laccases (TaLcc1, *r*MaL, and ThL) to cross-link model protein α-caseins was studied as described by Selinheimo et al. (2007b), with few modifications. α-Casein proteins were dissolved in

0.1 M phosphate buffer, pH 7.0, at a concentration of 3 g L^{-1}. Also studied was the potential of a cross-linking agent, i.e., a small phenolic molecule to induce cross-linking reactions. Catechol (2 mM) was used a cross-linking agent in the cross-linking experiments with catechol oxidase- or tyrosinase, whereas *p*-coumaric acid (2 mM) was the cross-linking agent in the experiments with laccase. The enzyme dosages were 10, 100 and 1000 nkat g^{-1} of α-caseins. The activity of AoCO4, TrT, and AbT was measured on catechol, while that of TaLcc1, *r*MaL, and ThL on ABTS. The corresponding amounts of protein used were 1, 10, and 100 mg of AoCO4 g^{-1} of α-caseins, 0.016, 0.16, and 1.6 mg of TrT g^{-1} of α-caseins, 0.011, 0.11, and 1.1 mg of AbT g^{-1} of α-caseins. The enzymatic incubations with α-caseins were performed at 25 ℃ for two and 24 hours, after which the samples were transferred to 95 °C for ten minutes for inactivation of the enzymes. The enzymes' efficiency in cross-linking the α-caseins was monitored through SDS-PAGE analysis with Criterion strain-free 4–20% gradient gels (BioRad).

4. Results and discussion

4.1 Biochemical and structural characterisation of a catechol oxidase from *Aspergillus oryzae* (Publication II and unpublished results)

4.1.1 Discovery and production of *Aspergillus oryzae* catechol oxidase

A bioinformatics approach was used to discover novel fungal extracellular tyrosinases. The VTT in-house data base of public genomes of fungi (Arvas et al. 2007) was searched for sequences containing the tyrosinase Interpro entry (IPR002227) and an N-terminal signal sequence. In total, 134 sequences were retrieved, of which 114 sequences had the HA1-X(n)-HA2-X(8)-HA3 and HB1-X(3)-HB2-X(n)-HB3 pattern for the CuA and CuB site of tyrosinases and catechol oxidases. The 114 sequences clearly fell into two groups on the basis of their length. Proteins with a length of 500–800 amino acids were named "long tyrosinases" while proteins with a length 300–500 amino acids as "short tyrosinases". Both long and short tyrosinases contained the conserved residues for tyrosinases, the conserved N-terminal Arg residue, the C-terminal "tyrosine motif" (Y-X-Y/F or Y/F-X-Y), and the two conserved His patterns, that co-ordinate the two copper ions at the active site of tyrosinases and catechol oxidases (Flurkey & Inlow 2008).

Three main differences between the long and short tyrosinases were found: a) the histidine pattern of CuA was HA1-X(7)-HA2-X(8)-HA3 in the short tyrosinases, whereas it was HA1-X(n)-HA2-X(8)-HA3, with n=20–23 amino acids in the long tyrosinases; b) the short tyrosinases presented a stop codon a few residues after the conserved C-terminal "tyrosine motif" and, accordingly, the linker region and the C-terminal domain were absent in these sequences; and c) a novel conserved pattern of six Cys was found in the short tyrosinases, but not in the long tyrosinases (**Figure 9**). The short tyrosinase sequence from *Aspergillus oryzae* (AoCO4; UniProtKB: Q2UNF9; Entrez gene ID: 5990879) had 14.7% overall amino acid sequence homology with the sequence of *Trichoderma reesei* tyrosinase (TrT), which has been shown to have excellent cross-linking properties (Selinheimo et al. 2007b). The *A. oryzae* short tyrosinase gene was chosen for cloning and expression as a representative of short putative tyrosinase proteins.
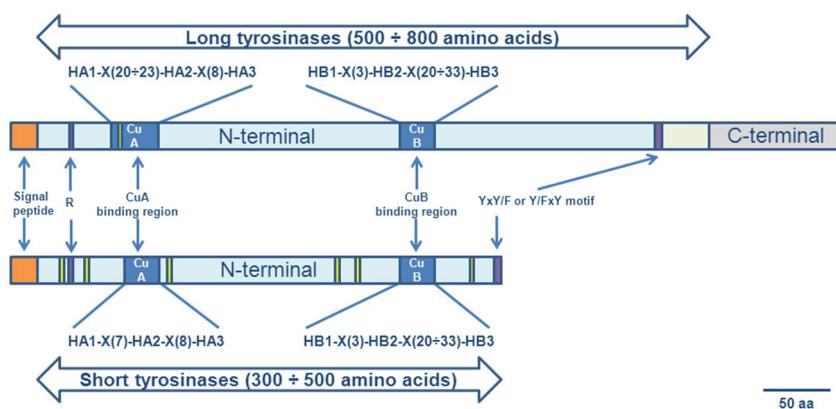
Figure 9. Schematic representation of the domains and the conserved residues of long and short tyrosinase sequences. Colour legend: signal peptide in orange, N-terminal domain in light blue, linker region in light green, C-terminal domain in light grey, copper-binding regions in blue, conserved Arg (R), conserved "tyrosine motif" (Y-X-Y/F or Y/F-X-Y) in purple, conserved Cys in yellow. HA1, HA2, and HA3 are the His residues co-ordinating the first copper ion (CuA), HB1, HB2, and HB3 are the His residues co-ordinating the second copper ion (CuB), and X is a generic amino acid residue.

The *A. oryzae* short tyrosinase gene was heterologously expressed in *T. reesei*. Transformants were first selected on plates containing L-tyrosine (55 mM) and copper (0.1 mM) and subsequently grown in shake flasks in medium supplemented with copper. Positive transformants grown on plates showed a black colour around the colonies, which indicates an oxidation of L-tyrosine. Differently, the transformants grown in liquid cultures did not show a significant activity on L-tyrosine and a high background was found in measurement of the activity of the parental strain on L-DOPA. Liquid cultures were thus selected on the basis of their activity on catechol. Despite the indications of tyrosine oxidation on the plate cultivation, the protein produced did not in the end show activity on L-tyrosine and L-DOPA, which are typical substrates for tyrosinases. Instead, it showed activity on catechol, which is a typical substrate of catechol oxidases. Therefore, the protein was classified as a catechol oxidase and named AoCO4. Production of AoCO4 was first optimised in shake flasks in different copper concentrations (0–6 mM), and subsequently AoCO4 was produced in a 10 L bioreactor in inducing medium at pH 5.5 with 0.5 mM copper. Expression of AoCO4 resulted in high yields, approximately 1.5 g L^{-1} protein.

4.1.2 Purification and characterisation of *Aspergillus oryzae* catechol oxidase

AoCO4 was purified with a two-step ion-exchange chromatography. The buffer-exchanged culture filtrate was applied to cation-exchange chromatography, with the catechol-active protein eluted in two fractions, at 60 and 90 mM salt concentration. The

main activity was eluted at 90 mM salt concentration. Both fractions were further purified via strong anion-exchange chromatography. The MALDI-TOF MS analysis of purified AoCO4 from the fraction eluted at 90 mM resulted in a molecular weight (MW) of 39.3 kDa, and the fraction eluted at 60 mM in a mixture of MWs 39.3 and 40.5 kDa. The first amino acid in the N-terminus of the shorter form of AoCO4 was Gly45, whereas the fraction eluted at 60 mM salt concentration contained equimolar concentrations of proteins starting with Ala1 and Gly45. Interestingly, a Kex2/furin-type cleavage site was identified in AoCO4 (Lys43-Arg44). Kex2/furin-type proteases are known to be involved in the maturation of proteins in the Golgi apparatus of *T. reesei* (Goller et al. 1998). The processed form had the conserved Arg residue (Arg55) after the Kex2/furin-type cleavage site in the sequence. This conserved Arg residue is part of a short strand that forms a parallel β-sheet with the conserved "tyrosine motif". The parallel β-sheet is structurally conserved in tyrosinases and catechol oxidases and it is important for keeping together the N-and C-terminal ends of the polypeptide chain (Flurkey & Inlow 2008). The results suggest that AoCO4 is a processed protein, from which the first 69 amino acids are cleaved. However, for some reasons the processing was not complete and also a small fraction (0.2%) of non-processed protein was found from the culture supernatant. It should be pointed out that both forms had similar specific activity on catechol. The work was continued with the processed from of AoCO4.

AoCO4 had a pH optimum in the acidic pH range (pH optimum 5–7), which is similar to the pH optimum of the fungal catechol oxidase from *Alternaria tenuis,* but different from the pH optima of catechol oxidases of plant origin, e.g., the catechol oxidase from *Ipomea batatas* (IbCO) has the pH optimum at 7.8 (Eicken et al. 1998). AoCO4 retained 50% of its activity after two hours incubation at 60 °C (pH 7.2). Thus, in the tested conditions AoCO4 was more thermostable than *A. tenuis* catechol oxidase, which is inactivated after 30 minutes of incubation at 60 °C (pH 5.2) (Motoda 1979a, Motoda 1979b).

AoCO4 was found to be active on the diphenolic substrates catechol, caffeic acid, hydrocaffeic acid and 4-*tert*-buthylcatechol (TBC), and on the monophenolic substrates aminophenol and guaiacol. AoCO4 was found not to be active on L-tyrosine and L-DOPA. Furthermore, AoCO4 showed weak activity on phenol, tyrosol, *p*-creasol, and aniline. The affinity of AoCO4 for all tested substrates was generally low. AoCO4 showed the highest affinity to catechol and lower affinity to caffeic acid, hydrocaffeic acid and TBC. Similarly low affinity of plant catechol oxidases from *I. batatas* and *Lycopus europaeus* have been reported for catechol (K_M=5 mM for both catechol oxidases) (Eicken et al. 1998, Rompel et al. 1999b). The turnover number (k_{cat}) and the specificity constant (k_{cat}/K_M) of AoCO4 were significantly lower than the k_{cat} of *L. europeus* and *I. batatas* for these substrates (data not shown). Hydrocaffeic acid is a structural analogue to caffeic acid, but it lacks the conjugation in the lateral chain. It has been suggested that substrates lacking this conjugation act as substrate analogues of tyrosinases and catechol oxidases and inhibit the enzyme (Fan & Flurkey 2004). Rompel et al. (1999b) reported that a catechol oxidase from *L. europeus* has high affinity on hydrocaffeic acid (K_M=4.3 mM), which is lower than the K_M measured for AoCO4 by a factor ten. It is possible that hydrocaffeic acid is more a substrate analogue of AoCO4 rather than a substrate of AoCO4. A summary of the characteristics of AoCO4 is shown in **Table 9**.

Table 9. Characteristics of the *Aspergillus oryzae* catechol oxidase (AoCO4).

Property	Result	Methods
Molecular weight	39.3 kDa (40.5 kDa) [a]	MALDI TOF-MS
N terminus	Gly45 (Ala1) [a]	N-terminal sequencing
Isoelectric point	5.2	Isoelectric focusing
pH Optimum	pH 5–7	Activity measurements
pH Stability	> 75% Activity retained in the pH range 5–9 after 48 hours of incubation	Activity measurements
$T_{1/2}$	20 Hours at 50 °C (pH 7) 2 hours at 60 °C (pH 7)	Activity measurements
T_m	70 °C	Circular dichroism
Compounds with which AoCO4 showed activity	Catechol (K_M=3.3±0.9 mM), caffeic acid (K_M=7.9±1.2 mM), hydrocaffeic acid (K_M=52.4±6.5 mM), TBC, aminophenol and guaiacol	Activity measurements
Compounds with which AoCO4 did not show activity	L-DOPA, D-DOPA, dopamine, L-tyrosine, tyramine, phloretic acid, 4-mercaptoethanol and ABTS	Activity measurements
Active site	Type 3 copper site Absorption maximum at 350 nm A dioxygen moiety bridged to CuA and CuB	UV/VIS spectroscopy; crystallography
Secondary structure	Negative peaks at 200 nm and 230 nm in the far-UV spectrum, indicating a helical structure Maximum intensity at 330 nm when AoCO4 was excited at 280 nm, indicating the presence of exposed aromatic residues on the surface of the protein	Circular dichroism Fluorescence spectroscopy
Tertiary structure	2.5 Å Resolution Monomer Four-helix-bundle core domain Three disulphide bridges Four glycosylation sites (Asn30, Asn104, Asn222, and Asn238)	X-ray data of crystals formed in hanging-drop method

[a] The molecular weight and the N-terminus of the non-processed form of AoCO4 are shown in parenthesis.

4.1.3 Crystal structure and spectroscopic features of *Aspergillus oryzae* catechol oxidase

A crystal structure of AoCO4 was solved at 2.5 Å resolution from the crystals obtained from a preparation containing both the non-processed and the processed form of the enzyme. The overall structure of AoCO4 was mainly α-helical (**Figure 10**). The folding of AoCO4 was stabilised by the three disulphide bridges, which are conserved with the short tyrosinases (**Figure 9**, **Table 10**). The core of the enzyme was formed by a four-helix bundle (α3, α4, α8 and α9) where the catalytic site is found. The two copper ions in the catalytic centre each were co-ordinated each by three His residues. The first His residue that co-ordinates the CuA (HA1) was found to be His102, making the His pattern HA1-X(7)-HA2-X(8)-HA3, which is distinctive of the short-tyrosinase sequences. However, the sequence of AoCO4 contained two putative His residues (His86 and His102), and either of these His residues could hypothetically be the HA1 residue that co-ordinates the CuA copper ion. If the residue His86 co-ordinates CuA, the sequence of AoCO4 would be compatible with the long-tyrosinases.
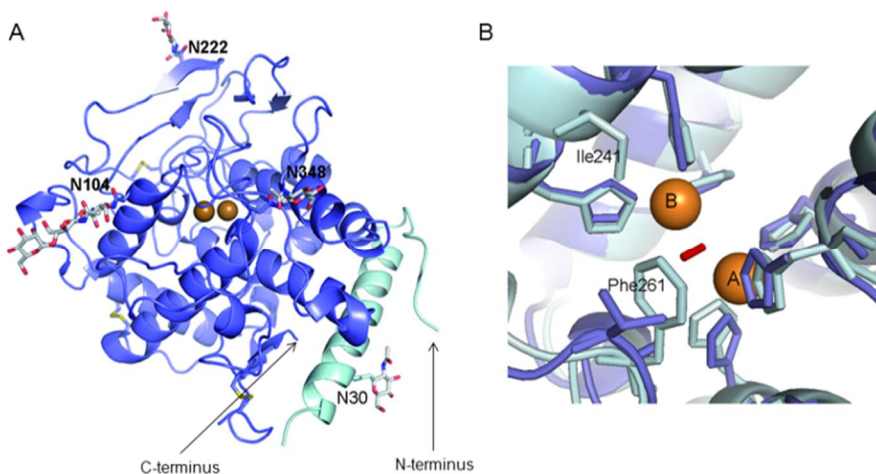
Figure 10. (A) A cartoon representation of the structure of the catechol oxidase from *Aspergillus oryzae* (AoCO4). The extra helix of the non-processed form of AoCO4 is shown in light blue. The copper ions at the active site are shown as orange spheres. (B) Comparison of the active site of *A. oryzae* catechol oxidase (blue) and *Ipomea batatas* catechol oxidase (light blue). The residues Phe241 and Ile241 of *I. batatas* catechol oxidase are also indicated. The copper ions in the active site are shown as orange spheres and the dioxygen moiety in red. (Unpublished results.)

The distance between CuA and CuB in the active site of AoCO4 was 4.1 Å and an elongated electron density was observed amidst CuA and CuB. A dioxygen moiety was found as bridging molecule between the two copper ions. The active site was studied also by spectroscopy. The UV/VIS absorption spectrum of AoCO4 showed a distinct maximum absorbance at 350 nm (**Figure 11**), suggesting that peroxide was present in the active

site of AoCO4 as reported for the *oxy*-forms (i.e., the enzyme form active on monophenols) of catechol oxidases and tyrosinases (Solomon et al. 1996). The *oxy*-form of catechol oxidases can be generated by addition of hydrogen peroxide (H_2O_2) to the enzyme, and it has been shown to give a maximum absorption at 350 nm, whereas the *met*-form of the enzyme does not show a clear maximum of absorbance at this wavelength (Jolley et al. 1974, Rompel et al. 1999a, Rompel et al. 2012). The UV/VIS absorption spectrum of AoCO4 showed a clear maximum at 350 nm without any H_2O_2 addition; however, addition of H_2O_2 transformed the enzyme totally to *oxy*-form. Five equivalents of H_2O_2 where needed for saturating AoCO4 to its oxidised form. From the ratio of the intensities of the absorbance at 350 nm of the untreated and treated enzyme, it was calculated that 35% of the untreated AoCO4 was in the *oxy*-form. This is significantly higher than the values reported for tyrosinases and catechol oxidases. For example, approximately 10–15% of AbT is in the *oxy*-form (Solomon et al. 1996). Despite 35% of the AoCO4 being in the *oxy*-form, AoCO4 did not have significant activity on monophenols, suggesting that the active site geometry and the essential amino acids around the active site determine the ability to oxidise monophenols.

The first three-dimensional structure of a catechol oxidase and of a tyrosinase were revealed from the plant *Ipomea batatas* (IbCO) (Klabunde et al. 1998) and the bacterium *Streptomyces castaneoglobisporus* (ScT) (Matoba et al. 2006), respectively. More recently, the three-dimensional structures of the bacterial tyrosinase from *Bacillus megaterium* (BmT) (Sendovski et al. 2011) and of the fungal tyrosinase from *Agaricus bisporus* (AbT) (Ismaya et al. 2011) were published. The reason catechol oxidases are not able to oxidise monophenols has been suggested to be related to a more restricted access of the substrates to the CuA site at the active site.

A Phe residue in IbCO (Phe261) clearly blocks the access of a substrate to the CuA at the active site, and it has been defined as a gatekeeper residue. Differently, in ScT the access to the active site is more open due to a smaller Gly residue (Gly203) instead of the Phe261 residue in IbCO. In the solved three-dimensional structure of ScT the active site was found to be occupied by a Tyr residue (Tyr98), which belongs to the co-crystallised caddie protein. This residue (Tyr98) was also suggested to function as a gatekeeper residue, keeping the active site of ScT occupied and thus preventing the binding of a substrate. A Val residue is present in BmT (Val218) and AbT (Val283) in the location of the gatekeeper residue (Phe261 in IbCO). A Val residue is less bulky than a Phe residue, so the active site of BmT and AbT is more accessible than the active site of IbCO. In the AoCO4 structure a Val residue (Val299) was found at the gatekeeper position, similarly to BmT and AbT. Despite of that, AoCO4 did not show a monophenolase activity on L-tyrosine, and t it was classified as a catechol oxidase.

Klabunde et al. (1998) suggested that a conserved Glu residue (Glu236 in IbCO) may assist in deprotonating the substrate at the active site of catechol oxidases and tyrosinases (**Figure 12** and **Table 10**). Similarly to IbCO, a Glu residue is present in the structures of BmT (Sendovski et al. 2011), ScT (Matoba et al. 2006), and AbT (Ismaya et al. 2011) tyrosinases, whereas a Gln residue is present in the human tyrosinase and a Leu residue in *Neurospora crassa* tyrosinase (Klabunde et al. 1998). In AoCO4 a Gln residue (i.e., Gln273) was found, as in human tyrosinase, but unlike BmT, ScT, and AbT.
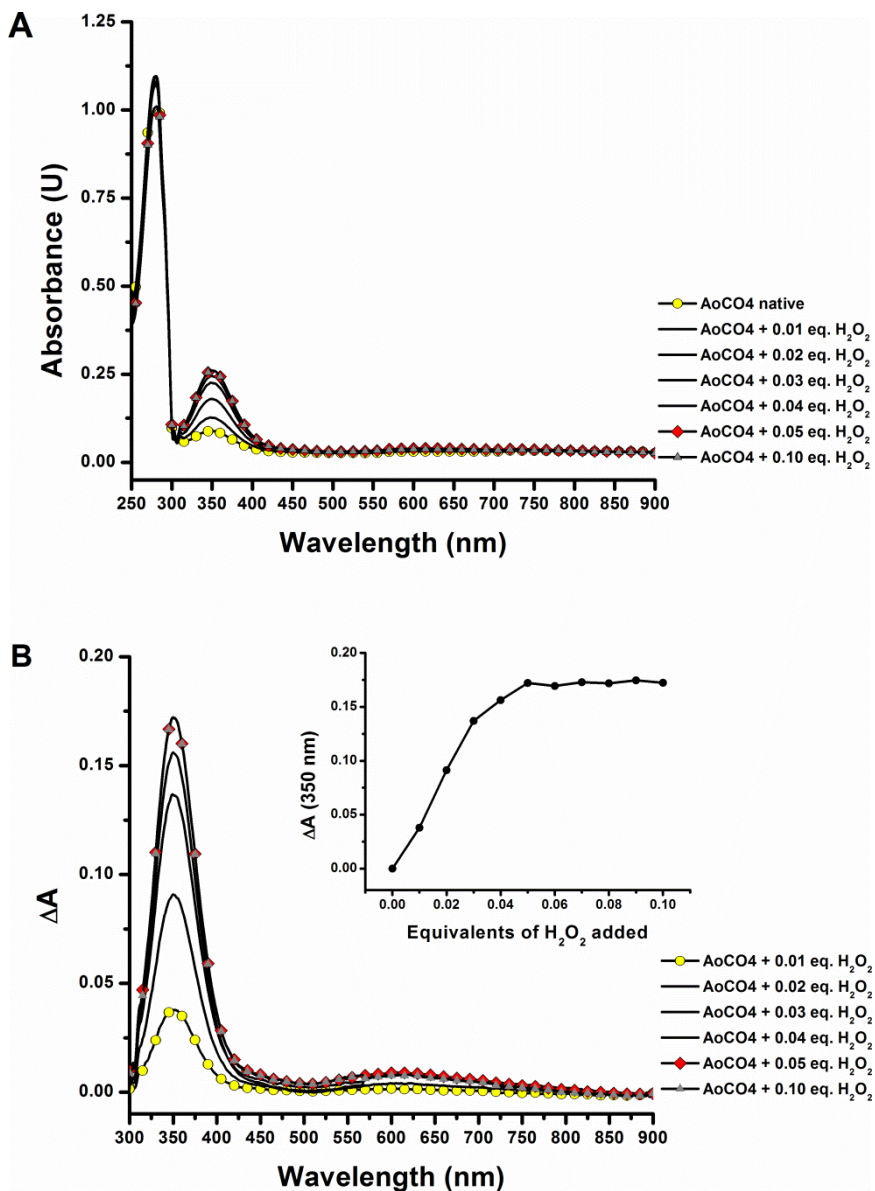
Figure 11. UV/VIS absorption spectra of *Aspergillus oryzae* catechol oxidase (AoCO4) and AoCO4 titrated with hydrogen peroxide (H_2O_2) (A) UV/VIS absorption spectra of the untreated AoCO4 and of the H_2O_2 treated AoCO4. The enzyme concentration was 30 µM and H_2O_2 was added to AoCO4 in 1–10 equivalents (eq.). Spectra were recorded in 10 mM Tris-HCl buffer, pH 7.2 by means of a Varian Cary 100 Bio spectrophotometer immediately after the addition of H_2O_2. (B) Difference spectra of AoCO4: H_2O_2 treated minus untreated enzyme. In the inset, the difference in absorbance (ΔA) at 350 nm is reported as a function of equivalents of H_2O_2 added. (Unpublished results)

```
AoCO4   MVALQALSLGLLASQALAFPAASQQAATATLPTTASSSTAVASSQLDQLANFAYNVTTDSV   36
BmT     ............................................................
ScT     ............................................................
IbCO    ...........................APIQAPEISKCVVPPADLPPGAVVDNCCPPV     31
VvCO    ...........................APIQAPDISKCGT..ATVPDGVTPTNCCPPV     29
AbT     ............................................................

AoCO4   AGGSESKRGGCTLQNLRVRRDWRAFSKTQ..KKDYINSVLCLQKLPSRTPAHLAPGARTRY   95
BmT     .........MSNKYRVRKNVLHLTDTE..KRDFVRTVLILKEKGIYD..............   36
ScT     ...MT......VRKNQATLTADE..KRRFVAAVLELKRSGRYD.................   32
IbCO    ASNIVDYKLP.AVTTMKVRPAAHTMDKDA..IAKFAKAVELMKALPADDP........RNF   81
VvCO    TTKIIDFQLPSSGSPMRTRPAAHLVSKEY..LAKYKKAIELQKALPDDDP........RSF   80
AbT     SDKKSLMPLVGIPGEIKNPLNILDFVKNDKFFTLYVRALQVLQARDQSDY........SSF   54

AoCO4   DDFVATHINQT..................QIIHYTGTFLAWHRYFIYEFEQALRDECS      135
BmT     .RYIAWHGAAG...........KFHTPPGSDRNAAHMSSAFLPWHREYLLRFERDLQSIN    84
ScT     .EFVRTHNEFIMSDTDSG.......ER.TGHRSPSFLPWHRRFLLDFEQALQSVD        78
IbCO    YQQALVHCAYCNGGYDQV...NFPDQEIQVHNSWLFFPFHRWYLYFYERILGKLIG..    134
VvCO    KQQANVHCTYCQGAYDQV...GYTDLELQVHASWLFLPFHRYYLYFNERILAKLID..    133
AbT     FQLGGIHGLPYTEWAKAQPQLHLYKANYCTHGTVLFPTWHRAYESTWEQTLWEAAGTVAQR  115

AoCO4   ..............YTGDYPYWNWGAD..........ADNMEKSQVFDGSETSMSGNGEYIPNQG  176
BmT     ..........PEVTLPYWEWETDAQMQDPSQSQIWSADFMGGNGNPIKD...FIVDTG      129
ScT     ..........SSVTLPYWDWSADRTVR......ASLWAPDFLGGTGRSTDG...RVMD.G    118
IbCO    ..........DPSFGLPFWNWDN.........PGGMVLPDFLNDSTSSLYD...SNRNQS    172
VvCO    ..........DPTFALPYWAWDN.........PDGMYMPTIYASSPSSLYD...EKRNAK    171
AbT     FTTSDQAEWIQAAKDLRQPFWDWGYWP......NDPDFLPDQVIRQDKVRPDIVTDYN      166

AoCO4   DIKLLLGNYPAIDLPPGSGGGCVTSGPFKDYKLNLGPAALSLPGGNMTAAANPLTYNPRCM  237
BmT     PFAA..GRWTTIDEQGNPSGGLKRN.........FGATKEAPTLPTRDDVLNALKITQYDTPP  181
ScT     PFAASTGNWP.INVRVDSRTYLRRS.........LGGS..VAELPTRAEVESVLAISAYDLPP  169
IbCO    HLPP.....VVVDLGYNGADTDVTD.........QQRITDNLALMYKQMVTNAGTAELFLGKA  221
VvCO    HLPP......TVIDLDYDGTEPTIPD.........DELKTDNLAIMYKQIVSGATTPKLFLGYP  220
AbT     GTKIEVEN.PILHYKFHPIEPTFEG...DFAQWQTTMRYPDVQKQENIEGMIAGIKAAAPG    223

AoCO4   KRSLTTEILQRYNTFPKIVELILDSDDIWDFQMTMQGVPGSGSIGVHGGGHYSMGGDP...  295
BmT     WDMTSQNSFR......................NQLEGFINGPQ..LHNRVHRWVG......  212
ScT     YNSASEG.FR......................NHLEGWR.GVN..LHNRVHVWVG......  198
IbCO    YRAGDAP.........................SPGAGSIETSP.....HIPIHRWVGD...PR  251
VvCO    YRAGDAI.........................DPGAGTLEHAP.....HNIVHKWTG....LA  249
AbT     FREWTFNMLTKNYTWELFSN...HGAVVGAHANSLE........MVHNTVHFLIGRDPTLD   273

AoCO4   ......GRDVYVSPGDTAFWLHHGMIDRVWWIWQNLDLRKRQNAISGTGTFMNNPASPNTT  350
BmT     ...GQMGVVPTAPNDPVFFLHHANVDRIWAVWQII...HRNQNYPGMKNGPFGQNFRDP.   265
ScT     ...GQMATG.VSPNDPVFWLHHAYVDKLWAEWQRR...HPDSAYVPTGGTPVDVDLNET.   250
IbCO    NTNNEDMGNFYSAGRDIAFYCHHSNVDRMWTIWQQLAGKPRKRDYTDSDWLNATFLFYDE.   311
VvCO    DKPSEDMGNFYTAGRDPIFGHHANVDRMWNIWKTIGGKNRK.DFTDTDWLDATFVFYDE.   308
AbT     PLVPGHMGSVPHAAFDPIFWMHHCNVDRLLALWQTMNYDVYVSEGMNREATMGLIPGQVLT   334

AoCO4   LDTVIDLGYANGGPIAMRDLMSTTAGPFCYVVYL.............              383
BmT     .........MYPWNTT.PEDVMHRKLGYVVYDIEL.......RKSKRSSHHHHHH.       303
ScT     .........MKPWNTVRPADLLDHTAY.YTFD.........ALEHHHHHH.            281
IbCO    .........NGQAVKVRIGDSLDNQKMGYKYAK.........TPLFWLDSKP.          345
VvCO    .........NKQLVKVKVSDCVDTSKLRYQYQD.........IPIPWLP.            339
AbT     EDS.PLEPFYTKNQDPWQSDDLEDWETLGFYPDFDPVKGKSKEEKSVYINDWVHKHYG     392
```

Figure 12. Amino acid sequence alignment of *Aspergillus oryzae* catechol oxidase (AoCO4; UniPROTKB: Q2UNF9), *Bacillus megaterium* tyrosinase (BmT; UniProtKB: B2ZB02), *Streptomyces castaneoglobisporus* tyrosinase (ScT; UniProtKB: Q83WS2), *Ipomea batatas* catechol oxidase (IbCO; UniProtKB: Q9ZP19), *Vitis vinifera* catechol oxidase (VvCO; UniProtKB: P43311), and *Agaricus bisporus* tyrosinase (AbT; UniProtKB: C7FF04). The copper-co-ordinating His residues are marked in red, the residues delimiting the core region in green, the disulphide bonds in yellow, the N-terminus in light blue. The triangles indicate the conserved residues involved in the interaction between C- and N-termini, the star the conserved proton donator residue. The numbering of the sequences was done according to their structures. The figure was produced by means of ALINE (Bond & Schuttelkopf 2009). (Unpublished results)

Siegbahn (2004) suggested a different reaction mechanism for catechol oxidases, wherein the oxygen could be bound to the active site as hydroperoxide during the catalytic cycle and in which no external base/acid is needed for proton acceptor and donor. The preliminary structure and the UV/VIS spectroscopic data for AoCO4 may support the reaction mechanism proposed by Siegbahn (2004). Further studies on the active site of AoCO4 are in progress and may elucidate the critical differences in the catalytic cycles of catechol oxidases and tyrosinases.

Table 10. Structural properties of *Aspergillus oryzae* catechol oxidase (AoCO4) as compared to the known structures of tyrosinases and catechol oxidases, i.e., *Bacillus megaterium* tyrosinase (BmT), *Streptomyces castaneoglobisporus* tyrosinase (ScT), *Ipomea batatas* (IbCO), *Vitis vinifera* catechol oxidase (VvCO) and *Agaricus bisporus* tyrosinase (AbT). (Unpublished results.)

Property	AoCO4	BmT	ScT	IbCO	VvCO	AbT
Sequence identity of AoCO4		27.2%	26.5%	21.8%	21.6%	21.6%
PDB ID		3NM8	1WX2	1BT1	2P3X	2Y9W
Core region	R55–Y382	R8–Y286	R4–Y333	R49–Y333	R48–Y330	R20–Y365
Four-helix bundle	α3 (R94–Q105) α4 (G113–C134) α8 (I281–290) α9 (T306–Q331)	α2 (I34–G46) α3 (F65–I83) α7 (L203–V211) α8 (V226–I244)	α2 (R30–S44) α3 (F59–V77) α6 (H190–V197) α7 (V211–R229)	α2 (F81–C92) α3 (F114–L132) α6 (H240–V247) α7 (I268–Q285)	α4 (F80–C91) α5 (F113–L131) α12 (H239–T246) α14 (F268–I285)	α3 (F54–H61) α4 (L89–R115) α10 (L255–D269) α11 (P290–M309)
His co-ordinating CuA	H102 (α3) H110 H119 (α4)	H42 (α2) H60 H69 (α3)	H38 (α2) H54 H63 (α3)	H88 (α2) H109 H118 (α3)	H87 (α4) H108 H117 (α5)	H61 (α3) H85 H94 (α4)
His co-ordinating CuB	H284 (α8) H288 (α8) H312 (α9)	H204 (α7) H208 (α7) H231 (α8)	H190 (α6) H194 (α6) H216 (α7)	H240 (α6) H244 (α6) H274 (α7)	H239 (α12) H243 (α12) H272 (α14)	H259 (α10) H263 (α10) H296 (α11)
Disulphide bonds	C39–C379 C75–C134 C198–C236	–	–	C11–C28 C27–C89	C11–C26 C25–C88	–
Thioether bond	–	–	–	C92–H109	C91–H108	C83–H85
Tyrosine motif	Y380–V381–Y382	Y284–V285–Y286	Y269–T270–F271	Y331–K332–Y333	Y328–Q329–Y330	F363–S364–Y365

Property	AoCO4	BmT	ScT	IbCO	VvCO	AbT
π Cation interaction	R55–Y380	R8–Y284	R4–Y269	R49–Y331	R48–Y328	R20–F363
Conserved R and residues forming a hydrogen bond with it	R55 D272	R8 D235	R4 D220	R49 D278	R48 D276	R20 D300, E102, Y365
Putative residues involved in assisting in the substrate deprotonation	Q273	E195	E181	E236	E235	E256

4.2 Detailed characterisation of the tyrosinase from *Trichoderma reesei* and comparison with the tyrosinase from *Agaricus bisporus* (Publications I and IV)

4.2.1 Characterisation of the substrate-specificity of tyrosinases from *Trichoderma reesei* and *Agaricus bisporus*

Among the studied tyrosinases, the extracellular tyrosinase from *Trichoderma reesei* (TrT) has been shown to have unique ability for cross-linking of proteins, and therefore TrT has potential application in protein cross-linking for industrial purposes (Selinheimo et al. 2007b). The fungal tyrosinase from *Pycnoporus sanguineus* can also cross-link proteins, but less efficiently than TrT. Unlike TrT, the commercial tyrosinase from *Agaricus bisporus* (AbT) and the plant tyrosinases from potato and apple can cross-link proteins only in the presence of a small diphenolic substrate acting as a cross-linking agent (Selinheimo et al. 2007b). A detailed characterisation of the substrate-specificity of TrT and AbT was carried out to increase understanding of the substrate-specificity of tyrosinases and thus also of the applicability of tyrosinase enzymes.

Tripeptides containing a Tyr residue in different positions (GGY, GYG, and YGG) and L-tyrosine (Y) were used as a model for peptide-bound Tyr residues. The K_M and V_{max} values for TrT and AbT for the tripeptides and L-tyrosine were determined by following of the consumption of the co-substrate molecular oxygen via polarography (**Table 11**). TrT and AbT performed differently on these model substrates. TrT showed very low affinity for Y and the kinetic parameters could not be determined (Figure 3 in Publication I), whereas the K_M values of AbT for the peptides were more than a factor ten lower than those of TrT. However, the turnover numbers (k_{cat}) were relatively similar between the two enzymes. TrT had lower affinity on YGG than on the GGY or GYG, and the highest k_{cat} was seen for GGY. With AbT the K_M and k_{cat} values were relatively similar to those with the three peptides. Clearly, therefore, the position of Y in the peptides affected the affinity and the reaction rate of TrT, whereas with AbT the position of Y in the peptide did not have a worthy effect. Furthermore, the lag periods (i.e., the time before the reaction products of monophenolase activity are produced at a linear/maximal rate) were shorter for TrT than for AbT and were not affected by the position of Y in the peptides.

The reaction end-products of the TrT- and AbT-catalysed oxidation of the three peptides were similar between the two tyrosinases. The reaction product of tyrosinase-catalysed oxidation of Y or YGG showed maximum absorbance at 475 nm, suggesting that the reaction end product is a dopachrome-related product. In contrast, the reaction product of tyrosinase-catalysed oxidation of GYG or GGY did not show a clear maximum at 475 nm, thus suggesting that a dopachrome-related product was not formed in significant quantities (Figure 4 in Publication I). The amino group of the Tyr residue of GYG and GGY is fixed in the peptide chain, so it is not free to take part in internal nucleophilic attack leading to the formation of a dopachrome-related product. However, it should be mentioned that analysis of the reaction end products based on the UV/VIS absorption spectra alone is not enough to elucidate the chemical structure of a reaction end product. Since the K_M of TrT for YGG was double the K_M of TrT for GGY and GYG,

the result suggested that the formation of a dopachrome-related product negatively affected the catalysis of TrT.

Overall, the oxidation capacity of TrT was lower than that of AbT on the peptide-bound tyrosine. Mattinen et al. (2008b) reported on the oxidation rates of TrT and AbT on a Tyr containing-peptide (EGVYVHPV) and GYG. The oxidation rate with TrT was faster on EGVYVHPV than on GYG, whereas with AbT it was the opposite. These results suggested that TrT can oxidise better Tyr residues in long peptides or proteins rather than short peptides, as also observed in the unique cross-linking ability of TrT (Selinheimo et al. 2007b).

Table 11. Kinetic parameters of the tyrosinases from *Trichoderma reesei* (TrT) and *Agaricus bisporus* (AbT) for L-tyrosine (Y) and the tripeptides GGY, GYG, and YGG, determined by oxygen consumption measurement. (Publication I).

Enzyme	Substrate	V_{max} (mg L^{-1} s^{-1}) $\ast$ 10^2	K_M (mM)	k_{cat} (s^{-1})	k_{cat} / K_M (s^{-1} mM^{-1})	Lag(s) with [S] 4 mM
TrT	Y		nd[a]			
TrT	GGY	11.9	3.1	7.2	2.4	120
TrT	GYG	6.9	3.9	4.2	1.1	120
TrT	YGG	5.2	6.0	3.2	0.5	120
AbT	Y		0.20			
AbT	GGY	2.7	0.11	3.0	26.5	600
AbT	GYG	2.8	0.18	3.1	17.1	240
AbT	YGG	3.6	0.21	4.0	19.0	180

[a] Could not be determined due to insolubility of the substrate.

The K_M and V_{max} values for TrT and AbT for small mono- and diphenolic substrates with different substitution of carboxyl and amino groups were determined by following of the oxidation products at specific wavelengths (**Table 12**). TrT and AbT were found to be very different in oxidation efficiency with small mono- and diphenolic substrates. Selinheimo et al. (2006b) reported on the K_M values of TrT for the substrates *p*-tyrosol (K_M=1.3 mM), *p*-coumaric acid (K_M=1.6 mM) and L-DOPA (K_M=3.0 mM), which were determined through monitoring of the consumption of the co-substrate molecular oxygen. In this work, in contrast, the K_M values were determined through following of the formation of the oxidation product at predetermined wavelengths. Slightly different K_M values were obtained. In general, the K_M values for AbT were observed to be the highest for those substrates whose structure contained a carboxyl group. The high K_M values of AbT for the substrates in which a carboxyl group was present in the structure suggest that these substrates are not real substrates for AbT (e.g., *p*-coumaric acid) but substrates analogues, as reported by several authors (Lim et al. 1999, Fan & Flurkey 2004, Kubo et al. 2004). A substrate analogue might bind the active site with the carboxylic group instead of with the hydroxyl group and thus compete with the substrate in binding of the active site of AbT (Fan & Flurkey 2004). In contrast, substrates in which a carboxyl group was present were real substrates for TrT. Again, unlike AbT, TrT showed low affinity for the substrates in which an amino group was present. A similar negative effect of an amine group on TrT was also found for the tripeptide YGG. In tyrosinases of fungi and

plant origin, several residues are highly conserved in the sequence region containing the His residues that co-ordinate the CuB (Marusek et al. 2006). However, these residues are not highly conserved in TrT: for instance, a Met residue (Met280 in AbT) is a Phe residue in TrT (Phe284) (Selinheimo et al. 2007b). Therefore, it seems possible from the results that the binding of substrates at the active site is different in TrT as compared to AbT and other tyrosinases. However, in the absence of a three-dimensional structure of TrT, this remains as a hypothesis.

Table 12. K_M (mM) and V_{max} (Abs/min) values of tyrosinases from *Trichoderma reesei* (TrT) and *Agaricus bisporus* (AbT) for mono- and diphenolic compounds, determined by spectrophotometric assay with substrate-specific wavelengths (Abs/min). TrT and AbT enzyme dosages were 3.8 and 3.1 µg mL^{-1} for the monophenols and 0.9 and 0.8 µg mL^{-1} for the diphenols, respectively. (Publication I).

Substrate	Detection absorbance (nm)	K_M (mM)		V_{max} (Abs/min)	
		TrT	AbT	TrT	AbT
L-Tyrosine	475	–	0.20	6[a]	13
L-DOPA	475	7.5	0.17	210	60
p-Coumaric acid	360	1.5	–	51	0
Caffeic acid	480	0.9	1.69	60	300
L-Tyramine	475	4.5	0.75	4	16
L-Dopamine	475	11	0.84	120	300
Phloretic acid	400	1.4	0.64	55	77
Hydrocaffeic acid	400/530[b]	3.0	0.91	450	840
Phenol	390	3.8	0.33	9	14
Catechol	400/540[c]	2.5	0.25	138	1500
p-Tyrosol	395	0.8	0.06	20	13

[a] Oxidation rate determined with L-tyrosine concentration of 2.5 mM, not the V_{max} value.

[b] Product formation followed at 400 and 530 nm with AbT and TrT, respectively.

[c] Product formation followed at 400 and 540 nm with AbT and TrT, respectively.

The lag periods were detected in both the TrT- and AbT-catalysed oxidation of small monophenols. The lag period relates to the transformation of the *met*-form to the *oxy*-form of tyrosinase. In the AbT-catalysed oxidation the lag period increased when the substrate concentration increased for the substrates on which AbT showed the highest affinity, but the lag period decreased with L-tyramine and phloretic acid when substrate concentration increased (Table 3 in Publication I). It has been shown that when AbT has high affinity on a monophenolic substrate, the substrate binds also to the *met*-form of AbT leading the enzyme to a dead-end pathway. Thus the transition of the *met*-form into the *oxy*-form, which is the only active form on monophenols, is retarded (Espín et al. 1999). In contrast to AbT, TrT had generally lower affinity than AbT on the monophenolic substrates tested and the lag periods measured generally decreased when the substrate concentrations were increased with all of the monophenolic substrates. Therefore, the results are logical; with low affinity on substrates, an increased lag period related to the dead-end pathway was not observed with TrT. Correspondingly AbT showed the lowest affinity for L-tyramine and phloretic acid, and thus the binding of these substrates to the *met*-form of AbT was less favourable and, in consequence, no prolonged lag period was measured with the increased substrate concentration. The duration of the lag period of a specific

substrate was not directly related to the K_M, suggesting that there are also other reasons for the differences in the lag periods other than the affinity of the enzyme on the substrate.

The UV/VIS absorption spectra of the products formed by TrT- and AbT-catalysed oxidation of monophenolic and diphenolic substrates indicated that the primary product from most of the substrates was similar with both enzymes. Unexpectedly, different end products accumulated in TrT- and AbT-catalysed oxidation when catechol or hydrocaffeic acid was used as the substrate (**Figure 13**). When L-DOPA was the substrate, the spectrum revealed that the accumulation of dopachrome (i.e., the oxidation product from L-DOPA) was different with the two enzymes. In the TrT-catalysed reaction, the intensity of absorbance was lower than in the AbT-catalysed reaction (Figure 5 in Publication IV). It is known that dopachrome, the end product of the proximal phase, is not a stable compound, and it undergoes non-enzymatic reactions leading to the formation of melanins (the distal phase of melanogenesis). The distal phase starts immediately after the proximal phase and a mixture of products is formed (Cooksey et al. 1997). The linear formation of dopachrome, i.e., the proximal phase of melanogenesis lasted only five minutes in the TrT-catalysed oxidation, whereas it was 15 minutes with AbT, a result similar to that reported by Cooksey et al. (1997). However, the UV/VIS absorption spectra of the products formed in the distal phase were similar between TrT and AbT, suggesting that both TrT and AbT generate similar melanins.
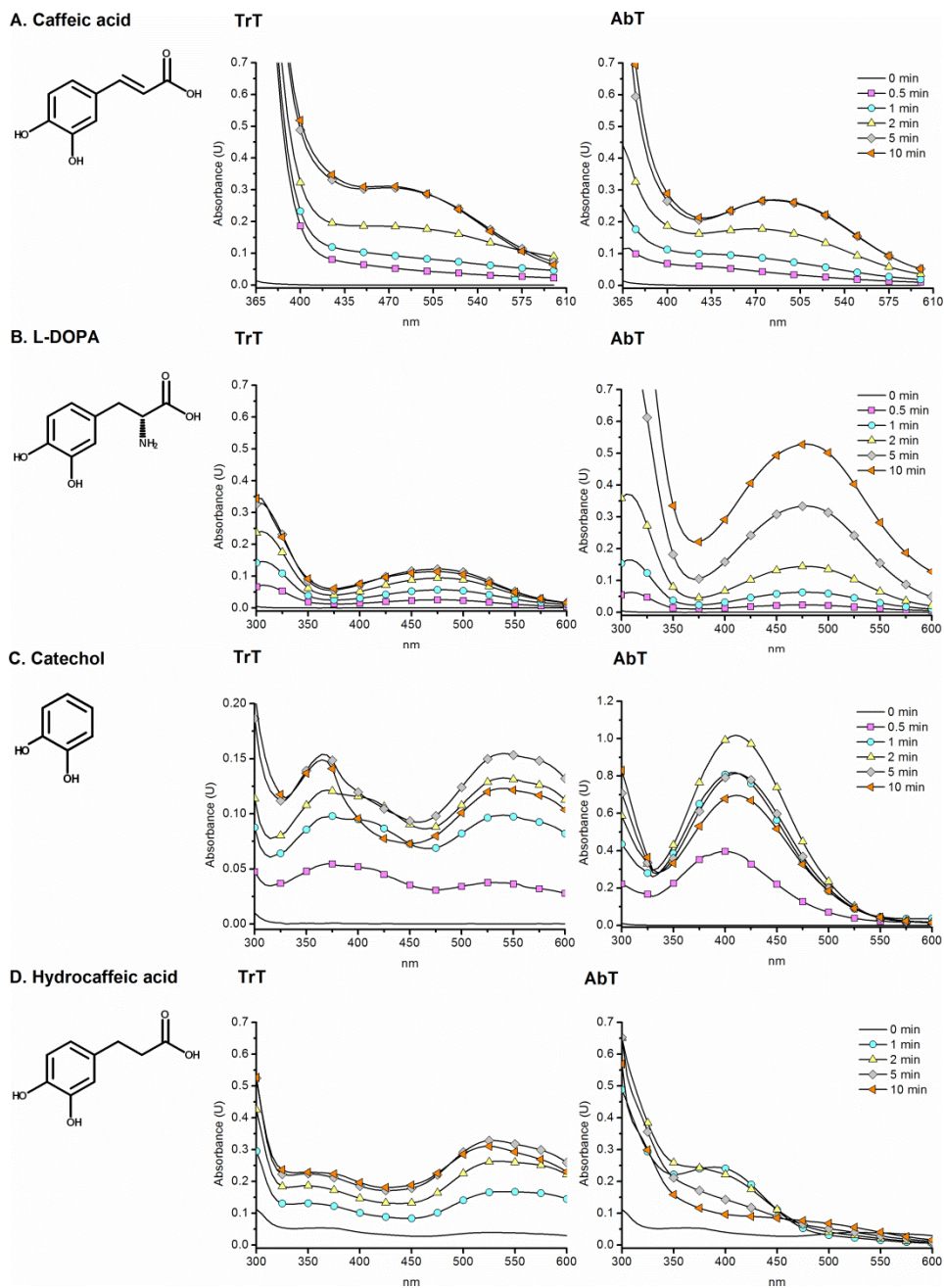
Figure 13. UV/VIS absorption product spectra of the oxidation of caffeic acid (A), L-DOPA (B), catechol (C) and hydrocaffeic acid (D) by tyrosinases from *Trichoderma reesei* (TrT) and *Agaricus bisporus* (AbT). Absorption spectra shown as a function of time.

4.2.2 *Trichoderma reesei* tyrosinase end product inhibition by dopachrome

The UV/VIS absorption spectra of products formed by TrT- and AbT-catalysed oxidation of L-DOPA suggested that TrT suffer from end product inhibition. It was detected that TrT produced dopachrome only in the first five minutes, despite the co-substrate molecular oxygen was still available in the reaction mixture. To confirm that dopachrome was the only product formed both by TrT- and AbT-catalysed oxidation of L-DOPA, the reaction end products of L-DOPA oxidation by the tyrosinases were analysed by means of ESI-FT-ICR mass spectrometry and through fragmentation analysis of the ions with IRMPD. Without enzyme, L-DOPA (MW 197 Da) gave an intense peak at *m/z* 196, corresponding to the [M – H]⁻ ion. In the TrT- and AbT-catalysed reactions, a new peak at *m/z* 192 was observed, corresponding to dopachrome (MW 193 Da) (**Figure 14**, and Figure 4 in Publication IV). Dopachrome was the only end product detected in the conditions studied. TrT-catalysed oxidation of L-DOPA clearly plateaued after a few minutes of reaction, and less than 0.1% of the L-DOPA was oxidised to dopachrome, whereas AbT catalysed the complete conversion of L-DOPA to dopachrome within 20 minutes of reaction in these reaction conditions (**Figure 14A and C**).
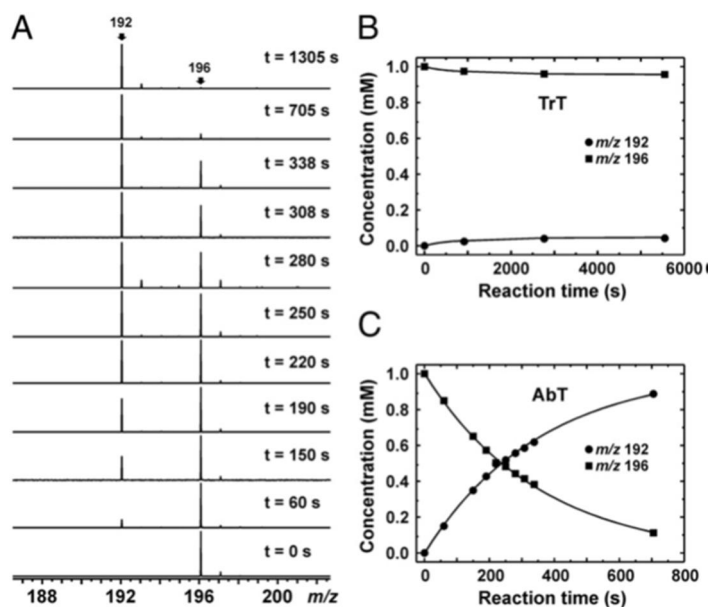
Figure 14. Negative mode ESI FT-ICR mass spectra from the oxidation of L-DOPA (*m/z* 196) to dopachrome (*m/z* 192) by *Agaricus bisporus* tyrosinase (AbT) at various time points (A). A plot of the respective ion intensities versus reaction time for *Trichoderma reesei* tyrosinase (TrT) (B) and AbT (C). (Publication IV).

The ESI-FT-ICR mass spectrometry result and the UV/VIS absorption spectra of products formed by TrT-catalysed oxidation of L-DOPA both suggested that TrT suffers from end product inhibition. With the aim of studying the TrT end product inhibition, dopachrome

was produced by AbT-catalysed oxidation of L-DOPA in acidic conditions to ensure the stability of dopachrome. Reaction mixtures containing 8 mM L-DOPA and dopachrome in various concentrations (0, 0.25, 0.75, and 1.5 mM) were used for calculation of the degree of inhibition. The oxygen consumption rate of TrT on 8 mM L-DOPA in the presence of 1.5 mM dopachrome was reduced by 50% (**Table 13**), indicating that dopachrome clearly inhibited the TrT-catalysed oxidation of L-DOPA,

Table 13. Degree of inhibition of *Trichoderma reesei* tyrosinase (TrT) by dopachrome on L-DOPA (8 mM), Reaction mixture was prepared in 0.1 M sodium acetate buffer pH 5.6 and analysed by oxygen consumption measurement, determined by oxygen consumption measurement. (Publication IV).

Dopachrome (mM)	Oxygen consumption rate ($\mu g \ L^{-1} \ min^{-1}$)	Inhibition (%)
0	18.2 ± 3.5	0
0.25	17.9 ± 2.7	2
0.75	12.0 ± 1.0	34
1.5	8.4 ± 1.7	54

The end product inhibition of TrT elucidates why the oxidation capacity of TrT with L-DOPA was reduced relative to AbT. Furthermore, it suggests that end product inhibition of TrT occurs also with the structurally related substrates L-tyrosine and YGG. According to the analysis of the end product absorption spectra, the end product of a TrT-oxidation of L-tyrosine or YGG is a dopachrome-related product with a maximum absorbance at 475 nm. Selinheimo et al. (2007b) reported that the cross-linking ability of TrT decreased when L-DOPA was added to the reaction mixture. The dopachrome end product inhibition elucidated in this work is most probably the explanation of the reduced cross-linking ability of TrT in the presence of L-DOPA as a cross-linking agent. Thus far, TrT is the first tyrosinase shown to suffer from end product inhibition. Several substrates of AbT have been shown to act as suicide substrates leading AbT towards the suicide inactivation pathway (Muñoz-Muñoz et al. 2010b), accordingly, the possibility of L-DOPA to act as a suicide substrate for TrT was evaluated. A suicide diphenolic substrate cannot bind the *oxy*-form of tyrosinase in a coplanar binding geometry, and it leads the enzyme toward the suicide inactivation pathway, with the consequent loss of one reduced copper ion (Cu^0) and hydrogen peroxide together with the reaction product. The stronger the withdrawing effect of the group in the C-4 position of the diphenolic substrate is, the greater is the likehood of leading to enzyme toward the suicide inactivation pathway (Muñoz-Muñoz et al. 2008). The ability of AoCO4 to recover its activity when dopachrome was removed from the reaction mixture was analysed by removing dopachrome by gel filtration. Interestingly, TrT retained its activity on L-DOPA (data not shown). The result indicated that dopachrome was not a suicide substrate of TrT.

4.2.3 Effect of inhibitors on the monophenolase and diphenolase activities of *Trichoderma reesei* tyrosinase

Since tyrosinases-catalysed browning reactions cause negative changes in organoleptic properties and in the appearance of fruits and vegetables, with subsequent shortening of product shelf life, research into inhibition of tyrosinases is of great interest. Among the large number of inhibitors of tyrosinases reported in the literature, kojic acid, arbutin, benzaldehyde, benzoic acid, potassium cyanide and sodium azide, were analysed as inhibitors of TrT with *p*-coumaric acid and caffeic acid used as monophenolic and diphenolic substrates (Table 2 in Publication IV). The inhibitory effect of the tested inhibitors on the monophenolase activity of TrT followed the order potassium cyanide > sodium azide > benzaldehyde > arbutin. No inhibitory effect was found for kojic acid or benzoic acid. However, kojic acid prolonged the lag period of TrT-catalysed oxidation of *p*-coumaric acid. The inhibitory effect of the tested inhibitors on the diphenolase activity of TrT followed the order kojic acid > potassium cyanide > sodium azide > arbutin. Benzaldehyde and benzoic acid did not have an effect on TrT-catalysed oxidation of caffeic acid, whereas they are known inhibitors for AbT (Wilcox et al. 1985, Chen et al. 1991, Cabanes et al. 1994, Conrad et al. 1994, Funayama et al. 1995, Espín & Wichers 1999). These results lend further support to the findings from the substrate-specificity determination that the amino acid residues close to the active sites of TrT and AbT are different.

The difference in the effect of kojic acid on mono- and diphenolase activity of TrT and AbT suggested that monophenols and diphenols bind differently to the active site of the tyrosinases. The three-dimensional structure of BmT in complex with kojic acid has recently shown that kojic acid is oriented with the hydroxymethyl group towards the CuB at the active site of *met*-tyrosinase and given rise to the hypothesis that monophenols bind to CuA whereas diphenols bind to CuB at the active site (Sendovski et al. 2011). Caffeic acid and kojic acid both bind to CuB, and indeed a clear competitive inhibition was observed for TrT. Differently, in the monophenolase cycle, *p*-coumaric acid binds to the CuA of TrT while kojic acid binds to the CuB, and no clear inhibition was observed. Furthermore, the lag time of TrT was greatly prolonged by kojic acid, suggesting that binding with the substrate is more difficult when the tyrosinase-kojic acid complex is present.

Interestingly, sodium azide was found to be a strong inhibitor of TrT, but it also had an effect on the reaction end products as analysed by UV/VIS absorption (Figure 6 in Publication IV). When catechol was used as a substrate, an absorption maximum at 400 nm was observed with AbT. In the presence of sodium azide the absorption maximum of products formed by AbT-catalysed oxidation of catechol was detected at 325 nm. Sugumaran (1995) reported on the formation of an azido-benzoquinone adduct at 325 nm after ten minutes of AbT-catalysed oxidation of catechol in the presence of sodium azide. The observed absorbance maximum at 325 nm indicated a formation of an azido-benzoquinone adduct with AbT. In contrast, the azido-benzoquinone adduct was not observed in product absorption spectra of TrT-catalysed oxidation of catechol in the presence of sodium azide. However, because the end-products formed by TrT- and AbT-catalysed oxidation of catechol were found to be different it is likely that the same adduct is not formed with TrT. A clear maximum at 375 nm was also observed in the product

absorption spectra of TrT- and AbT-catalysed oxidation of L-DOPA in the presence of sodium azide, suggesting that a dopachrome-adduct was formed with both the tyrosinases (Figure 5 in Publication IV).

4.3 Three-dimensional structure of a laccase from *Thielavia arenaria* and elucidation of the oxidation capacity of fungal laccases with different redox potential (Publication III)

4.3.1 Purification and three-dimensional structure of *Thielavia arenaria* laccase

The laccase 1 from the ascomycete fungus *Thielavia arenaria* (TaLcc1) has been shown to be an efficient enzyme in bleaching of denim at high temperatures, up to 80 °C and at neutral pH (Paloheimo et al. 2006). TaLcc1 was produced with *T. reesei* as a host at Roal Oy (Rajamäki, Finland). TaLcc1 was purified within a three-step purification procedure. First a culture supernatant was desalted and buffer-exchanged with gel filtration chromatography. Weak-affinity anion-exchange and subsequently high-affinity anion-exchange chromatography were used to purify TaLcc1 (**Table 14**).

Table 14. Purification of laccase 1 from *Thielavia arenaria*. Enzymatic activity was measured using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as a substrate.

Purification step	Total activity	Total protein	Specific activity	Activity yield	Purification factor
	(nkat)	(mg)	(nkat mg^{-1})	(%)	
Culture filtrate	382690	700	547		1.0
Desalting	366910	585	627	96	1.1
Weak-affinity anion exchange chromatography	163660	174	940	43	1.7
High-affinity anion exchange chromatography	22120	27	813	6	1.5

TaLcc1 was crystallised with the hanging drop diffusion method at room temperature. The three-dimensional structure of TaLcc1 was solved by X-ray crystallography at 2.5 Å resolution at University of Eastern Finland (PDB ID 3PPS). The overall structure of TaLcc1 was rather similar to the structures of the known laccases and in particular to the structure of the ascomycete laccase from *Melanocarpus albomyces* (MaL, PDB ID 1GW0 and *r*MaL, PDB ID 2Q9O), which was the first published three-dimensional structure of an ascomycete laccase (Hakulinen et al. 2002, Hakulinen et al. 2008). However, also clear differences between the three-dimensional structures of TaLcc1 and *r*MaL were found, including the loops near the T1 copper site and the amino acid thought to be responsible for catalytic proton transfer.

TaLcc1 was found to be a monomer composed of three cupredoxin-like domains, called domain A (1–160), domain B (161–340) and domain C (340–564) (**Figure 15**). The mononuclear T1 copper was located in domain C and it was co-ordinated by His432, Cys504, His509, and two non-bonding residues (Ile506 and Leu214). The trinuclear

T2/T3 copper cluster was located at the interface between domains A and C. The three copper ions of the T2/T3 copper cluster were co-ordinated in total by eight His residues and one molecule of solvent. The T2 copper ion was coordinated by His94, His437, and one molecule of solvent. The T3 copper ion by His141, His437, and His503, and the T3' copper ion by His96, His139, and His505. Differently from TaLcc1, in the nearly atomic resolution structure of *r*MaL the T2 copper ion is co-ordinated by two His residue and one chloride ion (Hakulinen et al. 2008).

In three-dimensional structures of ascorbate oxidase two solvent channels leading to the T2/T3 copper centre have been described. The broad solvent channel, leading to the T3 copper ions is called the T3 solvent channel. The narrower solvent channel leads to the T2 copper site, and it is called the T2 channel (Messerschmidt et al. 1992). The T3 and the T2 channels have been reported also in basidiomycete laccases, e.g., in the laccase from *Trametes versicolor* (Piontek et al. 2002). On the contrary, in the ascomycete laccase *r*MaL the T3 channel is blocked by the last four C-terminal amino acids (i.e., Asp-Ser-Gly-Leu, known as the C-terminal plug) and the T2 channel is very tight, with the residues His98, Gln131, and Asp439 limiting the access of the solvent molecules. Furthermore, in *r*MaL a triangle of amino acids (Ser, Ser and Asp), called the SDS-gate is found, but it is absent in the laccases from basidiomycete fungi (Hakulinen et al. 2002, Hakulinen et al. 2002, Hakulinen et al. 2008). Differently from *r*MaL, the T2 channel was found also in TaLcc1 (Figure 3 in Publication III). The residue corresponding to His98 in *r*MaL is Arg99 in TaLcc1, which is oriented such that it forms the surface of the solvent channel. In TaLcc1, as in *r*MaL, the T3 solvent channel was blocked by a C-terminal plug. The C-terminal plug is highly conserved in the sequences of ascomycete laccases. Divergently, basidiomycete laccases generally do not have this type of C-terminus. In another similarity to *r*MaL, the SDS-gate was found in TaLcc1 (Ser143, Ser511, Asp561).
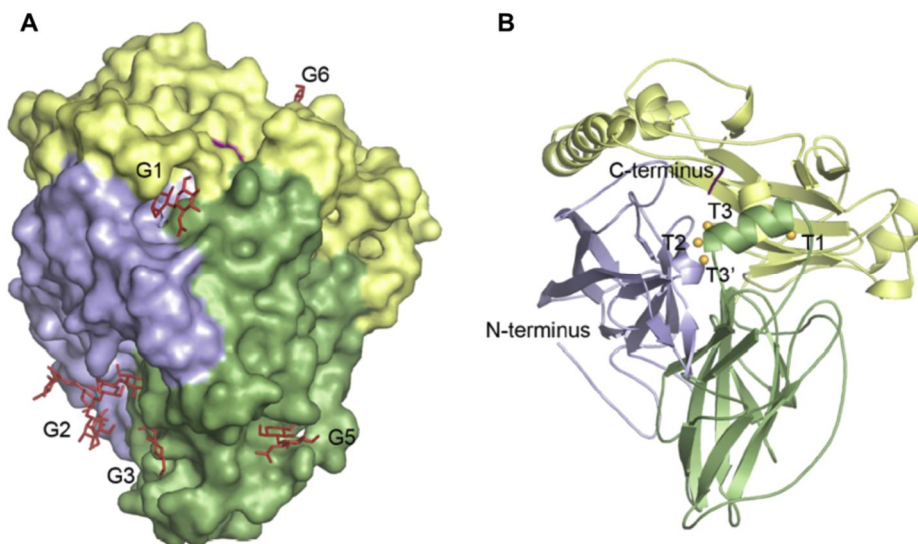
Figure 15. (A) The crystal structure of *Thielavia arenaria* laccase 1 (TaLcc1) as a surface model. Domain A is presented in blue, domain B in green, and domain C in yellow. The N-glycans are shown as red sticks. Glycans are named as G1 on Asn89, G2 on Asn202, G3 on Asn217, G4 on Asn247 (on the other side of the molecule), G5 on Asn290, and G6 on Asn376. (B) Cartoon representation of TaLcc1. The catalytic coppers are shown in orange, and the C-terminal plug in purple. (Publication III).

The substrate-binding site of TaLcc1 was found to be in a pocket between domains B and C, in a similarity to *r*MaL. The pocket was formed by ten hydrophobic residues (Ala193, Leu297, Leu363, Phe371, Trp373, Ile427, Val428, Leu430, Trp508, and His509) and one hydrophilic residue (Asp236). In general, the substrate-binding pocket of TaLcc1 was found to be similar to that one of *r*MaL. However, there are differences between the two enzymes, both in the shape and in the residues of the pocket. The pocket of TaLcc1 was found to be narrower than the pocket of *r*MaL in one direction, owing to the Leu297 in TaLcc1 (Ala297 in *r*MaL), and wider than the pocket of *r*MaL in the other direction, because of the Pro195 and Val428 in TaLcc1 (Phe194 and Phe427 in *r*MaL, respectively). The main difference between the substrate-binding pockets of TaLcc1 and *r*MaL was found to be in the residue Asp236 in TaLcc1, corresponding to Glu235 in *r*MaL (**Figure 16**).
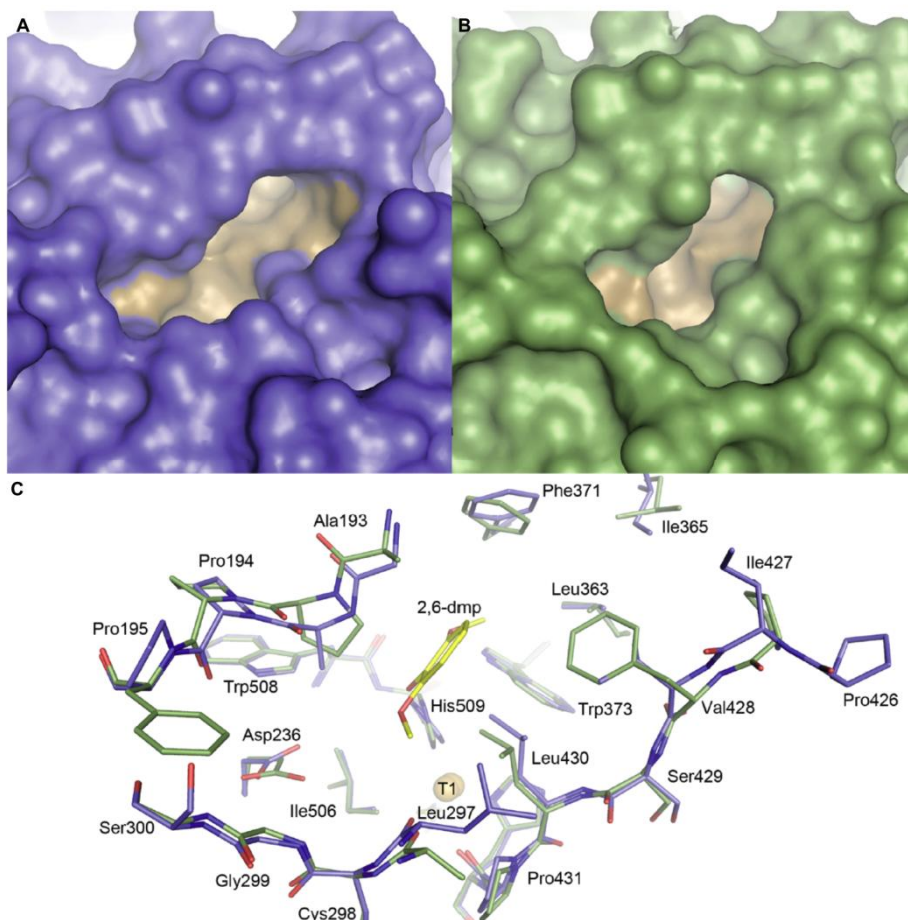
Figure 16. The substrate binding pockets of *Thielavia arenaria* laccase 1 (TaLcc1) (A) and the recombinant *Melanocarpus albomyces* laccase (*r*MaL) (B). (C) Superimposed amino acids forming the substrate-binding pockets. TaLcc1 is shown in blue, *r*MaL in green, and 2,6-dimethoxy phenol (2,6-DMP) from the complex structure (PDB ID 3FU8) in yellow. The amino acids of TaLcc1 are labelled. (Publication III).

The implications of the differences between the three-dimensional structures of TaLcc1, *r*MaL, and the basidiomycete laccase from *Trametes hirsuta* (ThL) for the capacity of these laccases to oxidise phenolic substrates is presented and discussed in the Subsection 4.3.2.

4.3.2 Elucidation of the oxidation capacity of fungal laccases with different redox potential as related to their available crystal structures

Both the affinity of the substrate to the enzyme and the difference in redox potential ($\Delta E°$) between the T1 copper site of the laccase and the substrate affect the oxidation rate of the substrate in laccase-catalysed reactions (Xu 1996a, Xu 1997). The rate of laccase-catalysed reactions is assumed to increase as the $\Delta E°$ between the T1 copper site of the laccase and the substrate increases. In turn, the pH affects the laccase-catalysed oxidation of a phenolic substrate, either positively or negatively. At higher pH values, the hydroxyl group of the phenolic substrate dissociates and inhibits the laccase, thus the rate of laccase-catalysed reactions decreases. At higher pH values, the $E°$ of the phenolic substrate is lower, whereas $E°$ for the T1 copper site is not pH-dependent, thus $\Delta E°$ increases and the rate of laccase-catalysed reaction is also expected to increase (Xu 1996a, Xu et al. 1996).

The enzyme kinetics with different p-substituted dimethoxy phenolic substrates of different $E°$ were analysed with three fungal laccases with different redox potential and examined in relation to their available three-dimensional structures. The novel laccase 1 from the ascomycete fungus *T. arenaria* (TaLcc1; $E°$ = 0.51 V) was compared to the other known laccase from ascomycete fungus *M. albomyces* (*r*MaL; $E°$ = 0.48 V) and to the high redox potential laccase from basidiomycete fungus *T. hirsuta* (ThL; $E°$ = 0.78 V). Three different p-substituted dimethoxy phenolic substrates, of different redox potential, were selected as substrates: 2,6-dimethoxy phenol (2,6-DMP), syringic acid and methyl syringate (**Table 15**). The kinetic of substrate oxidation by laccases was studied at two pHs, i.e., pH 4.5 and pH 6.0, where pH 4.5 is more optimal for ThL and pH 6.0 for TaLcc1 and *r*MaL (**Figure 17**).
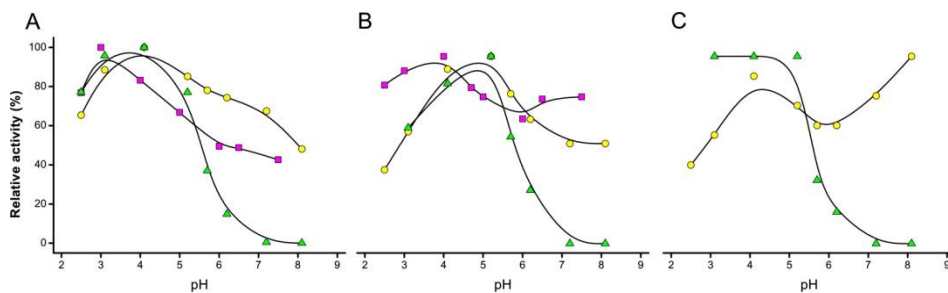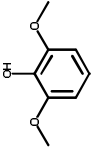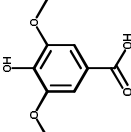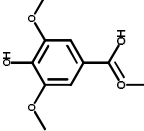

Figure 17. pH activity profiles of laccases from *Melanocarpus albomyces* (○), *Thielavia arenaria* (■), and *Trametes hirsuta* (▲), on 2,6-dimethoxy phenol (2,6-DMP) (A), syringic acid (B) and methyl syringate (C). (Publication III, supplementary data.)

Table 15. Kinetic parameters for laccases from *Melanocarpus albomyces* (*r*MaL), *Thielavia arenaria* (TaLcc1), and *Trametes hirsuta* (ThL), measured with 2,6-dimethoxy phenol (2,6-DMP), syringic acid and methyl syringate in 25 mM succinate buffer at pH 4.5 and in 40 mM MES buffer at pH 6.0 (25 °C). Structural formulas of the substrates are presented. Redox potentials (*E°*) of type 1 copper centre of the laccases and redox potentials of the substrates at pH 4.5 and 6.0 are provided, together with the redox potential differences (Δ*E°*) between the type 1 copper centre of the laccases and the substrates. n.d. not determined. (Publication III.)

	2,6-DMP		Syringic acid		Methyl syringate	
	pH 4.5 $E°$=0.53 V	pH 6 $E°$=0.40 V	pH 4.5 $E°$=0.57 V	pH 6 $E°$=0.51 V	pH 4.5 $E°$=0.69 V	pH 6 $E°$=0.65 V
***r*MaL ($E°$=0.48 V)**						
Δ$E°$ (V)	−0.05	0.08	−0.09	−0.03	−0.21	−0.17
K_M (µM)	18 ± 1	9.5 ± 0.9	122 ± 11	132 ± 22	n.d.	n.d.
V_{max} (dA min^{-1} nmol^{-1})	126 ± 2	119 ± 1	3.5 ± 0.1	12.1 ± 0.6	n.d.	n.d.
TaLcc1 ($E°$=0.51 V)						
Δ$E°$ (V)	−0.02	0.11	−0.06	0	−0.18	−0.14
K_M (µM)	45 ± 4	5.8 ± 0.6	128 ± 4	61 ± 4	n.d.	n.d.
V_{max} (dA min^{-1} nmol^{-1})	99 ± 2	87 ± 1	3.5 ± 0.1	2.7 ± 0.1	n.d.	n.d.
ThL ($E°$=0.78 V)						
Δ$E°$ (V)	0.25	0.38	0.21	0.27	0.09	0.13
K_M (µM)	18 ± 1	6.3 ± 1.3	35 ± 6	17 ± 4	168 ± 19	50 ± 16
V_{max} (dA min^{-1} nmol^{-1})	193 ± 3	91 ± 2	8.3 ± 0.4	2.0 ± 0.1	21 ± 1	2.6 ± 0.2

2,6-DMP and syringic acid were oxidised by all the three laccases in both of the pH conditions tested, while methyl syringate, the substrate of those tested that has the highest redox potential, was oxidised only by ThL. All three laccases were found to oxidise syringic acid at pH 6.0, despite the reaction not being facilitated by the $\Delta E°$ for TaLcc1 ($\Delta E°=-0.03$) and rMaL ($\Delta E°=0$). However, in these reaction conditions (pH=6.0) TaLcc1 and rMaL were able to oxidise syringic acid at pH 6.0 mainly on account of the favourable pH. Differently, a pH of 6.0 was not the optimal pH for ThL (about 30% of residual activity on syringic acid) and thus the ThL-catalysed oxidation of syringic acid was favourite largely through a high $\Delta E°$ ($\Delta E°=-0.27$). Nevertheless, the effect of $\Delta E°$ was found to prevail over the effect of pH for the substrates with high $E°$, i.e., in both pH conditions, methyl syringate was oxidised only by ThL, which is the laccase with the highest $E°$ among the three laccases.

Despite TaLcc1 and rMaL having shown similar pH profiles and $\Delta E°$ values, the affinity of TaLcc1 to 2,6-DMP was lower than the affinity of rMaL for the same substrate at pH 4.5. Furthermore, a three-fold increase in the reaction rate with syringic acid at pH 6 as compared with pH 4.5 was observed for rMaL only, not for TaLcc1. These differences in the TaLcc1 and rMaL-catalysed oxidation of phenolic substrates must be attributable to the differences in the residues forming the binding pockets of the two laccases. The most evident difference between the substrate-binding pockets of TaLcc1 and rMaL was found to be in the hydrophilic residue, which is also the putative catalytic amino acid. In TaLcc1, an Asp236 is found, whereas rMaL has a Glu235 in the correspondent position (**Figure 16**, **Figure 18**). Kallio et al. (2009) reported that a single mutation in the Glu catalytic residue (Glu235->Asp) of rMaL increases the K_M value for phenolic substrates, suggesting that a carboxylic group is important for the catalytic activity. Bento et al. (2010) have shown that in the bacterial laccase from *Bacillus subtilis* a Glu residue (Glu498) near the dinuclear T3 copper centre participates in the proton transfer at the trinuclear T2/T3 copper cluster. Basidiomycete laccases have a conserved Asp residue close to the dinuclear T3 copper centre, and this residue might function similarly to the Glu residue. Differently in the ascomycete laccases, the only acidic residue that might assist in the proton transfer is the carboxylate group from the C-terminus (Leu459 in TaLcc1 and Leu559 in rMaL). Therefore, it is likely that the proton transfer is assisted by different residues in the basidiomycete laccases (the Glu residue) and the ascomycete laccases (the carboxylate group in the C-terminus). Still, it is also possible that in the ascomycete laccases only the proton transfer from the T1 copper site to the trinuclear copper centre is assisted by the residues in the SDS-gate. The kinetics of ThL showed clearly differences from those of TaLcc1 and rMaL. As related to their three-dimensional structures, the loops forming the substrate-binding pocket of ThL are completely different from those in TaLcc1 and rMaL, most likely accounting for the strong differences in the oxidation capacity of laccases from basidiomycete and ascomycete fungi, in addition to the different $E°$.
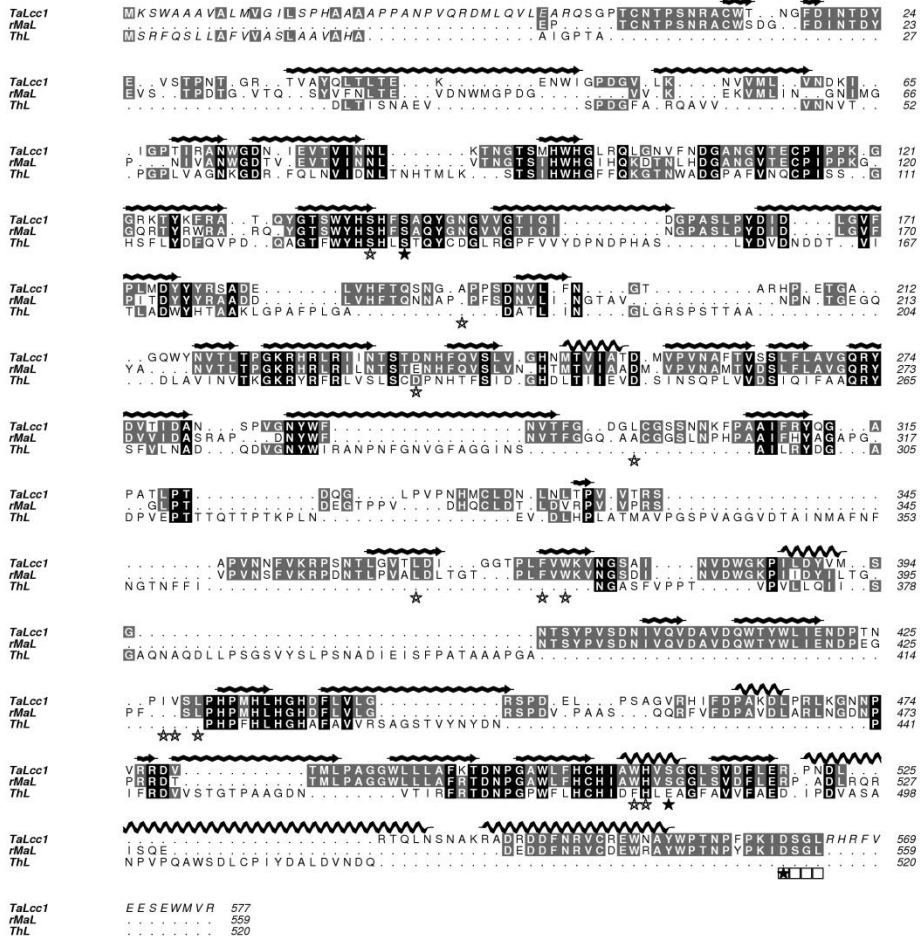
Figure 18. Amino acid sequence alignment of fungal laccases from *Thielavia arenaria* (TaLcc1, UniProtKB F6N9E7, PDB ID: 3PPS), *Melanocarpus albomyces* (*r*MaL, UniProtKB Q70KY3, PDB ID: 2Q9O), and *Trametes hirsuta* (ThL, UniProtKB Q02497). The N-terminally and C-terminally processed amino acid residues are indicated in italics. The secondary structure elements of TaLcc1 are also reported (α-helix as black helix, and strand as black strand). The hallow stars indicate the residues in the substrate binding pocket; the filled stars indicate the residues in the SDS-gate; the empty squares indicate the residues of the C-terminal tail. The figure was produced by means of ALINE (Bond & Schuttelkopf 2009). (Unpublished results)

4.4 Enzymatic cross-linking of α-casein proteins by means of catechol oxidase, tyrosinases, and laccases (Unpublished results)

All enzymes dealt with in this work were tested for their protein cross-linking ability with α-casein used as a model substrate with and without a cross-linking agent. Catechol was used as a cross-linking agent in the tyrosinases- and catechol oxidases-catalysed reactions, and *p*-coumaric acid in the laccases-catalysed reactions (**Figure 19**, **Figure 20**). *Trichoderma reesei* tyrosinase (TrT) efficiently cross-linked α-caseins without a cross-linking agent, whereas *Agaricus bisporus* tyrosinase (AbT) did not directly cross-link α-caseins, as also reported by Selinheimo et al. (2007b), nor was the newly discovered *Aspergillus oryzae* catechol oxidase (AoCO4) capable to cross-link α-caseins. AbT and AoCO4 clearly cross-linked α-caseins in the presence of the cross-linking agent; on the contrary, in the presence of catechol, the cross-linking ability of TrT was strongly reduced. Furthermore, AoCO4-catalysed (100 nkat g^{-1} caseins) cross-linking of caseins in the presence of catechol was more efficient than was AbT-catalysed (100 nkat g^{-1} caseins) cross-linking, as analysed by SDS-page. Selinheimo et al. (2007b) showed that tyrosinases from apple and potato have particularly low activity on L-tyrosine and are able to cross-link caseins in the presence of L-DOPA as a cross-linking agent. AoCO4 performed similarly to the tyrosinases from apple and potato in cross-linking of α-caseins. It is worthy of mention that, despite these enzymes from apple and potato being classified as tyrosinases, they have low activity on L-tyrosine and thereby resemble catechol oxidases more closely. In order to get a better understanding of the cross-linking abilities of catechol oxidases, more catechol oxidases should be discovered and characterised.

None of the three fungal laccases tested, i.e., those from *Thielavia arenaria* (TaLcc1), *Melanocarpus albomyces* (*r*MaL) and *Trametes hirsuta* (ThL), showed cross-linking ability of α-caseins without a cross-linking agent, in the reaction conditions studied. In the presence of *p*-coumaric acid (the cross-linking agent), the cross-linking efficiency of the three laccases tested followed the order TaLcc1 > *r*MaL > ThL. However, in contrast to the tyrosinases, none of the laccases were capable of fully cross-linking all of the α-caseins in the reaction conditions. The cross-linking abilities of *r*MaL and TaLcc1 had not been tested before. Previously, Selinheimo et al. (2008) reported on the cross-linking ability of ThL, showing that efficient cross-linking of α-caseins without any cross-linking agent was obtained with ThL (15000 nkat g^{-1} caseins). However, this was a higher dosage than the one used in the present experiment conditions (i.e., 1000 nkat g^{-1} caseins was the highest dosage tested). ThL was also found to cross-link α-caseins in the presence of *p*-coumaric acid, as already reported by Selinheimo et al. (2008).

Of the enzymes tested, TrT showed unique characteristics in protein cross-linking. The presence of a small diphenolic cross-linking agent (i.e., catechol) noticeably inhibited TrT-catalysed protein cross-linking. Similarly, TrT cross-linking

ability was greatly reduced when L-DOPA was used as a cross-linking agent, as reported by Selinheimo et al. (Selinheimo et al. 2007a, Selinheimo et al. 2007b, Mattinen et al. 2008a, Mattinen et al. 2008b, Selinheimo et al. 2008). The lower cross-linking efficiency of TrT in the presence of a cross-linking agent could be explained by the end product inhibition seen from the oxidation products of catechol and L-DOPA (Publication IV). Furthermore, it might be that the dopachrome formed by TrT-catalysed oxidation of L-DOPA could have reacted non-enzymatically with the protein-bound Tyr residues thus blocking these sites to further cross-linking of α-casein subunits.
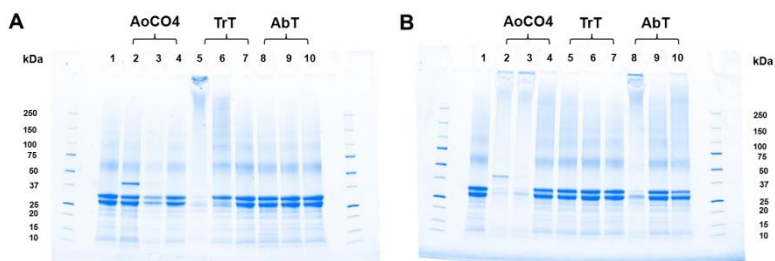
Figure 19. Cross-linking of α-casein proteins (3 g L^{-1}) by catechol oxidase from *Aspergillus oryzae* (AoCO4) and tyrosinases from *Trichoderma reesei* (TrT) and *Agaricus bisporus* (AbT) after 24 hours of incubation without a cross-linking agent (A) and with catechol (2 mM) as a cross-linking agent (B). The enzyme dosage were 1000, 100, and 10 nkat g^{-1} of α-caseins. Lanes for gel A: 1, α-caseins; 2, α-caseins + AoCO4 (1000 nkat); 3, α-caseins + AoCO4 (100 nkat); 4, α-caseins + AoCO4 (10 nkat); 5, α-caseins + TrT (1000 nkat); 6, α-caseins + TrT (100 nkat); 7, α-caseins + TrT (10 nkat); 8, α-caseins + AbT (1000 nkat); 9, α-caseins + AbT (100 nkat); 10, α-caseins + AbT (10 nkat). Lanes for gel B: the same as with gel A, except that catechol (2 mM) as a cross-linking agent was present in all of the experiments. (Unpublished results.)
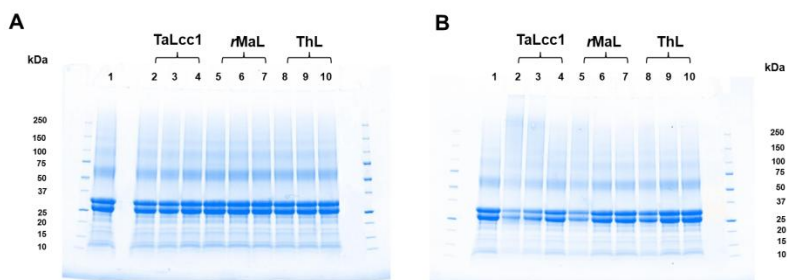
Figure 20. Cross-linking of α-casein proteins (3 g L^{-1}) by laccases from *Thielavia arenaria* (TaLcc1), *Melanocarpus albomyces* (*r*MaL) and *Trametes hirsuta* (ThL) after 24 hours of incubation without a cross-linking agent (A) and with *p*-coumaric acid (2 mM) as cross-linking agent (B). The enzyme dosage were 1000, 100 and 10 nkat g^{-1} of α-caseins. Lanes for gel A: 1, α-caseins; 2, α-caseins + TaLcc1 (1000 nkat); 3, α-caseins + TaLcc1 (100 nkat); 4, α-caseins + TaLcc1 (10 nkat); 5, α-caseins + *r*MaL (1000 nkat); 6, α-caseins + *r*MaL (100 nkat); 7, α-caseins + *r*MaL (10 nkat); 8, α-caseins + ThL (1000 nkat); 9, α-caseins + ThL (100 nkat); 10, α-caseins + ThL (10 nkat). Lanes for gel B: the same as with gel A, except that *p*-coumaric acid (2 mM) as a cross-linking agent was present in all of the experiments. (Unpublished results.)

5. Conclusions and future prospects

Food microstructure has an important role in the sensory perception of texture, water-binding, and rheological properties as well as in the digestibility and absorption of food components. Cross-linking enzymes may be used in food technology as specific tools for designed food properties and new products. This work was aimed at characterising copper-containing oxidative enzymes that can be utilised for cross-linking of proteins in food technology.

A novel family of short-tyrosinase sequences was identified in the available fungal genomes. Sequences belonging to this family are shorter in length and lack the C-terminal domain typical of long tyrosinase sequences. An extracellular catechol oxidase from *Aspergillus oryzae* (AoCO4) belonging to the newly discovered short-tyrosinase sequences was produced in *Trichoderma reesei* in high titre. AoCO4 was purified, and biochemically and structurally characterised. AoCO4 was observed to be a relatively thermostable enzyme, which is not typical for catechol oxidases and tyrosinases. AoCO4 was able to oxidise a limited range of diphenolic compounds, such as catechol, caffeic acid, hydrocaffeic acid, and 4-*tert*-buthylcatechol. AoCO4 oxidised also the monophenolic compounds aminophenol and guaiacol. However, AoCO4 did not oxidise L-DOPA and L-tyrosine, which are the characteristic substrates of tyrosinase, therefore it was classified as a catechol oxidase. Since generally catechol oxidases of fungal origin are not well-characterised enzymes, the characterisation of AoCO4 could provide a new contribution to the knowledge of these enzymes.

A preliminary crystal structure of AoCO4 was solved at 2.5 Å resolution and it is thus far the only three-dimensional structure of a catechol oxidase of fungal origin. The overall structure of AoCO4 was found to be similar to the known structures of plant catechol oxidases and fungal tyrosinases. AoCO4 was a monomer. The two copper ions of the type-3 copper site were found in a four-helix bundle, which is conserved in catechol oxidases and tyrosinases. The UV/VIS spectroscopic features of the enzyme indicated that 35% of AoCO4 was in the *oxy*-form (i.e., the form of tyrosinases active on monophenols). Nevertheless, AoCO4 did not show significant activity on monophenols. From the three-dimensional structure of AoCO4 there is no definite explanation for the lack of monophenolase activity of this enzyme. However, it could be hypothesised that the residues involved in the oxidation of monophenols and diphenols are different in AoCO4 in comparison to tyrosinases and catechol oxidases. Future structural studies might elucidate the

reaction mechanism of AoCO4 and catechol oxidases and reveal the differences with tyrosinases.

The extracellular tyrosinase from *Trichoderma reesei* (TrT) has been shown to have exclusive cross-linking ability of proteins. In this work a detailed characterisation of TrT was performed and the well characterised commercial tyrosinase from *Agaricus bisporus* (AbT) was used as a reference enzyme. The ability of TrT and AbT to oxidise small molecular weight monophenolic and diphenolic substrates was found to be strongly influenced by the substitution of the substrate molecule. In general, TrT had higher affinity on the substrates in which a carboxyl group was present in the structure, whereas AbT had higher affinity for the substrates in which an amino group was present in the structure. Furthermore, TrT showed lower affinity than AbT on L-tyrosine alone and for tripeptides varying in the position of Tyr residue.

Dopachrome was found to be the only reaction end product of both tyrosinases-catalysed oxidation of L-DOPA, which was used as representative diphenolic substrate with an amino group in the structure. Dopachrome was found to inhibit the TrT-catalysed oxidation of L-DOPA, but not the AbT-catalysed oxidation of L-DOPA. Thus far, this is the first report on a tyrosinase end product inhibition.

The effect of the redox potential on the oxidation capacity of fungal laccases was elucidated using a set of laccases and substituted phenolic substrates and related to the available crystal structures of these enzymes. For this purpose, a novel laccase 1 from the ascomycete fungus *Thielavia arenaria* (TaLcc1) and the laccases from *Melanocarpus albomyces* and *Trametes hirsuta* were used. The laccase-catalysed oxidation of phenolic substrates was found to be dependent on the difference in redox potential between the enzyme and the substrate ($\Delta E°$) and the pH. Indeed, the redox potential ($E°$) of a phenolic substrate is pH dependent and the laccase is inhibited by hydroxide ions. In general, both the $\Delta E°$ and the pH had an effect on the kinetics. However, the effect of $\Delta E°$ was found to prevail over the effect of the pH for substrates with high $E°$, such as methyl syringate.

The oxidative enzymes were shown to have potential cross-linking abilities. TrT was found to be the best enzyme for cross-linking of α-caseins, whereas AbT and AoCO4 were found to be the best enzymes for cross-linking α-caseins in the presence of catechol as a cross-linking agent. As a novel finding, AoCO4 was also found to cross-link α-caseins in the presence of catechol as a cross-linking agent.

References

Aaslyng, D., Rorbaek, K. & Sorensen, N. 1996. An enzyme for dying keratinous fibres. Patent No: WO9719998.

Abadulla, E., Tzanov, T., Costa, S., Robra, K., Cavaco-Paulo, A. & Gübitz, G.M. 2000. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol, Vol. 66, No. 8, pp. 3357–3362.

Ahmed, S.R., Lutes, A.T. & Barbari, T.A. 2006. Specific capture of target proteins by oriented antibodies bound to tyrosinase-immobilized Protein A on a polyallylamine affinity membrane surface. J Memb Sci, Vol. 282, No. 1–2, pp. 311–321.

Akyilmaz, E., Kozgus, O., Türkmen, H. & Çetinkaya, B. 2010. A mediated polyphenol oxidase biosensor immobilized by electropolymerization of 1,2-diamino benzene. Bioelectrochemistry, Vol. 78, No. 2, pp. 135–140.

Andberg, M., Hakulinen, N., Auer, S., Saloheimo, M., Koivula, A., Rouvinen, J. & Kruus, K. 2009. Essential role of the C-terminus in *Melanocarpus albomyces* laccase for enzyme production, catalytic properties and structure. FEBS J, Vol. 276, No. 21, pp. 6285–6300.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. & Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric Biol Chem, Vol. 53, No. 10, pp. 2613–2617.

Anghileri, A., Lantto, R., Kruus, K., Arosio, C. & Freddi, G. 2007. Tyrosinase-catalyzed grafting of sericin peptides onto chitosan and production of protein-polysaccharide bioconjugates. J Biotechnol, Vol. 127, No. 3, pp. 508–519.

Arias, M.E., Arenas, M., Rodriguez, J., Soliveri, J., Ball, A.S. & Hernandez, M. 2003. Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. Appl Environ Microbiol, Vol. 69, No. 4, pp. 1953–1958.

Aroca, P., Solano, F., García-Borrón, J.C. & Lozano, J.A. 1990. A new spectrophotometric assay for dopachrome tautomerase. J Biochem Biophys Methods, Vol. 21, No. 1, pp. 35–46.

Arvas, M., Kivioja, T., Mitchell, A., Saloheimo, M., Ussery, D., Penttila, M. & Oliver, S. 2007. Comparison of protein coding gene contents of the fungal phyla *Pezizomycotina* and *Saccharomycotina*. BMC Genomics, Vol. 8, pp. 325.

Baldrian, P. 2006. Fungal laccases – occurrence and properties. FEMS Microbiol Rev, Vol. 30, No. 2, pp. 215–242.

Bao, W., O'Malley, D.M., Whetten, R. & Sederoff, R.R. 1993. A laccase associated with lignification in loblolly pine xylem. Science, Vol. 260, No. 5108, pp. 672–674.

Bar Nun, N., Tal Lev, A., Harel, E. & Mayer, A.M. 1988. Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. Phytochemistry, Vol. 27, No. 8, pp. 2505–2509.

Baron, C.P., Børresen, T. & Jacobsen, C. 2007. Comparison of methods to reduce dioxin and polychlorinated biphenyls contents in fishmeal: extraction and enzymatic treatments. J Agric Food Chem, Vol. 55, No. 4, pp. 1620–1626.

Basnar, B., Xu, J., Li, D. & Willner, I. 2007. Encoded and enzyme-activated nanolithography of gold and magnetic nanoparticles on silicon. Langmuir, Vol. 23, No. 5, pp. 2293–2296.

Battistuzzi, G., Di Rocco, G., Leonardi, A. & Sola, M. 2003. ^{1}H NMR of native and azide-inhibited laccase from *Rhus vernicifera*. J Inorg Biochem, Vol. 96, No. 4, pp. 503–506.

Bell, A.A. & Wheeler, M.H. 1986. Biosynthesis and functions of fungal melanins. Annu Rev Phytopathol, Vol. 24, pp. 411–451.

Beltramini, M. & Lerch, K. 1982. Fluorescence properties of *Neurospora* tyrosinase. Biochem J, Vol. 205, No. 1, pp. 173–180.

Bento, I., Silva, C.S., Chen, Z., Martins, L.O., Lindley, P.F. & Soares, C.M. 2010. Mechanisms underlying dioxygen reduction in laccases. Structural and modelling studies focusing on proton transfer. BMC Struct Biol, Vol. 10, pp. 28.

Bergman, P.J., McKnight, J., Novosad, A., Charney, S., Farrelly, J., Craft, D., Wulderk, M., Jeffers, Y., Sadelain, M., Hohenhaus, A.E., et al. 2003. Long-term survival of dogs with advanced malignant melanoma after

DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res, Vol. 9, No. 4, pp. 1284–1290.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K. & Xu, F.F. 1997. Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspergillus oryzae*. Appl Environ Microbiol, Vol. 63, No. 8, pp. 3151–3157.

Bernan, V., Filpula, D., Herber, W., Bibb, M. & Katz, E. 1985. The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterization of the gene product. Gene, Vol. 37, No. 1–3, pp. 101–110.

Bertrand, T., Jolivalt, C., Briozzo, P., Caminade, E., Joly, N., Madzak, C. & Mougin, C. 2002. Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry, Vol. 41, No. 23, pp. 7325–7333.

Bi, J.L. & Felton, G.W. 1995. Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. J Chem Ecol, Vol. 21, pp. 1511–1530.

Bittner, S. 2006. When quinones meet amino acids: chemical, physical and biological consequences. Amino Acids, Vol. 30, No. 3, pp. 205–224.

Boer, H. & Koivula, A. 2003. The relationship between thermal stability and pH optimum studied with wild-type and mutant *Trichoderma reesei* cellobiohydrolase Cel7A. Eur J Biochem, Vol. 270, No. 5, pp. 841–848.

Bollag, J.-M. & Leonowicz, A. 1984. Comparative studies of extracellular fungal laccases. Appl Environ Microbiol, Vol. 48, No. 4, pp. 849–854.

Bond, C.S. & Schuttelkopf, A.W. 2009. ALINE: a WYSIWYG protein-sequence alignment editor for publication-quility alignments. Acta Cryst, Vol. D65, pp. 510–512.

Borthakur, D., Lamb, J.W. & Johnston, A.W. 1987. Identification of two classes of *Rhizobium phaseoli* genes required for melanin synthesis, one of which is required for nitrogen fixation and activates the transcription of the other. Mol Gen Genet, Vol. 207, No. 1, pp. 155–160.

Bourbonnais, R. & Paice, M.G. 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl Environ Microbiol, Vol. 36, No. 6, pp. 823–827.

Bourbonnais, R., Paice, M.G., Freiermuth, B., Bodie, E. & Borneman, S. 1997. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol, Vol. 63, No. 12, pp. 4627–4632.

Brändén, R. & Deinum, J. 1977. Type 2 copper(II) as a component of the dioxygen reducing site in laccase: evidence from EPR experiments with [17]O. FEBS Lett, Vol. 73, No. 2, pp. 144–146.

Brändén, R., Deinum, J. & Coleman, M. 1978. A mass spectrometric investigation of the reaction between [18]O_2 and reduced tree laccase. A differentiation between the two water molecules formed. FEBS Lett, Vol. 89, No. 1, pp. 180–182.

Breathnach, A. 1996. Melanin hyperpigmentation of skin: melasma, topical treatment with azelaic acid, and other therapies. Cutis, Vol. 57, No. 1, pp. 36–45.

Bubacco, L., Van Gastel, M., Groenen, E.J., Vijgenboom, E. & Canters, G.W. 2003. Spectroscopic characterization of the electronic changes in the active site of *Streptomyces antibioticus* tyrosinase upon binding of transition state analogue inhibitors. J Biol Chem, Vol. 278, No. 9, pp. 7381–7389.

Buchert, J., Selinheimo, E., Kruus, K., Mattinen, M.-L., Lantto, R. & Autio, K. 2007. Using crosslinking enzymes to improve textural and other properties of food. In: Rastall, R. (ed.) Novel enzyme technology for food applications. CRC press, pp. 101–139.

Buchert, J., Ercili Cura, D., Ma, H., Gasparetti, C., Monogioudi, E., Faccio, G., Boer, H., Partanen, R., Selinheimo, E., Lantto, R., et al. 2010. Cross linking food proteins for improved functionality. Annu Rev Food Sci Technol, Vol. 1, No. 1, pp. 113–138.

Busch, J.L.H.C., Hrncirik, K., Bulukin, E., Boucon, C. & Mascini, M. 2006. Biosensor measurements of polar phenolics for the assessment of the bitterness and pungency of virgin olive oil. J Agric Food Chem, Vol. 54, No. 12, pp. 4371–4377.

Cabanes, J., Chazarra, S. & García Carmona, F. 1994. Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. J Pharm Pharmacol, Vol. 46, pp. 982–985.

Call, H.P. & Mücke, I. 1997. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process). J Biotechnol, Vol. 53, No. 2–3, pp. 163–202.

Camarero, S., Ibarra, D., Martínez, Á.T., Romero, J., Gutiérrez, A. & del Río, J.C. 2007. Paper pulp delignification using laccase and natural mediators. Enzyme Microb Technol, Vol. 40, No. 5, pp. 1264–1271.

Canfora, L., Iamarino, G., Rao, M.A. & Gianfreda, L. 2008. Oxidative transformation of natural and synthetic phenolic mixtures by *Trametes versicolor* laccase. J Agric Food Chem, Vol. 56, No. 4, pp. 1398–1407.

Canovas, F.G., García-Carmona, F., Sanchez, J.V., Pastor, J.L. & Teruel, J.A. 1982. The role of pH in the melanin biosynthesis pathway. J Biol Chem, Vol. 257, No. 15, pp. 8738–8744.

Carvalho, G.M., Alves, T.L. & Freire, D.M. 2000. L-DOPA production by immobilized tyrosinase. Appl Biochem Biotechnol, Vol. 84–86, pp. 791–800.

Chang, T.S. 2009. An updated review of tyrosinase inhibitors. Int J Mol Sci, Vol. 10, No. 6, pp. 2440–2475.

Chao, A., Shyu, S., Lin, Y. & Mi, F. 2004. Enzymatic grafting of carboxyl groups on to chitosan – to confer on chitosan the property of a cationic dye adsorbent. Bioresour Technol, Vol. 91, No. 2, pp. 157–162.

Chedekel, M.R., Agin, P.P. & Sayre, R.M. 1980. Photochemistry of pheomelanin: action spectrum for superoxide production. Photochem Photobiol, Vol. 31, pp. 553–555.

Chefetz, B., Chen, Y. & Hadar, Y. 1998. Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl Environ Microbiol, Vol. 64, No. 9, pp. 3175–3179.

Chen, J.S., Wei, C. & Marshall, M.R. 1991. Inhibition mechanism of kojic acid on polyphenol oxidase. J Agric Food Chem, Vol. 39, pp. 1897–1901.

Chen, Y.T., Stockert, E., Tsang, S., Coplan, K.A. & Old, L.J. 1995. Immunophenotyping of melanomas for tyrosinase: implications for

vaccine development. Proc Natl Acad Sci U S A, Vol. 92, No. 18, pp. 8125–8129.

Chen, S., Ge, W. & Buswell, J.A. 2004. Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*. Eur J Biochem, Vol. 271, No. 2, pp. 318–328.

Choi, M. & Davidson, V.L. 2011. Cupredoxins-A study of how proteins may evolve to use metals for bioenergetic processes. Metallomics, Vol. 2, pp. 140–151.

Claus, H. & Decker, H. 2006. Bacterial tyrosinases. Syst Appl Microbiol, Vol. 29, No. 1, pp. 3–14.

Coll, P.M., Fernandez-Abalos, J.M., Villanueva, J.R., Santamaria, R. & Perez, P. 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol, Vol. 59, No. 8, pp. 2607–2613.

Conrad, J.S., Dawso, S.R., Hubbard, E.R., Meyers, T.E. & Strothkamp, K.G. 1994. Inhibitor binding to the binuclear active site of tyrosinase: temperature, pH, and solvent deuterium isotope effects. Biochemistry, Vol. 33, No. 19, pp. 5739–5744.

Conrad, L.S., Sponholz, W.R. & Berker, O. 2000. Treatment of cork woth a phenol oxidazing enzyme. USA. Patent No: US 6152966.

Cooksey, C.J., Garratt, P.J., Land, E.J., Pavel, S., Ramsden, C.A., Riley, P.A. & Smit, N.P. 1997. Evidence of the indirect formation of the catecholic intermediate substrate responsible for the autoactivation kinetics of tyrosinase. J Biol Chem, Vol. 272, No. 42, pp. 26226–26235.

Coyne, V.E. & Al-Harthi, L. 1992. Induction of melanin biosynthesis in *Vibrio cholerae*. Appl Environ Microbiol, Vol. 58, No. 9, pp. 2861–2865.

Cuff, M.E., Miller, K.I., van Holde, K.E. & Hendrickson, W.A. 1998. Crystal structure of a functional unit from *Octopus* hemocyanin. J Mol Biol, Vol. 278, No. 4, pp. 855–870.

D'Annibale, A., Stazi, S.R., Vinciguerra, V. & Giovannozzi Sermanni, G. 2000. Oxirane-immobilized *Lentinula edodes* laccase: stability and phenolics removal efficiency in olive mill wastewater. J Biotechnol, Vol. 77, No. 2–3, pp. 265–273.

Dawley, R.M. & Flurkey, W.H. 1993. Differentiation of tyrosinase and laccase using 4-hexyl-resorcinol, a tyrosinase inhibitor. Phytochemistry, Vol. 33, No. 2, pp. 281–284.

De La Mora, E., Valderrama, B., Horjales, E. & Rudino-Pinera, E. 2008. Crystal structure of *Coriolopsis gallica* laccase at 1.7 Å resolution. DOI:10.2210/pdb2vdz/pdb.

De Leeuw, S.M., Smit, N.P., Van Veldhoven, M., Pennings, E.M., Pavel, S., Simons, J.W. & Schothorst, A.A. 2001. Melanin content of cultured human melanocytes and UV-induced cytotoxicity. J Photochem Photobiol B, Vol. 61, No. 3, pp. 106–113.

de Marco, A. & Roubelakis-Angelakis, K.A. 1997. Laccase activity could contribute to cell-wall reconstitution in regenerating protoplasts. Phytochemistry, Vol. 46, No. 3, pp. 421–425.

Decker, H., Schweikardt, T. & Tuczek, F. 2006. The first crystal structure of tyrosinase: all questions answered? Angew Chem Int Ed Engl, Vol. 45, No. 28, pp. 4546–4550.

Dedeyan, B., Klonowska, A., Tagger, S., Tron, T., Iacazio, G., Gil, G. & Le Petit, J. 2000. Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. Appl Environ Microbiol, Vol. 66, No. 3, pp. 925–929.

Dervall, B.J. 1961. Phenolase and peptic enzyme activity in the chocolate spot disease of beans. Nature, Vol. 189, p. 311.

Dinckaya, E., Akyilmaz, E., Akgol, S., Tatar Önal, S., Zihnioglu, F. & Telefoncu, A. 1998. A novel catechol oxidase enzyme electrode for the specific determination of catechol. Biosci Biotechnol Biochem, Vol. 62, No. 11, pp. 2098–2100.

DiSpirito, A.A., Taaffe, L.R., Lipscomb, J.D. & Hooper, A.B. 1985. A 'blue' copper oxidase from *Nitrosomonas europaea*. Biochim Biophys Acta, Vol. 827, No. 3, pp. 320–326.

Dittmer, N.T., Suderman, R.J., Jiang, H., Zhu, Y., Gorman, M.J., Kramer, K.J. & Kanost, M.R. 2004. Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. Insect Biochem Mol Biol, Vol. 34, No. 1, pp. 29–41.

Dong, S. & Chen, X. 2002. Some new aspects in biosensors. J Biotechnol, Vol. 82, No. 4, pp. 303–323.

Draelos, Z.D. 2007. Skin lightening preparations and the hydroquinone controversy. Dermatol Ther, Vol. 20, No. 5, pp. 308–313.

Dubé, E., Shareck, F., Hurtubise, Y., Daneault, C. & Beauregard, M. 2008. Homologous cloning, expression, and characterisation of a laccase from *Streptomyces coelicolor* and enzymatic decolourisation of an indigo dye. Appl Microbiol Biotechnol, Vol. 79, No. 4, pp. 597–603.

Duckworth, H.W. & Coleman, J.E. 1970. Physicochemical and kinetic properties of mushroom tyrosinase. J Biol Chem, Vol. 245, No. 7, pp. 1613–1625.

Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown, S.H., Ostergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H. & Davies, G.J. 1998. Crystal structure of the type-2 Cu depleted laccase from *Coprinus cinereus* at 2.2 Å resolution. Nat Struct Biol, Vol. 5, No. 4, pp. 310–316.

Dwivedi, U.N., Singh, P., Pandey, V.P. & Kumar, A. 2011. Structure-function relationship among bacterial, fungal and plant laccases. J Molec Catal B, Vol. 68, No. 2, pp. 117–128.

Eggert, C., Temp, U. & Eriksson, K.-E.L. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. Appl Environ Microbiol, Vol. 62, No. 4, pp. 1151–1158.

Eicken, C., Zippel, F., Büldt-Karentzopoulos, K. & Krebs, B. 1998. Biochemical and spectroscopic characterization of catechol oxidase from sweet potatoes (*Ipomoea batatas*) containing a type-3 dicopper center. FEBS Lett, Vol. 436, No. 2, pp. 293–299.

Eickman, N.C., Solomon, E.I., Larrabee, J.A., Spiro, T.G. & Lerch, K. 1978. Ultraviolet resonance Raman study of oxytyrosinase. Comparison with oxyhemocyanins. J Am Chem Soc, Vol. 100, No. 20, pp. 6529–6531.

Enguita, F.J., Martins, L.O., Henriques, A.O. & Carrondo, M.A. 2003. Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. J Biol Chem, Vol. 278, No. 21, pp. 19416–19425.

Ercili Cura, D., Lille, M., Partanen, R., Kruus, K., Buchert, J. & Lantto, R. 2010. Effect of *Trichoderma reesei* tyrosinase on rheology and microstructure of acidified milk gels. Int Dairy J, Vol. 20, No. 12, pp. 830–837.

Espín, J.C., García-Ruíz, P.A., Tudela, J. & García-Cánovas, F.G. 1998. Study of stereospecificity in mushroom tyrosinase. Biochem J, Vol. 331, No. 2, pp. 547–551.

Espín, J.C., Jolivet, S. & Wichers, H.J. 1999. Kinetic study of the oxidation of γ-L-glutaminyl-4-hydroxybenzene catalyzed by mushroom (*Agaricus bisporus*) tyrosinase. J Agric Food Chem, Vol. 47, No. 9, pp. 3495–3502.

Espín, J.C. & Wichers, H.J. 1999. Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. J Agric Food Chem, Vol. 47, No. 7, pp. 2638–2644.

Espín, J.C., Varón, R., Fenoll, L.G., Gilabert, M.A., García-Ruíz, P.A., Tudela, J. & García-Cánovas, F.G. 2000. Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase. Eur J Biochem, Vol. 267, No. 5, pp. 1270–1279.

Færgemand, M., Otte, J. & Qvist, K.B. 1998. Cross-linking of whey proteins by enzymatic oxidation. J Agric Food Chem, Vol. 46, pp. 1326–1333.

Fan, Y. & Flurkey, W.H. 2004. Purification and characterization of tyrosinase from gill tissue of Portabella mushrooms. Vol. 65, No. 6, pp. 671–678.

Fatibello-Filho, O. & da Cruz Vieira, I. 1997. Flow injection spectrophotometric determination of L-dopa and carbidopa in pharmaceutical formulations using a crude extract of sweet potato root [*Ipomoea batatas* (L.) Lam.] as enzymatic source. Analyst, Vol. 122, No. 4, pp. 345–350.

Faure, D., Bouillant, M.L. & Bally, R. 1994. Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. Appl Environ Microbiol, Vol. 60, No. 9, pp. 3413–3415.

Faure, D., Bouillant, M. & Bally, R. 1995. Comparative study of substrates and inhibitors of *Azospirillum lipoferum* and *Pyricularia oryzae* laccases. Appl Environ Microbiol, Vol. 61, No. 3, pp. 1144–1146.

Felton, G.W., Donato, K., Del Vecchio, R.J. & Duffey, S.S. 1989. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J Chem Ecol, Vol. 15, pp. 2267–2694.

Fenoll, L.G., Rodríguez-López, J.N., Garcia-Molina, F.J., García-Cánovas, F.G. & Tudela, J. 2002. Michaelis constants of mushroom tyrosinase with respect to oxygen in the presence of monophenols and diphenols. Int J Biochem Cell Biol, Vol. 34, No. 4, pp. 332–336.

Fernandez-Larrea, J. & Stahl, U. 1996. Isolation and characterization of a laccase gene from *Podospora anserina*. Mol Gen Genet, Vol. 252, No. 5, pp. 539–551.

Ferraroni, M., Myasoedova, N., Schmatchenko, V., Leontievsky, A., Golovleva, L., Scozzafava, A. & Briganti, F. 2007. Crystal structure of a blue laccase from *Lentinus tigrinus*: evidences for intermediates in the molecular oxygen reductive splitting by multicopper oxidases. BMC Struct Biol, Vol. 7, No. 60.

Fling, M., Horowitz, N.H. & Heinenmann, S.F. 1963. The isolation and properties of crystalline tyrosinase from *Neurospora*. J Biol Chem, Vol. 238, pp. 2045–2053.

Flurkey, W.H., Ratcliffl, B., Lopez, L., Kuglin, J. & Dawley, R.M. 1995. Differentiation of fungal tyrosinases and laccases using selective inhibitors and substrates. In: Lee, C. & Whitaker, J.R. (eds.) Enzymatic browning and its prevention. ACS Symposium Series; American Chemical Society, Washington, DC, pp. 81–89.

Flurkey, W.H. & Inlow, J.K. 2008. Proteolytic processing of polyphenol oxidase from plants and fungi. J Inorg Biochem, Vol. 102, No. 12, pp. 2160–2170.

Folk, J.E. & Finlayson, J.S. 1977. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv Protein Chem, Vol. 31, pp. 1–133.

Freddi, G., Anghileri, A., Sampaio, S., Buchert, J., Monti, P. & Taddei, P. 2006. Tyrosinase-catalyzed modification of *Bombyx mori* silk fibroin: grafting of chitosan under heterogeneous reaction conditions. J Biotechnol, Vol. 125, No. 2, pp. 281–294.

Froehner, S.C. & Eriksson, K.-E.L. 1974. Purification and properties of *Neurospora crassa* laccase. J Bacteriol, Vol. 120, No. 1, pp. 458–465.

Funayama, M., Arakawa, H., Yamamoto, R., Nishino, T., Shin, T. & Murao, S. 1995. Effects of alpha- and beta-arbutin on activity of tyrosinases from

mushroom and mouse melanoma. Biosci Biotechnol Biochem, Vol. 59, No. 1, pp. 143–144.

Galante, Y.M. & Formantici, C. 2003. Enzyme applications in detergency and in manufacturing industries. Curr Org Chem, Vol. 7, No. 13, pp. 1399–1422.

Galhaup, C., Wagner, H., Hinterstoisser, B. & Haltrich, D. 2002. Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb Technol, Vol. 30, No. 4, pp. 529–536.

Garavaglia, S., Cambria, M.T., Miglio, M., Ragusa, S., Iacobazzi, V., Palmieri, F., D'Ambrosio, C., Scaloni, A. & Rizzi, M. 2004. The structure of *Rigidoporus lignosus* laccase containing a full complement of copper ions, reveals an asymmetrical arrangement for the T3 copper pair. J Mol Biol, Vol. 342, No. 5, pp. 1519–1531.

Garzillo, A.M., Colao, M.C., Buonocore, V., Oliva, R., Falcigno, L., Saviano, M., Santoro, A.M., Zappala, R., Bonomo, R.P., Bianco, C., et al. 2001. Structural and kinetic characterization of native laccases from *Pleurotus ostreatus*, *Rigidoporus lignosus*, and *Trametes trogii*. J Protein Chem, Vol. 20, No. 3, pp. 191–201.

Gasowska, B., Kafarski, P. & Wojtasek, H. 2004. Interaction of mushroom tyrosinase with aromatic amines, *o*-diamines and *o*-aminophenols. Biochim Biophys Acta, Vol. 1673, No. 3, pp. 170–177.

van Gastel, M., Bubacco, L., Groenen, E.J.J., Vijgenboom, E. & Canters, G.W. 2000. EPR study of the dinuclear active copper site of tyrosinase from *Streptomyces antibioticus*. FEBS Lett, Vol. 474, No. 2-3, pp. 228-232.

Gavnholt, B. & Larsen, K. 2002. Molecular biology of plant laccases in relation to lignin formation. Physiol Plant, Vol. 116, pp. 273–280.

Ge, H., Gao, Y., Hong, Y., Zhang, M., Xiao, Y., Teng, M. & Niu, L. 2010. Structure of native laccase B from *Trametes* sp. AH28-2. Acta Crystallogr Sect F Struct Biol Cryst Commun, Vol. 66, pp. 254–258.

Gerdemann, C., Eicken, C. & Krebs, B. 2002. The crystal structure of catechol oxidase: new insight into the function of type-3 copper proteins. Acc Chem Res, Vol. 35, No. 3, pp. 183–191.

Germann, U.A. & Lerch, K. 1986. Isolation and partial nucleotide sequence of the laccase gene from *Neurospora crassa*: amino acid sequence homology

of the protein to human ceruloplasmin. Proc Natl Acad Sci U S A, Vol. 83, No. 23, pp. 8854–8858.

Gheibi, N., Saboury, A.A., Haghbeen, K. & Moosavi-Movahedi, A.A. 2005. Activity and structural changes of mushroom tyrosinase induced by *n*-alkyl sulfates. Colloids Surf B Biointerfaces, Vol. 45, No. 2, pp. 104–107.

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G. & Sannia, G. 1999. Protein and gene structure of a blue laccase from *Pleurotus ostreatus*1. Biochem.J, Vol. 341 (Pt 3), No. Pt 3, pp. 655–663.

Gill, R., Freeman, R., Xu, J., Willner, I., Winograd, S., Shweky, I. & Banin, U. 2006. Probing biocatalytic transformations with CdSe-ZnS QDs. J Am Chem Soc, Vol. 128, No. 48, pp. 15376–15377.

Goller, S.P., Schoisswohl, D., Baron, M., Parriche, M. & Kubicek, C.P. 1998. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. Appl Environ Microbiol, Vol. 64, No. 9, pp. 3202–3208.

Griffin, M., Casadio, R. & Bergamini, C.M. 2002. Transglutaminases: nature's biological glues. Biochem J, Vol. 368, No. 2, pp. 377–396.

Gukasyan, G.S. 1999. Purification and some properties of tyrosinase from *Aspergillus flavipes* 56003. Biokhimiya, Vol. 64, No. 4, pp. 497–501.

Gutiérrez, A., Rencoret, J., Ibarra, D., Molina, S., Camarero, S., Romero, J., del Río, J.C. & Martínez, Á.T. 2007. Removal of lipophilic extractives from paper pulp by laccase and lignin-derived phenols as natural mediators. Environ Sci Technol, Vol. 41, No. 11, pp. 4124–4129.

Hakulinen, N., Kiiskinen, L.-L., Kruus, K., Saloheimo, M., Paananen, A., Koivula, A. & Rouvinen, J. 2002. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. Nat Struct Biol, Vol. 9, No. 8, pp. 601–605.

Hakulinen, N., Kruus, K., Koivula, A. & Rouvinen, J. 2006. A crystallographic and spectroscopic study on the effect of X-ray radiation on the crystal structure of *Melanocarpus albomyces* laccase. Biochem Biophys Res Commun, Vol. 350, No. 4, pp. 929–934.

Hakulinen, N., Andberg, M., Kallio, J., Koivula, A., Kruus, K. & Rouvinen, J. 2008. A near atomic resolution structure of a *Melanocarpus albomyces* laccase. J Struct Biol, Vol. 162, No. 1, pp. 29–39.

Halaban, R., Patton, R.S., Cheng, E., Svedine, S., Trombetta, E.S., Wahl, M.L., Ariyan, S. & Hebert, D.N. 2002. Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway. J Biol Chem, Vol. 277, No. 17, pp. 14821–14828.

Halaouli, S., Asther, M., Kruus, K., Guo, L., Hamdi, M., Sigoillot, J.C., Asther, M. & Lomascolo, A. 2005. Characterization of a new tyrosinase from *Pycnoporus* species with high potential for food technological applications. J Appl Microbiol, Vol. 98, No. 2, pp. 332–343.

Halaouli, S., Asther, M., Sigoillot, J.C., Hamdi, M. & Lomascolo, A. 2006a. Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. J Appl Microbiol, Vol. 100, No. 2, pp. 219–232.

Halaouli, S., Record, E., Casalot, L., Hamdi, M., Sigoillot, J.C., Asther, M. & Lomascolo, A. 2006b. Cloning and characterization of a tyrosinase gene from the white-rot fungus *Pycnoporus sanguineus*, and overproduction of the recombinant protein in *Aspergillus niger*. Appl Environ Microbiol, Vol. 70, No. 5, pp. 580–589.

Harjinder, S. 1991. Modification of food proteins by covalent crosslinking. Trends Food Sci Technol, Vol. 2, No. 0, pp. 196–200.

Hepp, A.F., Himmelwright, R.S., Eickman, N.C. & Solomon, E.I. 1979. Ligand displacement reactions of oxyhemocyanin: comparison of reactivities of arthropods and molluscs. Biochem Biophys Res Commun, Vol. 89, No. 4, pp. 1050–1057.

Hildén, K., Hakala, T.K. & Lundell, T. 2009. Thermotolerant and thermostable laccases. Biotechnol Lett, Vol. 31, No. 8, pp. 1117–1128.

Himmelwright, R.S., Eickman, N.C., LuBien, C.D., Lerch, K. & Solomon, E.I. 1980. Chemical and spectroscopic studies of the binuclear copper active site of *Neurospora* tyrosinase: comparison to hemocyanins. J Am Chem Soc, Vol. 102, pp. 7339–7344.

Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R. & Kües, U. 2006. Phylogenetic comparison and classification of laccase and related

multicopper oxidase protein sequences. Vol. 273, No. 10, pp. 2308–2326.

Hoffmann, P.F. & Esser, K. 1977. The phenol oxidases of the ascomycete *Podospora anserina*. XII. Affinity of laccases II and III to substrates with different substitution patterns. Arch Microbiol, Vol. 112, pp. 111–114.

Huang, K.X., Fujii, I., Ebizuka, Y., Gomi, K. & Sankawa, U. 1995. Molecular cloning and heterologous expression of the gene encoding dihydrogeodin oxidase, a multicopper blue enzyme from *Aspergillus terreus*. J Biol Chem, Vol. 270, No. 37, pp. 21495–21502.

Ismaya, W.T., Rozeboom, H.J., Weijn, A., Mes, J.J., Fusetti, F., Wichers, H.J. & Dijkstra, B.W. 2011. Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. Biochemistry, Vol. 50, No. 24, pp. 5477–5486.

Ito, S., Kato, T., Shinpo, K. & Fujita, K. 1984. Oxidation of tyrosine residues in proteins by tyrosinase. Biochem J, Vol. 222, pp. 407–411. Itoh, S., Kumei, H., Taki, M., Nagatomo, S., Kitagawa, T. & Fukuzumi, S. 2001. Oxygenation of phenols to catechols by a (μ-η^2:η^2-peroxo)dicopper(II) complex: mechanistic insight into the phenolase activity of tyrosinase. J Am Chem Soc, Vol. 123, No. 27, pp. 6708–6709.

Ito, S. & Wakamatsu, K. 2008. Chemistry of mixed melanogenesis-pivotal roles of dopaquinone. Photochem Photobiol, Vol. 84, No. 3, pp. 582–592.

Jacobson, E.S. 2000. Pathogenic roles for fungal melanins. Clin Microbiol Rev, Vol. 13, No. 4, pp. 708–717.

Johnson, D.L., Thompson, J.L., Brinkmann, S.M., Schuller, K.A. & Martin, L.L. 2003. Electrochemical characterization of purified *Rhus vernicifera* laccase: voltammetric evidence for a sequential four-electron transfer. Biochemistry, Vol. 42, No. 34, pp. 10229–10237.

Jolley, R.L.,Jr., Evans, L.H., Makino, N. & Mason, H.S. 1974. Oxytyrosinase. J Biol Chem, Vol. 249, No. 2, pp. 335–345.

Jordan, A.M., Khan, T.H., Osborn, H.M., Photiou, A. & Riley, P.A. 1999. Melanocyte-directed enzyme prodrug therapy (MDEPT): development of a targeted treatment for malignant melanoma. Bioorg Med Chem, Vol. 7, No. 9, pp. 1775–1780.

Jordan, A.M., Khan, T.H., Malkin, H., Osborn, H.M., Photiou, A. & Riley, P.A. 2001. Melanocyte-directed enzyme prodrug therapy (MDEPT): development of second generation prodrugs for targeted treatment of malignant melanoma. Bioorg Med Chem, Vol. 9, No. 6, pp. 1549–1558.

Jung, H., Xu, F.F. & Li, K. 2002. Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. Enzyme Microb Technol, Vol. 30, No. 2, pp. 161–168.

Jus, S., Kokol, V. & Gübitz, G.M. 2008. Tyrosinase-catalysed coupling of functional molecules onto protein fibres. Vol. 42, No. 7, pp. 535–542.

Käärik, A. 1965. The identification of the mycelia of wood-decay fungi by their oxidation reactions with phenolic compounds. Studia forestalia suecica, Vol. 31, pp. 1–80.

Kaljunen, H., Gasparetti, C., Kruus, K., Rouvinen, J. & Hakulinen, N. 2011. Crystallization and preliminary X-ray analysis of *Aspergillus oryzae* catechol oxidase. Acta Crystallogr Sect F Struct Biol Cryst Commun, Vol. 67, No. 6, pp. 672–674.

Kallio, J.P., Auer, S., Jänis, J., Andberg, M., Kruus, K., Rouvinen, J., Koivula, A. & Hakulinen, N. 2009. Structure-function studies of a *Melanocarpus albomyces* laccase suggest a pathway for oxidation of phenolic compounds. J Mol Biol, Vol. 392, No. 4, pp. 895–909.

Kanda, K., Sato, T., Ishii, S., Enei, H. & Ejiri, S. 1996. Purification and properties of tyrosinase isozymes from the gill of *Lentinus edodes* fruiting body. Biosci Biotechnol Biochem, Vol. 60, No. 8, pp. 1273–1278.

Karahanian, E., Corsini, G., Lobos, S. & Vicuña, R. 1998. Structure and expression of a laccase gene from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Biochim Biophys Acta, Vol. 1443, No. 1–2, pp. 65–74.

Kato, A., Wada, T., Kobayashi, K., Seguro, K. & Motoki, M. 1991. Ovomulcin-food protein conjugates prepared through the transglutaminase reaction. Agric Biol Chem, Vol. 55, pp. 1027–1031.

Kato, A., Minaki, K. & Kobayashi, K. 1993. Improvement of emulsifying properties of egg white proteins by the attachment of polysaccharide through maillard reaction in a dry state. Vol. 41, No. 4, pp. 540–543.

Kiiskinen, L.-L., Viikari, L. & Kruus, K. 2002. Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*. Appl Environ Microbiol, Vol. 59, No. 2–3, pp. 198–204.

Kiiskinen, L.-L., Kruus, K., Bailey, M., Ylösmäki, E., Siika-Aho, M. & Saloheimo, M. 2004. Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. Microbiology, Vol. 150, No. 9, pp. 3065–3074.

Kim, Y.J. & Uyama, H. 2005. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. Cell Mol Life Sci, Vol. 62, No. 15, pp. 1707–1723.

Klabunde, T., Eicken, C., Sacchettini, J.C. & Krebs, B. 1998. Crystal structure of a plant catechol oxidase containing a dicopper center. Nat Struct Biol, Vol. 5, No. 12, pp. 1084–1090.

Klonowska, A., Gaudin, C., Fournel, A., Asso, M., Le Petit, J., Giorgi, M. & Tron, T. 2002. Characterization of a low redox potential laccase from the basidiomycete C30. Eur J Biochem, Vol. 269, No. 24, pp. 6119–6125.

Knowles, W.S. 2003. Asymmetric hydrogenations (Nobel Lecture 2001). Vol. 345, No. 1–2, pp. 3–13.

Ko, E.M., Leem, Y.E. & Choi, H.T. 2001. Purification and characterization of laccase isozymes from the white-rot basidiomycete *Ganoderma lucidum*. Appl Microbiol Biotechnol, Vol. 57, No. 1–2, pp. 98–102.

Komori, H., Miyazaki, K. & Higuchi, Y. 2009. X-ray structure of a two-domain type laccase: A missing link in the evolution of multi-copper proteins. FEBS Lett, Vol. 583, No. 7, pp. 1189–1195.

Kong, K.-H., Lee, J., Park, H. & Cho, S. 1998. Purification and characterization of the tyrosinase isozymes of pine needles. Biochem Mol Biol Int, Vol. 45, No. 4, pp. 717–724.

Kong, K.-H., Hong, M.P., Choi, S.S., Kim, Y.T. & Cho, S.H. 2000. Purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. Biotechnol Appl Biochem, Vol. 31, No. 2, pp. 113–118.

Kubo, I., Nihei, K. & Tsujimoto, K. 2004. Methyl *p*-coumarate, a melanin formation inhibitor in B16 mouse melanoma cells. Vol. 12, No. 20, pp. 5349–5354.

Kupper, U., Niedermann, D.M., Travaglini, G. & Lerch, K. 1989. Isolation and characterization of the tyrosinase gene from *Neurospora crassa*. J Biol Chem, Vol. 264, No. 29, pp. 17250–17258.

Kwon, B.S., Haq, A.K., Pomerantz, S.H. & Halaban, R. 1987. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc Natl Acad Sci U S A, Vol. 84, No. 21, pp. 7473–7477.

Labat, E., Morel, M.H. & Rouau, X. 2000. Effects of laccase and ferulic acid on wheat flour doughs. Cereal Chem, Vol. 77, No. 6, pp. 823–828.

Labat, E., Morel, M.H. & Rouau, X. 2001. Effect of laccase and manganese peroxidase on wheat gluten and pentosans during mixing. Food Hydrocoll, Vol. 15, No. 1, pp. 47–52.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, Vol. 227, No. 5259, pp. 680–685.

Land, E.J., Ramsden, C.A. & Riley, P.A. 2007. The mechanism of suicide-inactivation of tyrosinase: a substrate structure investigation. Tohoku J Exp Med, Vol. 212, No. 4, pp. 341–348.

Lang, G. & Cotteret, J. 1998. Hair dye composition containing a laccase. Patent No: WO9936036.

Langfelder, K., Streibel, M., Jahn, B., Haase, G. & Brakhage, A.A. 2003. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol, Vol. 38, No. 2, pp. 143–158.

Lantto, R., Heine, E., Freddi, G., Lappalainen, A., Miettinen-Oinonen, A., Niku-Paavola, M. & Buchert, J. 2005a. Enzymatic modification of wool with tyrosinase and peroxidase. J Textile Inst, Vol. 96, No. 2, pp. 109–116.

Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J. & Autio, K. 2005b. Enzyme-aided modification of chicken-breast myofibril proteins: effect of laccase and transglutaminase on gelation and thermal stability. J Agric Food Chem, Vol. 53, No. 23, pp. 9231–9237.

Lantto, R., Puolanne, E., Kruus, K., Buchert, J. & Autio, K. 2007. Tyrosinase-aided protein cross-linking: effects on gel formation of chicken breast myofibrils and texture and water-holding of chicken breast meat homogenate gels. J Agric Food Chem, Vol. 55, No. 4, pp. 1248–1255.

Lawton, T.J., Sayavedra-Soto, L.A., Arp, D.J. & Rosenzweig, A.C. 2009. Crystal structure of a two-domain multicopper oxidase: implications for the evolution of multicopper blue proteins. J Biol Chem, Vol. 284, No. 15, pp. 10174–10180.

Lee, S.-K., George, S.D., Antholine, W.E., Hedman, B., Hodgson, K.O. & Solomon, E.I. 2002. Nature of the intermediate formed in the reduction of O_2 to H_2O at the trinuclear copper cluster active site in native laccase. J Am Chem Soc, Vol. 124, No. 21, pp. 6180–6193.

Lee, Y., Whittaker, M. & Whittaker, J. 2008. The electronic structure of the Cys-Tyr(*) free radical in galactose oxidase determined by EPR spectroscopy. Biochemistry (N.Y.), Vol. 47, No. 25, pp. 6637–6649.

Lerch, K. & Ettinger, L. 1972. Purification and characterization of a tyrosinase from *Streptomyces glaucescens*. Eur J Biochem, Vol. 31, No. 3, pp. 427–437.

Lerch, K. 1982. Primary structure of tyrosinase from *Neurospora crassa*. II. Complete amino acid sequence and chemical structure of a tripeptide containing an unusual thioether. J Biol Chem, Vol. 257, No. 11, pp. 6414–6419.

Lerch, K. 1983. *Neurospora* tyrosinase: structural, spectroscopic and catalytic properties. Mol Cell Biochem, Vol. 52, No. 2, pp. 125–138.

Lertsiri, S., Phontree, K., Thepsingha, W. & Bhumiratana, A. 2003. Evidence of enzymatic browning due to laccase-like enzyme during mash fermentation in Thai soybean paste. Food Chem, Vol. 80, No. 2, pp. 171–176.

Li, X., Wei, Z., Zhang, M., Peng, X., Yu, G., Teng, M. & Gong, W. 2007. Crystal structures of *E. coli* laccase CueO at different copper concentrations. Biochem Biophys Res Commun, Vol. 354, No. 1, pp. 21–26.

Liao, J.C., Gregor, P., Wolchok, J.D., Orlandi, F., Craft, D., Leung, C., Houghton, A.N. & Bergman, P.J. 2006. Vaccination with human tyrosinase DNA induces antibody responses in dogs with advanced melanoma. Cancer Immun, Vol. 6, p. 8.

Lim, J.Y., Ishiguro, K. & Kubo, I. 1999. Tyrosinase inhibitory *p*-coumaric acid from ginseng leaves. Phytother Res, Vol. 13, No. 5, pp. 371–375.

Lim, K.G. & Palmore, G.T.R. 2007. Microfluidic biofuel cells: The influence of electrode diffusion layer on performance. Biosens Bioelectron, Vol. 22, No. 6, pp. 941–947.

Littoz, F. & McClements, D.J. 2008. Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. Food Hydrocoll, Vol. 22, No. 7, pp. 1203–1211.

Litvintseva, A.P. & Henson, J.M. 2002. Cloning, characterization, and transcription of three laccase genes from *Gaeumannomyces graminis* var. *tritici*, the take-all fungus. Appl Environ Microbiol, Vol. 68, No. 3, pp. 1305–1311.

Liu, Y. & Dong, S. 2007. A biofuel cell with enhanced power output by grape juice. Vol. 9, No. 7, pp. 1423–1427.

Lundell, T.K., Mäkelä, M.R. & Hildén, K. 2010. Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. J Basic Microbiol, Vol. 50, No. 1, pp. 5–20.

Lyashenko, A.V., Zhukhlistova, N.E., Gabdoulkhakov, A.G., Zhukova, Y.N., Voelter, W., Zaitsev, V.N., Bento, I., Stepanova, E.V., Kachalova, G.S., Koroleva, O.V., Betzel, C., Lindley, P.F., Mikhailov, A.M., Tishkov, V.I. & Morgunova, E.Y. 2007. Crystal structure of laccase from *Coriolus zonatus* at 2.6 Å resolution. 10.2210/pdb2hzh/pdb.

Marbach, I., Harel, E. & Mayer, A.M. 1984. Molecular properties of extracellular *Botrytis cinerea* laccase. Phytochemistry, Vol. 23, No. 12, pp. 2713–2717.

Martin, C., Moeder, M., Daniel, X., Krauss, G. & Schlosser, D. 2007. Biotransformation of the polycyclic musks HHCB and AHTN and metabolite formation by fungi occurring in freshwater environments. Environ Sci Technol, Vol. 41, No. 15, pp. 5395–5402.

Martins, L.O., Soares, C.M., Pereira, M.M., Teixeira, M., Costa, T., Jones, G.H. & Henriques, A.O. 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J Biol Chem, Vol. 277, No. 21, pp. 18849–18859.

Marusek, C.M., Trobaugh, N.M., Flurkey, W.H. & Inlow, J.K. 2006. Comparative analysis of polyphenol oxidase from plant and fungal species. J Inorg Biochem, Vol. 100, No. 1, pp. 108–123.

Mason, H.S. 1948. The chemistry of melanin; mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. J Biol Chem, Vol. 172, No. 1, pp. 83–99.

Masse, M.-O., Duvallet, V., Borremans, M. & Goeyens, L. 2001. Identification and quantitative analysis of kojic acid and arbutine in skin-whitening cosmetics. Int J Cosmet Sci, Vol. 23, No. 4, pp. 219–232.

Matera, I., Gullotto, A., Tilli, S., Ferraroni, M., Scozzafava, A. & Briganti, F. 2008. Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with *p*-toluate. Inorganica Chim Acta, Vol. 361, No. 14–15, pp. 4129–4137.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H. & Sugiyama, M. 2006. Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. J Biol Chem, Vol. 281, No. 13, pp. 8981–8990.

Matoba, Y., Bando, N., Oda, K., Noda, M., Higashikawa, F., Kumagai, T. & Sugiyama, M. 2011. A molecular mechanism for copper transportation to tyrosinase that is assisted by a metallochaperone, caddie protein. J Biol Chem, Vol. 286, No. 34, pp. 30219–30231.

Mattinen, M.-L., Hellman, M., Steffensen, C.L., Selinheimo, E., Permi, P., Kalkkinen, N., Kruus, K. & Buchert, J. 2008a. Laccase and tyrosinase catalysed polymerization of proteins and peptides. J Biotechnol, Vol. 136, No. 1, pp. S318–S318.

Mattinen, M.-L., Lantto, R., Selinheimo, E., Kruus, K. & Buchert, J. 2008b. Oxidation of peptides and proteins by *Trichoderma reesei* and *Agaricus bisporus* tyrosinases. J Biotechnol, Vol. 133, No. 3, pp. 395–402.

Mayer, A.M. & Harel, E. 1979. Polyphenol oxidases in plants. Phytochemistry, Vol. 18, No. 2, pp. 193–215.

Mayer, A.M. 1987. Polyphenol oxidases in plants-recent progress. Phytochemistry, Vol. 26, No. 1, pp. 11–20.

Mayer, A.M. & Staples, R.C. 2002. Laccase: new functions for an old enzyme. Phytochemistry, Vol. 60, No. 6, pp. 551–565.

McMahon, A.M., Doyle, E.M., Brooks, S. & O'Connor, K.E. 2007. Biochemical characterisation of the coexisting tyrosinase and laccase in the soil

bacterium *Pseudomonas putida* F6. Enzyme Microb Technol, Vol. 40, pp. 1435–1441.

Melo, G.A., Shimizu, M.M. & Mazzafera, P. 2006. Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust. Phytochemistry, Vol. 67, No. 3, pp. 277–285.

Mensah, C.A., Adamafio, N.A., Amaning-Kwarteng, K. & Rodrigues, F.K. 2012. Reduced tannin content of laccase-treated cocoa (*Theobroma cacao*) pod husk. IJBC, Vol. 6, pp. 31–36.

Messerschmidt, A., Ladenstein, R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzelli, R., Rossi, A. & Finazzi-Agro, A. 1992. Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. J Mol Biol, Vol. 224, No. 1, pp. 179–205.

Mikolasch, A., Niedermeyer, T.H., Lalk, M., Witt, S., Seefeldt, S., Hammer, E., Schauer, F., Gesell, M., Hessel, S., Julich, W.D., et al. 2006. Novel penicillins synthesized by biotransformation using laccase from *Trametes* spec. Chem Pharm Bull (Tokyo), Vol. 54, No. 5, pp. 632–638.

Mikolasch, A., Wurster, M., Lalk, M., Witt, S., Seefeldt, S., Hammer, E., Schauer, F., Julich, W.D. & Lindequist, U. 2008. Novel beta-lactam antibiotics synthesized by amination of catechols using fungal laccase. Chem Pharm Bull (Tokyo), Vol. 56, No. 7, pp. 902–907.

Minussi, R.C., Pastore, G.M. & Durán, N. 2002. Potential applications of laccase in the food industry. Trends Food Sci Technol, Vol. 13, No. 6–7, pp. 205–216.

Minussi, R.C., Rossi, M., Bologna, L., Rotilio, D., Pastore, G.M. & Durán, N. 2007. Phenols removal in musts: strategy for wine stabilization by laccase. J Molec Catal B, Vol. 45, No. 3–4, pp. 102–107.

Mita, D.G., Attanasio, A., Arduini, F., Diano, N., Grano, V., Bencivenga, U., Rossi, S., Amine, A. & Moscone, D. 2007. Enzymatic determination of BPA by means of tyrosinase immobilized on different carbon carriers. Biosens Bioelectron, Vol. 23, No. 1, pp. 60–65.

Monogioudi, E., Creusot, N., Kruus, K., Gruppen, H., Buchert, J. & Mattinen, M.,-L. 2009. Cross-linking of β-casein by *Trichoderma reesei* tyrosinase and *Streptoverticillium mobaraense* transglutaminase followed by SEC–MALLS. Food Hydrocoll, Vol. 23, No. 7, pp. 2008–2015.

Montiel, A.M., Fernandez, F.J., Marcial, J., Soriano, J., Barrios-Gonzalez, J. & Tomasini, A. 2004. A fungal phenoloxidase (tyrosinase) involved in pentachlorophenol degradation. Biotechnol Lett, Vol. 26, No. 17, pp. 1353–1357.

Morpurgo, L., Graziani, M.T., Desideri, A. & Rotilio, G. 1980. Titrations with ferrocyanide of japanese-lacquer-tree (*Rhus vernicifera*) laccase and of the type 2 copper-depleted enzyme. Interrelation of the copper sites. Biochem J, Vol. 187, No. 2, pp. 367–370.

Morrison, M.E., Yagi, M.J. & Cohen, G. 1985. In vitro studies of 2,4-dihydroxyphenylalanine, a prodrug targeted against malignant melanoma cells. Proc Natl Acad Sci U S A, Vol. 82, No. 9, pp. 2960–2964.

Motoda, S. 1979a. Properties of polyphenol oxidase from *Alternaria tenuis*. J Ferment Technol, Vol. 57, No. 2, pp. 79–85.

Motoda, S. 1979b. Purification and some properties of polyphenol oxidase from *Alternaria tenuis*. J Ferment Technol, Vol. 57, No. 2, pp. 71–78.

Muñoz-Muñoz, J.L., Garcia-Molina, F.J., García-Ruíz, P.A., Molina-Alarcón, M., Tudela, J., García-Cánovas, F.G. & Rodríguez-López, J.N. 2008. Phenolic substrates and suicide inactivation of tyrosinase: kinetics and mechanism. Biochem J, Vol. 416, No. 3, pp. 431–440.

Muñoz-Muñoz, J.L., Acosta-Motos, J.R., Garcia-Molina, F.J., Varón, R., García-Ruíz, P.A., Tudela, J., García-Cánovas, F.G. & Rodríguez-López, J.N. 2010a. Tyrosinase inactivation in its action on dopa. Biochim Biophys Acta, Vol. 1804, No. 7, pp. 1467–1475.

Muñoz-Muñoz, J.L., Garcia-Molina, F.J., Varón, R., García-Ruíz, P.A., Tudela, J., García-Cánovas, F.G. & Rodríguez-López, J.N. 2010b. Suicide inactivation of the diphenolase and monophenolase activities of tyrosinase. IUBMB Life, Vol. 62, No. 7, pp. 539–547.

Muñoz-Muñoz, J.L., Garcia-Molina, F., García-Ruíz, P.A., Varon, R., Tudela, J., Rodríguez-López, J.N. & García-Cánovas, F. 2011. Catalytic oxidation of *o*-aminophenols and aromatic amines by mushroom tyrosinase. Biochim Biophys Acta, Vol. 1814, No. 12, pp. 1974–1983.

Nagai, M., Sato, T., Watanabe, H., Saito, K., Kawata, M. & Enei, H. 2002. Purification and characterization of an extracellular laccase from the

edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. Appl Environ Microbiol, Vol. 60, No. 3, pp. 327–335.

Nagai, M., Kawata, M., Watanabe, H., Ogawa, M., Saito, K., Takesawa, T., Kanda, K. & Sato, T. 2003. Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. Microbiology, Vol. 149, No. 9, pp. 2455–2462.

Nakamura, M., Nakajima, T., Ohba, Y., Yamauchi, S., Lee, B.R. & Ichishima, E. 2000. Identification of copper ligands in *Aspergillus oryzae* tyrosinase by site-directed mutagenesis. Biochem J, Vol. 350, No. 2, pp. 537–545.

Nakamura, T., Sho, S. & Ogura, Y. 1966. On the purification and properties of mushroom tyrosinase. J Biochem, Vol. 59, No. 5, pp. 481–486.

Niku-Paavola, M.L., Karhunen, E., Salola, P. & Raunio, V. 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem J, Vol. 254, No. 3, pp. 877–884.

Niladevi, K.N., Jacob, N. & Prema, P. 2008. Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticu*s: purification and characterization. Process Biochem, Vol. 43, No. 6, pp. 654–660.

Niladevi, K.N. 2009. Ligninolytic enzymes. In: Singh nee' Nigam, P. & Pandey, A. (eds.) Biotechnology for agro-industrial residues utilisation. Springer Netherlands, The Netherlands. pp. 397–414.

Nishizawa, Y., Nakabayashi, K. & Shinagawa, E. 1995. Purification and characterization of laccase from white rot fungus *Trametes sanguinea* M85-2. J Ferment Bioeng, Vol. 80, No. 1, pp. 91–93.

Olivares, C. & Solano, F. 2009. New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. Pigment Cell Melanoma Res, Vol. 22, No. 6, pp. 750–760.

O'Malley, D.M., Whetten, R., Bao, W., Chen, C.-L. & Sederoff, R.R. 1993. The role of laccase in lignification. Vol. 4, No. 5, pp. 751–757.

Ossola, M. & Galante, Y.M. 2004. Scouring of flax rove with the aid of enzymes. Enzyme Microb Technol, Vol. 34, No. 2, pp. 177–186.

Palmer, A.E., Randall, D.W., Xu, F.F. & Solomon, E.I. 1999. Spectroscopic studies and electronic structure description of the high potential type 1 copper

site in fungal laccase: Insight into the effect of the axial ligand. J Am Chem Soc, Vol. 121, No. 30, pp. 7138–7149.

Palmieri, G., Giardina, P., Marzullo, L., Desiderio, B., Nitti, G., Cannio, R. & Sannia, G. 1993. Stability and activity of a phenol oxidase from the ligninolytic fungus *Pleurotus ostreatus*. Appl Microbiol Biotechnol, Vol. 39, No. 4–5, pp. 632–636.

Palmieri, G., Giardina, P., Bianco, C., Fontanella, B. & Sannia, G. 2000. Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. Appl Environ Microbiol, Vol. 66, No. 3, pp. 920–924.

Palmieri, G., Cennamo, G., Faraco, V., Amoresano, A., Sannia, G. & Giardina, P. 2003. Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures. Enzyme Microb Technol, Vol. 33, No. 2–3, pp. 220–230.

Palmore, G.T.R. & Kim, H.-H. 1999. Electro-enzymatic reduction of dioxygen to water in the cathode compartment of a biofuel cell. J Electroanal Chem, Vol. 464, No. 1, pp. 110–117.

Paloheimo, M., Valtakari, L., Puranen, T., Kruus, K., Kallio, J., Mäntylä, A., Fagerström, R., Ojapalo, P. & Vehmaanperä, J. 2006. Novel laccase enzyme and use thereof. Patent No: WO 2006 032723 A1.

Palonen, H. & Viikari, L. 2004. Role of oxidative enzymatic treatments on enzymatic hydrolysis of softwood. Biotechnol Bioeng, Vol. 86, No. 5, pp. 550–557.

Palumbo, A., d'Ischia, M., Misuraca, G. & Prota, G. 1987. Effect of metal ions on the rearrangement of dopachrome. Biochim Biophys Acta, Vol. 925, No. 2, pp. 203–209.

Parrish, F.W., Wiley, B.J., Simmons, E.G. & Long, L.,Jr 1966. Production of aflatoxins and kojic acid by species of *Aspergillus* and *Penicillium*. Appl Microbiol, Vol. 14, No. 1, p. 139.

Parsons, M.R., Convery, M.A., Wilmot, C.M., Yadav, K.D., Blakeley, V., Corner, A.S., Phillips, S.E., McPherson, M.J. & Knowles, P.F. 1995. Crystal structure of a quinoenzyme: copper amine oxidase of *Escherichia coli* at 2 A resolution. Structure, Vol. 3, No. 11, pp. 1171–1184.

Parvez, S., Kang, M., Chung, H., Cho, C., Hong, M., Shin, M. & Bae, H. 2006. Survey and mechanism of skin depigmenting and lightening agents. Phytother Res, Vol. 20, No. 11, pp. 921–934.

Pazarlioğlu, N.K., Sariişik, M. & Telefoncu, A. 2005. Laccase: production by *Trametes versicolor* and application to denim washing. Process Biochem, Vol. 40, pp. 1673–1678.

Penttila, M., Nevalainen, H., Ratto, M., Salminen, E. & Knowles, J. 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene, Vol. 61, No. 2, pp. 155–164.

Peralta-Zamora, P., Pereira, C.M., Tiburtius, E.R.L., Moraes, S.G., Rosa, M.A., Minussi, R.C. & Durán, N. 2003. Decolorization of reactive dyes by immobilized laccase. Appl Catal B, Vol. 42, No. 2, pp. 131–144.

Philipp, S., Held, T. & Kutzner, H.J. 1991. Purification and characterization of the tyrosinase of *Streptomyces michiganensis* DSM 40015. J Basic Microbiol, Vol. 31, pp. 293–300.

Piontek, K., Antorini, M. & Choinowski, T. 2002. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90 Å resolution containing a full complement of coppers. J Biol Chem, Vol. 277, No. 40, pp. 37663–37669.

Polyakov, K.M., Fedorova, T.V., Stepanova, E.V., Cherkashin, E.A., Kruzeev, S.A., Strokopytov, B.V., Lamzin, V.S. & Koroleva, O.V. 2009. Structure of native laccase from *Trametes hirsuta* at 1.8 Å resolution. Acta Crystallogr Sect F Struct Biol Cryst Commun, Vol. 65, pp. 611–617.

Poppius-Levlin, K., Wang, W., Tamminen, T., Hortling, B., Viikari, L. & Niku-Paavola, M. 1999. Effects of laccase/HBT treatment on pulp and lignin structures. J Pulp Pap Sci, Vol. 25, No. 3, pp. 90–94.

Prota, G. 1980. Recent advances in the chemistry of melanogenesis in mammals. J Investig Dermatol, Vol. 75, No. 1, pp. 122–127.

Prota, G. 1988. Progress in the chemistry of melanins and related metabolites. Med Res Rev, Vol. 8, No. 4, pp. 525–556.

Ramirez, E.D., Whitaker, J.R. & Virador, V.M. 2004. Polyphenol oxidase. In: Whitaker, J.R., Voragen, A.G.J. & Wong, D.W.S. (eds.) Handbook of food enzymology. CRC Press, pp. 509–523.

Raper, H.S. 1927. The tyrosinase-tyrosine reaction: production from tyrosine of 5:6-dihydroxyindole and 5:6-dihydroxyindole-2-carboxylic acid – the precursors of melanin. Biochem J, Vol. 21, No. 1, pp. 89–96.

Rescigno, A., Sanjust, E., Soddu, G., Rinaldi, A.C., Sollai, F., Curreli, N. & Rinaldi, A. 1998. Effect of 3-hydroxyanthranilic acid on mushroom tyrosinase activity. Biochim Biophys Acta, Vol. 1384, No. 2, pp. 268–276.

Reyes, P., Pickard, M.A. & Vazquez-Duhalt, R. 1999. Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. Biotechnol Lett, Vol. 21, No. 10, pp. 875–880.

Rigling, D. & Van Alfen, N.K. 1993. Extra- and intracellular laccases of the chestnut blight fungus, *Cryphonectria parasitica*. Appl Environ Microbiol, Vol. 59, No. 11, pp. 3634–3639.

Rittstieg, K., Suurnäkki, A., Suortti, T., Kruus, K., Gübitz, G.M. & Buchert, J. 2002. Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. Enzyme Microb Technol, Vol. 31, No. 4, pp. 403–410.

Robb, D.A. 1984. Tyrosinase. In: Lontie, R. (ed.) Copper proteins and copper enzymes. CRC Press, Inc., Boca Raton, FL, pp. 207–241.

Rodgers, C.J., Blanford, C.F., Giddens, S.R., Skamnioti, P., Armstrong, F.A. & Gurr, S.J. 2009. Designer laccases: a vogue for high-potential fungal enzymes? Trends Biotechnol, Vol. 28, No. 2, pp. 63–72.

Rodriguez Couto, S. & Toca Herrera, J.L. 2006. Laccases in the textile industry. BMBR, Vol. 1, No. 4, pp. 115–120.

Rodríguez-López, J.N., Tudela, J., Varón, R. & García-Cánovas, F.G. 1991. Kinetic study on the effect of pH on the melanin biosynthesis pathway. Biochim Biophys Acta, Vol. 1076, No. 3, pp. 379–386.

Rodríguez-López, J.N., Fenoll, L.G., García-Ruíz, P.A., Varon, R., Tudela, J., Thorneley, R.N. & García-Cánovas, F. 2000. Stopped-flow and steady-state study of the diphenolase activity of mushroom tyrosinase. Biochemistry, Vol. 39, No. 34, pp. 10497–10506.

Rogers, M.S. & Dooley, D.M. 2003. Copper-tyrosyl radical enzymes. Curr Opin Chem Biol, Vol. 7, No. 2, pp. 189–196.

Rokhsana, D., Howells, A.E., Dooley, D.M. & Szilagyi, R.K. 2012. Role of the Tyr-Cys cross-link to the active site properties of galactose oxidase. Inorg Chem, Vol. 51, pp. 3513–3524.

Rompel, A., Fischer, H., Meiwes, D., Büldt-Karentzopoulos, K., Dillinger, R., Tuczek, F., Witzel, H. & Krebs, B. 1999a. Purification and spectroscopic studies on catechol oxidases from *Lycopus europaeus* and *Populus nigra*: evidence for a dinuclear copper center of type 3 and spectroscopic similarities to tyrosinase and hemocyanin. J Biol Inorg Chem, Vol. 4, No. 1, pp. 56–63.

Rompel, A., Fischer, H., Meiwes, D., Büldt-Karentzopoulos, K., Magrini, A., Eicken, C., Gerdemann, C. & Krebs, B. 1999b. Substrate specificity of catechol oxidase from *Lycopus europaeus* and characterization of the bioproducts of enzymic caffeic acid oxidation. FEBS Lett, Vol. 445, No. 1, pp. 103–110.

Rompel, A., Büldt-Karentzopoulos, K., Molitor, C. & Krebs, B. 2012. Purification and spectroscopic studies on catechol oxidase from lemon balm (*Melissa officinalis*). Phytochemistry, Vol. 81, pp. 19–23.

Roure, M., Delattre, P. & Froger, H. 1992. Composition for an enzymic coloration of keratin fibres, especially for hair and its use in a dyeing process. Patent No: EP0504005.

Samokhvalov, A., Hong, L., Liu, Y., Garguilo, J., Nemanich, R.J., Edwards, G.S. & Simon, J.D. 2005. Oxidation potentials of human eumelanosomes and pheomelanosomes. Photochem Photobiol, Vol. 81, No. 1, pp. 145–148.

Sanchez-Amat, A. & Solano, F. 1997. A pluripotent polyphenol oxidase from the melanogenic *Marine alteromonas* sp. shares catalytic capabilities of tyrosinases and laccases. Biochem Biophys Res Commun, Vol. 240, No. 3, pp. 787–792.

Sanjust, E., Cecchini, G., Sollai, F., Curreli, N. & Rescigno, A. 2003. 3-Hydroxykynurenine as a substrate/activator for mushroom tyrosinase. Arch Biochem Biophys, Vol. 412, No. 2, pp. 272–278.

Sannia, G., Giardina, P. & Luna, M. 1986. Laccase from *Pleurotus ostreatus*. Biotechnol Lett, Vol. 8, No. 11, pp. 797–800.

Schneider, P., Caspersen, M.B., Mondorf, K., Halkier, T., Skov, L.K., Østergaard, P.R., Brown, K.M., Brown, S.H. & Xu, F.F. 1999. Characterization of a

Coprinus cinereus laccase. Enzyme Microb Technol, Vol. 25, No. 6, pp. 502–508.

Schoot Uiterkamp, A.J.M., Evans, L.H., Jolley, R.L.,Jr. & Mason, H.S. 1976. Absorption and circular dichroism spectra of different forms of mushroom tyrosinase. Biochim Biophys Acta, Vol. 453, No. 1, pp. 200–204.

Schouten, A., Wagemakers, L., Stefanato, F.L., van der Kaaij, R.M. & van Kan, J.A.L. 2002. Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. Mol Microbiol, Vol. 43, No. 4, pp. 883–894.

Selamat, M.J., Bakar, J. & Nazamid, S. 2002. Oxidation of polyphenols in unfermented and partly fermented cocoa beans by cocoa polyphenol oxidase and tyrosinase. J Sci Food Agric, Vol. 82, No. 5, pp. 559–566.

Selinheimo, E., Kruus, K., Buchert, J., Hopia, A. & Autio, K. 2006a. Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. Vol. 43, No. 2, pp. 152–159.

Selinheimo, E., Saloheimo, M., Ahola, E., Westerholm-Parvinen, A., Kalkkinen, N., Buchert, J. & Kruus, K. 2006b. Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus *Trichoderma reesei*. FEBS J, Vol. 273, No. 18, pp. 4322–4335.

Selinheimo, E., Autio, K., Kruus, K. & Buchert, J. 2007a. Elucidating the mechanism of laccase and tyrosinase in wheat bread making. J Agric Food Chem, Vol. 55, No. 15, pp. 6357–6365.

Selinheimo, E., NiEidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'Beirne, D., Buchert, J. & Kruus, K. 2007b. Comparison of the characteristics of fungal and plant tyrosinases. J Biotechnol, Vol. 130, No. 4, pp. 471–480.

Selinheimo, E., Lampila, P., Mattinen, M.-L. & Buchert, J. 2008. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. J Agric Food Chem, Vol. 56, No. 9, pp. 3118–3128.

Sendovski, M., Kanteev, M., Ben-Yosef, V.S., Adir, N. & Fishman, A. 2011. First structures of an active bacterial tyrosinase reveal copper plasticity. J Mol Biol, Vol. 405, No. 1, pp. 227–237.

Seo, S.Y., Sharma, V.K. & Sharma, N. 2003. Mushroom tyrosinase: recent prospects. J Agric Food Chem, Vol. 51, No. 10, pp. 2837–2853.

Sethuraman, A., Akin, D.E. & Eriksson, K.-E.L. 1999. Production of ligninolytic enzymes and synthetic lignin mineralization by the bird's nest fungus *Cyathus stercoreus*. Appl Environ Microbiol, Vol. 52, No. 5, pp. 689–697.

Setti, L., Giuliani, S., Spinozzi, G. & Pifferi, P.G. 1999. Laccase catalyzed-oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. Enzyme Microb Technol, Vol. 25, No. 3–5, pp. 285–289.

Shin, W., Sundaram, U.M., Cole, J.L., Zhang, H.H., Hedman, B., Hodgson, K.O. & Solomon, E.I. 1996. Chemical and spectroscopic definition of the peroxide-level intermediate in the multicopper oxidases: relevance to the catalytic mechanism of dioxygen reduction to water. J Am Chem Soc, Vol. 118, No. 13, pp. 3202–3215.

Shuster Ben-Yosef, V., Sendovski, M. & Fishman, A. 2010. Directed evolution of tyrosinase for enhanced monophenolase/diphenolase activity ratio. Enzyme Microb Technol, Vol. 47, No. 7, pp. 372–376.

Si, J.Q. 1994. Use of laccase in baking industry. Patent No: PCT/DK94/00232.

Siegbahn, P.E. 2004. The catalytic cycle of catechol oxidase. J Biol Inorg Chem, Vol. 9, No. 5, pp. 577–590.

Sigoillot, J.-C., Herpoël, I., Frasse, P., Moukha, S., Lesage-Messen, L. & Marcel, A. 1999. Laccase production by a monokaryotic strain of *Pycnoporus cinnabarinus* derived from a dikaryotic strain. World J Microbiol Biotechnol, Vol. 15, No. 4, pp. 481–484.

Sjoblad, R. & Bollag, J. 1981. Oxidative coupling of aromatic compounds by enzymes from soil microorganisms. In: Paul, E. & Ladd, J. (eds.) Soil biochemistry. Marcel Dekker, New York. Pp. 113–152.

Skálová, T., Dohnálek, J., Østergaard, L.H., Østergaard, P.R., Kolenko, P., Dušková, J., Štěpánková, A. & Hašek, J. 2009. The structure of the small laccase from *Streptomyces coelicolor* reveals a link between laccases and nitrite reductases. J Mol Biol, Vol. 385, No. 4, pp. 1165–1178.

Skálová, T., Duskova, J., Hasek, J., Stepankova, A., Koval, T., Ostergaard, L.H. & Dohnalek, J. 2011. Structure of laccase from *Streptomyces coelicolor*

after soaking with potassium hexacyanoferrate and at an improved resolution of 2.3 Å. Acta Crystallogr Sect F Struct Biol Cryst Commun, Vol. 67, pp. 27–32.

Smolander, M., Boer, H., Valkiainen, M., Roozeman, R., Bergelin, M., Eriksson, J.-E., Zhang, X.-C., Koivula, A. & Viikari, L. 2008. Development of a printable laccase-based biocathode for fuel cell applications. Enzyme Microb Technol, Vol. 43, No. 2, pp. 93–102.

Soler-Rivas, C., Arpin, N., Olivier, J.M. & Wichers, H.J. 1997. Activation of tyrosinase in *Agaricus bisporus* strains following infection by *Pseudomonas tolaasii* or treatment with a tolaasin-containing preparation. Mycol Res, Vol. 101, No. 3, pp. 375–382.

Solomon, E.I., Sundaram, U.M. & Machonkin, T.E. 1996. Multicopper oxidases and oxygenases. Chem Rev, Vol. 96, No. 7, pp. 2563–2606.

Solomon, E.I., Chen, P., Metz, M., Lee, S.-K. & Palmer, A.E. 2001. Oxygen binding, activation, and reduction to water by copper proteins. Angew Chem Int Ed Engl, Vol. 40, No. 24, pp. 4570–4590.

Streffer, K., Vijgenboom, E., Tepper, A.W.J.W., Makower, A., Scheller, F.W., Canters, G.W. & Wollenberger, U. 2001. Determination of phenolic compounds using recombinant tyrosinase from *Streptomyces antibioticus*. Anal Chim Acta, Vol. 427, No. 2, pp. 201–210.

Subramanian, N., Venkatesh, P., Ganguli, S. & Sinkar, V.P. 1999. Role of polyphenol oxidase and peroxidase in the generation of black tea theaflavins. J Agric Food Chem, Vol. 47, No. 7, pp. 2571–2578.

Sugumaran, M., Giglio, L., Kundzicz, H., Saul, S. & Semensi, V. 1992. Studies on the enzymes involved in puparial cuticle sclerotization in *Drosophila melanogaster*. Arch Insect Biochem Physiol, Vol. 19, No. 4, pp. 271–283.

Sugumaran, M. 1995. A caution about the azide inhibition of enzymes associated with electrophilic metabolites. Biochem Biophys Res Commun, Vol. 212, No. 3, pp. 834–839.

Sugumaran, M. 2002. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res, Vol. 15, No. 1, pp. 2–9.

Suzuki, T., Endo, K., Ito, M., Tsujibo, H., Miyamoto, K. & Inamori, Y. 2003. A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. Biosci Biotechnol Biochem, Vol. 67, No. 10, pp. 2167–2175.

Swan, G.A. & Waggott, A. 1970. Studies related to the chemistry of melanins. X. Quantitative assessment of different types of units present in dopa-melanin. J Chem Soc Perkin 1, Vol. 10, pp. 1409–1418.

Swan, G.A. 1974. Structure, chemistry, and biosynthesis of the melanins. Fortschr Chem Org Naturst, Vol. 31, No. 0, pp. 521–582.

Takada, K., Uozumi, T., Kimurja, A., Someya, K. & Yoshino, T. 2003. Influence of oxidative and/or reductive treatment on human hair (III) oxidative reaction of polyphenol oxidase (laccase) to hair dyeing. J Oleo Sci, Vol. 52, No. 10, pp. 557–563.

Takeuchi, S., Zhang, W., Wakamatsu, K., Ito, S., Hearing, V.J., Kraemer, K.H. & Brash, D.E. 2004. Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin. Proc Natl Acad Sci U S A, Vol. 101, No. 42, pp. 15076–15081.

Tepper, A.W.J.W. 2005. Structure and mechanism of the type-3 copper protein tyrosinase. Doctoral Thesis Leiden University. Pp. 1–165.

Thalmann, C.R. & Lötzbeyer, T. 2002. Enzymatic cross-linking of proteins with tyrosinase. Eur Food Res Technol, Vol. 214, pp. 276–281.

Torres, E., Bustos-Jaimes, I. & Le Borgne, S. 2003. Potential use of oxidative enzymes for the detoxification of organic pollutants. Appl Catal B, Vol. 46, No. 1, pp. 1–15.

Toussaint, O. & Lerch, K. 1987. Catalytic oxidation of 2-aminophenols and ortho hydroxylation of aromatic amines by tyrosinase. Biochemistry, Vol. 26, No. 26, pp. 8567–8571.

Tsukamoto, K., Jackson, I.J., Urabe, K., Montague, P.M. & Hearing, V.J. 1992. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO J, Vol. 11, No. 2, pp. 519–526.

Valderrama, B., Oliver, P., Medrano-Soto, A. & Vazquez-Duhalt, R. 2003. Evolutionary and structural diversity of fungal laccases. Vol. 84, No. 4,

pp. 289–299. Virador, V.M., Reyes Grajeda, J.P., Blanco-Labra, A., Mendiola-Olaya, E., Smith, G.M., Moreno, A. & Whitaker, J.R. 2009. Cloning, sequencing, purification, and crystal structure of grenache (*Vitis vinifera*) polyphenol oxidase. J Agric Food Chem, Vol. 58, pp. 1189–1201.

Wahleithner, J.A., Xu, F., Brown, K.M., Brown, S.H., Golightly, E.J., Halkier, T., Kauppinen, S., Pederson, A. & Schneider, P. 1996. The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*. Curr Genet, Vol. 29, No. 4, pp. 395–403.

Waite, J.H. 1976. Calculating extinction coefficients for enzymatically produced *o*-quinones. Anal Biochem, Vol. 75, No. 1, pp. 211–218.

Walker, J.R. & Ferrar, P.H. 1998. Diphenol oxidases, enzyme-catalysed browning and plant disease resistance. Biotechnol Genet Eng Rev, Vol. 15, pp. 457–498.

Wang, N. & Hebert, D.N. 2006. Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. Pigment Cell Res, Vol. 19, No. 1, pp. 3–18.

Wilcox, D.E., Porras, A.G., Hwang, Y.T., Lerch, K., Winkler, M.E. & Solomon, E.I. 1985. Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. J Am Chem Soc, Vol. 107, pp. 4015–4027.

Williamson, P.R. 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J Bacteriol, Vol. 176, No. 3, pp. 656–664.

Wong, Y. & Yu, J. 1999. Laccase-catalyzed decolorization of synthetic dyes. Water Res, Vol. 33, No. 16, pp. 3512–3520.

Wood, D.A. 1980a. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. J Gen Microbiol, Vol. 117, pp. 327–338.

Wood, D.A. 1980b. Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. J Gen Microbiol, Vol. 117, No. 2, pp. 339–345.

Xiao, Y.Z., Chen, Q., Hang, J., Shi, Y.Y., Xiao, Y.Z., Wu, J., Hong, Y.Z. & Wang, Y.P. 2004. Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes* sp. AH28-2. Mycologia, Vol. 96, pp. 26–35.

Xie, J., Song, K., Qiu, L., He, Q., Huang, H. & Chen, Q. 2007. Inhibitory effects of substrate analogues on enzyme activity and substrate specificities of mushroom tyrosinase. Food Chem, Vol. 103, No. 4, pp. 1075–1079.

Xu, F.F. 1996a. Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry, Vol. 35, No. 23, pp. 7608–7614.

Xu, F.F. 1996b. Catalysis of novel enzymatic iodide oxidation by fungal laccase. Appl Biochem Biotechnol, Vol. 59, No. 3, pp. 221–230.

Xu, F.F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M. & Solomon, E.I. 1996. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochim Biophys Acta, Vol. 1292, No. 2, pp. 303–311.

Xu, F.F. 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J Biol Chem, Vol. 272, No. 2, pp. 924–928.

Xu, F.F., Palmer, A.E., Yaver, D.S., Berka, R.M., Gambetta, G.A., Brown, S.H. & Solomon, E.I. 1999. Targeted mutations in a *Trametes villosa* laccase. Axial perturbations of the T1 copper. J Biol Chem, Vol. 274, No. 18, pp. 12372–12375.

Xu, F.F., Kulys, J.J., Duke, K., Li, K., Krikstopaitis, K., Deussen, H.J., Abbate, E., Galinyte, V. & Schneider, P. 2000. Redox chemistry in laccase-catalyzed oxidation of N-hydroxy compounds. Appl Environ Microbiol, Vol. 66, No. 5, pp. 2052–2056.

Xu, F.F., Deussen, H.J., Lopez, B., Lam, L. & Li, K. 2001. Enzymatic and electrochemical oxidation of N-hydroxy compounds. Redox potential, electron-transfer kinetics, and radical stability. Eur J Biochem, Vol. 268, No. 15, pp. 4169–4176.

Yagi, A., Kanbara, T. & Morinobu, N. 1987. Inhibition of mushroom tyrosinase by *Aloe* extract. Planta Medica, Vol. 53, No. 6, pp. 515–517.

Yagüe, S., Terrón, M.C., González, T., Zapico, E., Bocchini, P., Galletti, G.C. & González, A.E. 2000. Biotreatment of tannin-rich beer-factory wastewater with white-rot basidiomycete *Coriolopsis gallica* monitored by pyrolysis/gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom, Vol. 14, No. 10, pp. 905–910.

Yaropolov, A.I., Kharybin, A.N., Emnéus, J., Marko-Varga, G. & Gorton, L. 1995. Flow-injection analysis of phenols at a graphite electrode modified with co-immobilised laccase and tyrosinase. Anal Chim Acta, Vol. 308, No. 1–3, pp. 137–144.

Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K. & Dalboge, H. 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. Appl Environ Microbiol, Vol. 62, No. 3, pp. 834–841.

Yaver, D.S., Overjero, M.D., Xu, F., Nelson, B.A., Brown, K.M., Halkier, T., Bernauer, S., Brown, S.H. & Kauppinen, S. 1999. Molecular characterization of laccase genes from the basidiomycete *Coprinus cinereus* and heterologous expression of the laccase lcc1. Appl Environ Microbiol, Vol. 65, No. 11, pp. 4943–4948.

Yoon, J., Liboiron, B.D., Sarangi, R., Hodgson, K.O., Hedman, B. & Solomon, E.I. 2007. The two oxidized forms of the trinuclear Cu cluster in the multicopper oxidases and mechanism for the decay of the native intermediate. Proc Natl Acad Sci U S A, Vol. 104, No. 34, pp. 13609–13614.

Yoruk, R. & Marshall, M.R. 2003. Physocochemical properties and function of plant polyphenol oxidase: a review. J Food Biochem, Vol. 27, No. 5, pp. 361–422.

Yoshida, H. 1883. Chemistry of lacquer (Urushi), part 1. J Chem Soc, Vol. 43, pp. 472–486.

Zhukova, Y.N., Lyashenko, A.V., Zhukhlistova, N.E., Voelter, W., Gabdoulkhakov, A.G., Bento, I., Zaitsev, V.N., Stepanova, E.V., Kachalova, G.S., Koroleva, O.V., et al. 2006. Purification, crystallization and preliminary X-ray study of the fungal laccase from *Cerrena maxima*. Acta Crystallogr Sect F Struct Biol Cryst Commun, Vol. 62, No. 10, pp. 954–957.

Zille, A., Gornacka, B., Rehorek, A. & Cavaco-Paulo, A. 2005. Degradation of azo dyes by *Trametes villosa* laccase over long periods of oxidative conditions. Appl Environ Microbiol, Vol. 71, No. 11, pp. 6711–6718.

Publications II is not included in the PDF version.
Please order the printed version to get the complete publication
(http://www.vtt.fi/publications/index.jsp).

Title	**Biochemical and structural characterisation of the copper containing oxidoreductases catechol oxidase, tyrosinase, and laccase from ascomycete fungi**
Author(s)	Chiara Gasparetti
Abstract	Catechol oxidase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), and laccase (EC 1.10.3.2) are copper-containing metalloenzymes. They oxidise substituted phenols and use molecular oxygen as a terminal electron acceptor. Catechol oxidases and tyrosinases catalyse the oxidation of *p*-substituted *o*-diphenols to the corresponding *o*-quinones. Tyrosinases also catalyse the introduction of a hydroxyl group in the *ortho* position of *p*-substituted monophenols and the subsequent oxidation to the corresponding *o*-quinones. Laccases can oxidise a wide range of compounds by removing single electrons from the reducing group of the substrate and generate free radicals. The reaction products of these oxidases can react further non-enzymatically and lead to formation of polymers and cross-linking of proteins and carbohydrates, in certain conditions. The work focused on examination of the properties of catechol oxidases, tyrosinases and laccases. A novel catechol oxidase from the ascomycete fungus *Aspergillus oryzae* was characterised from biochemical and structural point of view. Tyrosinases from *Trichoderma reesei* and *Agaricus bisporus* were examined in terms of substrate specificity and inhibition. The oxidation capacity of laccases was elucidated by using a set of laccases with different redox potential and a set of substituted phenolic substrates with different redox potential. Finally, an evaluation of the protein cross-linking ability of catechol oxidase from *A. oryzae*, tyrosinases from *T. reesei* and *A. bisporus* and laccases from *Trametes hirsuta*, *Thielavia arenaria*, and *Melanocarpus albomyces* was performed.
ISBN, ISSN	
Date	October 2012
Language	English
Pages	124 p. + app 55 p.
Keywords	*Trichoderma reesei, Agaricus bisporus*, *Aspergillus oryzae*, *Thielavia arenaria*, tyrosinase, catechol oxidase, laccase, purification, characterisation, oxidation capacity, inhibition, three-dimensional structure, cross-linking
Publisher	

VTT Technical Research Centre of Finland is a globally networked multitechnological contract research organization. VTT provides high-end technology solutions, research and innovation services. We enhance our customers' competitiveness, thereby creating prerequisites for society's sustainable development, employment, and wellbeing.

Turnover: EUR 300 million
Personnel: 3,200

VTT publications

VTT employees publish their research results in Finnish and foreign scientific journals, trade periodicals and publication series, in books, in conference papers, in patents and in VTT's own publication series. The VTT publication series are VTT Visions, VTT Science, VTT Technology and VTT Research Highlights. About 100 high-quality scientific and professional publications are released in these series each year. All the publications are released in electronic format and most of them also in print.

VTT Visions
This series contains future visions and foresights on technological, societal and business topics that VTT considers important. It is aimed primarily at decision-makers and experts in companies and in public administration.

VTT Science
This series showcases VTT's scientific expertise and features doctoral dissertations and other peer-reviewed publications. It is aimed primarily at researchers and the scientific community.

VTT Technology
This series features the outcomes of public research projects, technology and market reviews, literature reviews, manuals and papers from conferences organised by VTT. It is aimed at professionals, developers and practical users.

VTT Research Highlights
This series presents summaries of recent research results, solutions and impacts in selected VTT research areas. Its target group consists of customers, decision-makers and collaborators.

Biochemical and structural characterisation of the copper containing oxidoreductases catechol oxidase, tyrosinase, and laccase from ascomycete fungi

Catechol oxidase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), and laccase (EC 1.10.3.2) are copper-containing metalloenzymes. They oxidise substituted phenols and use molecular oxygen as a terminal electron acceptor. Catechol oxidases and tyrosinases catalyse the oxidation of *p*-substituted *o*-diphenols to the corresponding *o*-quinones. Tyrosinases also catalyse the introduction of a hydroxyl group in the *ortho* position of *p*-substituted monophenols and the subsequent oxidation to the corresponding *o*-quinones. Laccases can oxidise a wide range of compounds by removing single electrons from the reducing group of the substrate and generate free radicals. The reaction products of these oxidases can react further non-enzymatically and lead to formation of polymers and cross-linking of proteins and carbohydrates, in certain conditions.

The work focused on examination of the properties of catechol oxidases, tyrosinases and laccases. A novel catechol oxidase from the ascomycete fungus *Aspergillus oryzae* was characterised from biochemical and structural point of view. Tyrosinases from *Trichoderma reesei* and *Agaricus bisporus* were examined in terms of substrate specificity and inhibition. The oxidation capacity of laccases was elucidated by using a set of laccases with different redox potential and a set of substituted phenolic substrates with different redox potential. Finally, an evaluation of the protein cross-linking ability of catechol oxidase from *A. oryzae*, tyrosinases from *T. reesei* and *A. bisporus* and laccases from *Trametes hirsuta*, *Thielavia arenaria*, and *Melanocarpus albomyces* was performed.