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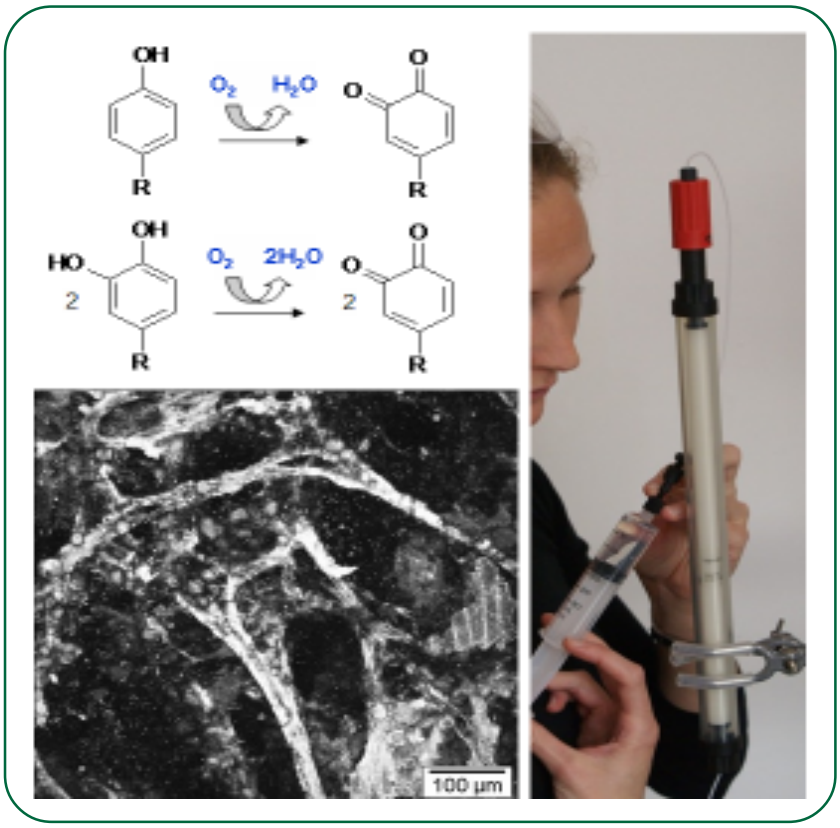

Emilia Selinheimo

Tyrosinase and laccase as novel crosslinking tools for food biopolymers

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Emilia Selinheimo

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Keywords *Trichoderma reesei*, tyrosinase, characterization, crosslinking, laccase, protein, xylan, phenolic acid, xylanase, wheat, dough, bread, rheology

Abstract

Tyrosinases and laccases are copper-containing oxidoreductases, which catalyze oxidation of various mono- and polyphenolic compounds. Tyrosinases oxidize *p*-monophenols and *o*-diphenols to quinones, whereas laccases are capable of oxidizing a larger variety of aromatic compounds, such as substituted mono- and polyphenols, aromatic amines and thiol compounds, with subsequent production of radicals. Both tyrosinase and laccase generate reaction products which are prone to react further non-enzymatically, which may lead to polymerization. In addition to the low molecular mass phenolic compounds, the phenolic moieties present in certain biopolymers are susceptible to oxidation by tyrosinase and laccase, which enables crosslinking of the biopolymers.

The biochemical properties of a novel fungal tyrosinase from *Trichoderma reesei* (TrT) were characterized in this work. The substrate specificity and protein crosslinking ability of TrT were compared to other tyrosinases of plant and fungal origin. Furthermore, the suitability of TrT and laccase from *Trametes hirsuta* (ThL) was examined for hetero-crosslinking of carbohydrates and proteins and for improving wheat breadmaking quality.

TrT was over-expressed in its original host under a strong *cbh1* promoter and purified with a three step purification procedure, consisting of desalting by gel filtration, and cation exchange and gel filtration chromatography. The purified TrT showed a molecular weight of 43.2 kDa as analyzed by mass spectrometry. TrT was found to be processed from the C-terminus by cleavage of a peptide fragment of about 20 kDa. TrT was active both on L-tyrosine and L-dopa, thus showing typical characteristics of a true tyrosinase. TrT had broad substrate specificity, and the enzyme showed the highest activity and stability in the

neutral and alkaline pH range, with an optimum at pH 9. TrT retained its activity relatively well at temperatures of 40 ºC and below.

When tyrosinases from apple (AT), potato (PT), the white rot fungus *Pycnoporus sanguineus* (PsT) and the edible mushroom *Agaricus bisporus* (AbT) were compared to TrT, it was found that the tyrosinases had clearly different features in terms of substrate specificity, inhibition and their ability to crosslink the model protein α-casein. Generally the tyrosinases had lower activity on monophenols than on di- or triphenols. PsT had the highest monophenolase/diphenolase ratio for the oxidation of monophenolic L-tyrosine and diphenolic L-dopa. However, TrT had the highest activity on most of the tested monophenols, and showed clearly shorter lag periods prior to the oxidation of the monophenols than the other enzymes. The activity of AT and PT on tyrosine was undetectable which explains the poor crosslinking ability of α-casein by these enzymes. AbT was also unable to crosslink α-casein, although it could oxidize tyrosine of di- and tri-peptides. Conversely, the activity of PsT on the model peptides turned out to be relatively low, although the enzyme could crosslink α-casein. Of the analyzed tyrosinases, TrT clearly had the best ability to directly crosslink α-casein. However, by after addition of a small molecular weight phenolic compound, L-dopa, to the reaction mixture, the other tyrosinases were also able to crosslink α-casein. It is assumed that L-dopa acted as a bridging compound between the α-casein subunits.

The capability of the two different fungal oxidative enzymes, the TrT tyrosinase and the ThL laccase, to catalyze formation of hetero-conjugates between tyrosine side-chains of α-casein and phenolic acids of hydrolyzed oat spelt xylan (hOSX) was studied. TrT was able to crosslink α-casein more efficiently than ThL, whereas only ThL was able to polymerize hOSX. The radical- and quinone-mediated protein crosslinking clearly differed, which was indicated by enhancement of crosslinking by the presence of phenolic acids with ThL, and by inhibition with TrT. Despite the notable differences between the oxidative enzymes in their ability to crosslink the biopolymers, both ThL and TrT were observed to be able to catalyze oxidative hetero-crosslinking of α-casein and xylan.

The effects of TrT and ThL were also compared in wheat flour breadmaking. The enzymes were found to act in wheat dough and bread via different crosslinking mechanisms. Both ThL and TrT improved the bread quality, especially when

used in combination with xylanase, as indicated by an increase in bread volume and bread crumb softness during storage. The effect of ThL is assumed to be based mainly on the crosslinking of ferulic acid -substituted arabinoxylan with subsequent arabinoxylan network formation, and thus indirectly also strengthening the gluten network of dough. ThL may also have directly oxidized the tyrosyl residues of gluten proteins or enhanced the disulphide bridge formation in gluten polymers via ferulic acid-derived radicals, thus assisting protein aggregation in dough. The effects of TrT in dough and bread are suggested to be due mainly to polymerization of gluten proteins via production of reactive quinones by oxidation of the protein-bound tyrosine residues with consequent formation of crosslinks in the gluten proteins. Tyrosinase may also have influenced the texture properties of dough and bread by oxidizing other phenolic compounds as well as tyrosine of wheat flour, such as *p*-coumaric and caffeic acids.

The oxidative enzymes, tyrosinase and laccase, were shown to have potential in crosslinking of food biopolymers. The *T. reesei* tyrosinase was found to be an efficient protein crosslinker, especially when compared to the *T. hirsuta* laccase or to the tyrosinases of plant and fungal origin. On the other hand, ThL was observed to be more efficient in catalyzing the formation of hetero-crosslinks between proteins and carbohydrates, as compared to TrT. It was shown in this work that both types of oxidative enzymes, tyrosinase and laccase, can be applied to generate food biopolymers with added functionalities or novel food structures from diverse raw materials.

Selinheimo, Emilia. Tyrosinase and laccase as novel crosslinking tools for food biopolymers [Elintarvikebiopolymeerien ristisilloittaminen tyrosinaasilla ja lakkaasilla]. Espoo 2008. VTT Publications 693. 114 s. + liitt. 62 s.

Avainsanat *Trichoderma reesei*, tyrosinase, characterization, crosslinking, laccase, protein, xylan, phenolic acid, xylanase, wheat, dough, bread, rheology

Tiivistelmä

Tyrosinaasit ja lakkaasit ovat kuparia sisältäviä entsyymejä, jotka katalysoivat mono- ja polyfenolisten yhdisteiden hapettumisreaktioita. Tyrosinaasien substraatteja ovat sekä *p*-monofenolit että *o*-di-fenolit, ja reaktiotuotteena muodostuu reaktiivisia kinoneita. Lakkaasit hapettavat mono- ja polyfenolien lisäksi muitakin aromaattisia yhdisteitä, kuten aromaattisia amiineita ja tioleita. Lakkaasien katalysoimissa hapetusreaktioissa syntyy radikaaleja, jotka kinonien tavoin ovat erittäin reaktiivisia yhdisteitä. Kinonien ja radikaalien jatkoreaktiot voivat johtaa substraattien polymeroitumiseen sekä kovalenttisten sidosten muodostumiseen reaktiivisten ryhmien kautta. Tyrosinaasit ja lakkaasit pystyvät hapettamaan myös biopolymeerirakenteissa esiintyviä fenolisia yhdisteitä, minkä johdosta kyseisten biopolymeerien entsyymiavusteinen ristisilloittaminen on mahdollista.

Tässä työssä karakterisoitiin uutta *Trichoderma reesei* -homeesta peräisin olevaa tyrosinaasia. *T. reesei* -tyrosinaasin biokemiallisia ominaisuuksia tutkittiin ja verrattiin muihin home- ja kasviperäisiin tyrosinaaseihin. Entsyymien substraattispesifisyyttä sekä kykyä ristisilloittaa maitoproteiinia tutkittiin. *T. reesei* -tyrosinaasin kykyä muodostaa heterokonjugaatteja proteiinien ja hiilihydraattien välille verrattiin *Trametes hirsuta* -lakkaasiin. Lisäksi tutkittiin *T. reesei* -tyrosinaasin ja *T. hirsuta* -lakkaasin soveltuvuutta vehnätaikinan ja -leivän rakenneominaisuuksien parantamiseen.

T. reesei -tyrosinaasi tuotettiin ylituottomenetelmällä alkuperäisessä tuottoisännässään. Entsyymi puhdistettiin kasvuliuoksesta kolmivaiheisella kromatografisella prosessilla. Ensin tehtiin kasvuliuoksen suolanpoisto geelisuodatuksella. Entsyymiliuosta puhdistettiin kationivaihtokromatografiaa hyödyntäen, jonka jälkeen geelisuodatuksella tyrosinaasi oli mahdollista erottaa täysin muista

T. reesei -homeen proteiineista. *T. reesei* -tyrosinaasin molekyylipainoksi määritettiin 43,2 kDa. Todettiin, että aktiivinen entsyymi oli prosessoitunut proteiini: polypeptidin C-terminaalisesta päästä oli katkennut noin 20 kDa -kokoinen peptidi. Entsyymi katalysoi sekä L-tyrosiinin että L-dopan hapettumista *o*-kinoneiksi, mikä osoitti proteiinin olevan aito tyrosinaasi. *T. reesei* -tyrosinaasi oli aktiivinen neutraalilla ja emäksisellä pH-alueella, ja entsyymin pH-optimiksi määritettiin pH 9.0. Tyrosinaasin todettiin olevan stabiili 40 ºC:ssa ja sen alapuolella, mutta korkeammissa lämpötiloissa entsyymi menetti aktiivisuutensa suhteellisen nopeasti.

Verrattaessa *T. reesei* -tyrosinaasia omenasta, perunasta, *Pycnoporus sanguineus* -valkolahottajasienestä sekä *Agaricus bisporus* -herkkusienestä peräisin oleviin tyrosinaaseihin havaittiin entsyymeiden substraatti- ja stereospesifisyyden, inhiboitumisen ja kyvyn ristisilloittaa maitoproteiinia eroavan toisistaan huomattavasti. Kaikki tyrosinaasit katalysoivat tehokkaammin di-fenolien kuin monofenolien hapettumista, mutta yleisesti ottaen *T. reesei* -tyrosinaasin havaittiin hapettavan monofenolisia yhdisteitä tehokkaimmin. Omenan ja perunan tyrosinaasit eivät hapettaneet L-tyrosiinia, minkä oletettiin olevan myös yhteydessä entsyymien kyvyttömyyteen ristisilloittaa maitoproteiini α-kaseiinia. Herkkusienen tyrosinaasi ei myöskään pystynyt ristisilloittamaan maitoproteiinia, vaikka sen havaittiin hapettavan di- ja tripeptidien tyrosiinisivuketjuja. *P. sanguineus* -tyrosinaasin kyky hapettaa peptidejä taas oli verrattain heikko, mutta entsyymi kykeni silti polymeroimaan maitoproteiinia. Tutkituista entsyymeistä *T. reesei* -tyrosinaasi ristisilloitti maitoproteiinia selvästi tehokkaimmin. Kun reaktioseokseen lisättiin pienimolekyylipainoista di-fenolia, L-dopaa, kaikkien tyrosinaasien havaittiin ristisilloittavan α-kaseiinia. L-dopan oletettiin toimineen silloittavana molekyylinä maitoproteiinien välillä.

T. reesei -tyrosinaasin ja *T. hirsuta* -lakkaasin kykyä ristisilloittaa proteiinia ja hiilihydraattia tutkittiin. Entsyymejä verrattiin suhteessa niiden kykyyn katalysoida polymerointireaktioita maitoproteiini α-kaseiinin ja kauran ksylaanin fenolisten sivuryhmien kautta. Radikaali- ja kinonivälitteisten reaktioiden havaittiin eroavan toisistaan selvästi biopolymeerien ristisilloitusreaktioissa. Tyrosinaasi kykeni polymeroimaan α-kaseiinia lakkaasia paremmin. Toisaalta lakkaasi kykeni polymeroimaan kauran ksylaania, mutta tyrosinaasi ei. Lisäksi reaktioseokseen lisätyt fenoliset hapot indusoivat proteiinin polymerointireaktioita lakkaasilla, kun taas tyrosinaasin katalysoimissa reaktioissa tulos oli päinvastainen, ja fenolihapot inhiboivat maitoproteiinin ristisilloittumista. Huolimatta havaituista eroista

entsyymien välillä sekä lakkaasin että tyrosinaasin osoitettiin muodostavan heteroristisidoksia maitoproteiinin ja kauran ksylaanin välillä.

T. reesei -tyrosinaasin ja *T. hirsuta* -lakkaasin katalysoimia reaktioita verrattiin myös vehnäleivonnassa. Entsyymien havaittiin vaikuttavan vehnätaikinan ja -leivän rakenneominaisuuksiin eri komponenttien polymeroinnin välityksellä. Kumpikin entsyymi paransi vehnäleivän rakennetta lisäämällä leivän ominaistilavuutta sekä sisuksen pehmeyttä. Parhaat tulokset leivän rakenneominaisuuksien osalta saavutettiin, kun tyrosinaasia ja lakkaasia käytettiin yhdessä ksylanaasin kanssa. Lakkaasin vaikutus johtui ilmeisesti pääosin arabinoksylaanin ristisiloittumisesta arabinoksylaanin ferulahapposivuryhmien hapettumisen ja sitä seuraavien radikaalireaktioiden kautta. Mahdollisesti lakkaasi polymeroi vehnätaikinassa myös gluteeniproteiineja joko suoraan tai ferulahappoperäisten radikaalien kautta vaikuttaen siten gluteenin rikkisidoksiin ja taikinan gluteeniverkon muodostumiseen. Tyrosinaasin vaikutusmekanismi taikinassa perustui pääosin vehnän proteiinien ristisitoutumiseen proteiinien tyrosiinisivuryhmien hapettumisen ja muodostuneiden kinonien polymerointireaktioiden kautta. Tyrosinaasi saattoi vaikuttaa vehnäleivän rakenneominaisuuksiin myös hapettamalla proteiinien lisäksi vehnäjauhon fenoliyhdisteitä kuten *p*-kumaarihappoa ja kahvihappoa.

Tässä työssä osoitettiin, että tyrosinaasi ja lakkaasi kykenevät ristisitomaan maito- ja viljaperäisiä biopolymeerejä. *T. reesei* -tyrosinaasin todettiin olevan tehokas maito- ja gluteeniproteiineja polymeroiva entsyymi verrattuna *T. hirsuta* -lakkaasiin tai muihin tutkittuihin home- ja kasviperäisiin tyrosinaaseihin. Toisaalta lakkaasin havaittiin olevan *T. reesei* -tyrosinaasia tehokkaampi katalysoimaan heteropolymerointireaktioita maitoproteiinin ja kauran ksylaanin välillä. Tulosten perusteella voidaan todeta, että tutkitut oksidatiiviset entsyymit, *T. reesei* -tyrosinaasi ja *T. hirsuta* -lakkaasi, soveltuvat elintarvikkeiden rakenne- ja funktionaalisten ominaisuuksien muokkaamiseen.

Preface

The work described in this thesis was carried out at VTT Technical Research Centre of Finland during the years 2004–2007. The research was conducted with financial support from the Research Foundation of Raisiogroup (Raisio, Finland), the project "Controlled modification of carbohydrates and proteins" of the Finnish Funding Agency for Technology and Innovation (TEKES), and the Commission of the European Communities, specific RTD programme "Quality of Life and management of Living Resources, project number QLK1-2002-02208". I thank Dr. Anu Kaukovirta-Norja and Prof. Juha Ahvenainen for providing me with the facilities to carry out the work at VTT. I acknowledge Professor Matti Leisola at Helsinki University of Technology for his advice and encouragement during the course of the work.

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I warmly thank all my colleagues and co-authors for their knowledge and support during the work. In particularly, Maija-Liisa Mattinen, I appreciate the broadening scientific discussions throughout the work. Piritta Lampila, I thank you for your patient work and friendship. Markku Saloheimo, Nisse Kalkkinen, Deirdre NiEidhin, Charlotte Steffensen and Chiara Gasparetti, I am grateful for your valuable contributions to the publications. My special gratitude goes to Outi Liehunen: "We never gave up!" I thank Ritva Heinonen, Airi Hyrkäs, Riitta Isoniemi, Leena Pulkki and Liisa Änäkäinen for their valuable assist and in the laboratory for practical advice on many occasions.

I am grateful to my mother and father for all their loving support during the work. All my dear friends, sisters and brothers, I am thankful to you for keeping me smiling and connected to the real world during this overwhelming journey. You are priceless.

In Espoo, September 2008

Emilia Selinheimo

List of bublications

This thesis consists of an overview and of the following publications, which are referred to in the text using Roman numerals, as **Publications I–V**:

Publication I Selinheimo, E., Saloheimo, M., Ahola, E., Westerholm-Parvinen, A., Kalkkinen, N., Buchert, J., Kruus, K. Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus *Trichoderma reesei*. FEBS J 2006; 273:4322–4335.

Publication II Selinheimo, E., Nieidhin, D., Steffensen, C., Nielsen, J., Lomascolo, A., Halaouli, S., Record, E., O'beirne, D., Buchert, J., Kruus, K. Comparison of the characteristics of fungal and plant tyrosinases. J Biotechnol 2007; 130:471–480.

Publication III Selinheimo, E., Lampila, P., Mattinen, M.-L., Buchert, J. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. J Agric Food Chem 2008; 56:3118–3128.

Publication IV Selinheimo, E., Kruus, K., Buchert, J., Hopia A., Autio K. Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. J Cereal Sci 2006; 43:152–159.

Publication V Selinheimo, E., Kruus, K., Buchert, J., Autio, K. Elucidating the mechanism of laccase and tyrosinase in wheat bread making. J Agric Food Chem 2007; 55:6357–6365.

The author's contribution to the appended publications

Publication I — The author was responsible for the purification and biochemical characterization of the novel tyrosinase. Dr. Markku Saloheimo had the main responsibility for production of the enzyme and Prof. Nisse Kalkkinen for the mass spectrometric analyses. The author interpreted the results and wrote the publication together with Dr. Kristiina Kruus and the other authors.

Publication II — The author was responsible for planning the work together with Dr. Kristiina Kruus. The author conducted the laboratory work, analyzed the data and had the main responsibility in writing the publication.

Publication III — The author had the main responsibility for the experimental design, interpretation of the results and writing the publication. The publication was part of the MSc (Tech) thesis of Piritta Lampila. The author supervised the work together with Dr. Maija-Liisa Mattinen.

Publication IV — The author planned the research work together with the other authors. The author carried out the experimental part of the work, and had the main responsibility for analyzing the results and writing the publication.

Publication V — The author was responsible for planning the work together with the other authors. The author conducted the laboratory work for the study and interpreted the data. The author had the main responsibility for writing the publication.

Contents

Appendices of this publication are not included in the PDF version.
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Abbreviations

AbT	tyrosinase from *Agaricus bisporus*
AT	tyrosinase from apple
AX	arabinoxylan
BsX	xylanase from *B. subtilis*
EPR	electron paramagnetic resonance
E_x	extensibility
FA	ferulic acid
hOSX	enzymatically solubilised oat spelt xylan
L-dopa	3,4-dihydroxy-L-phenylalanine
p-CA	*para*-coumaric acid
POX	peroxidase
PsT	tyrosinase from *Pycnopous sanguineus*
PT	tyrosinase from potato
R_{max}	resistance to stretching
ThL	laccase from *Trametes hirsuta*
TlX	xylanase from *Thermomyces lanuginosus*
TrT	tyrosinase from *Trichoderma reesei*
Y	tyrosine

1. Introduction

Texture plays a major role in food product quality. Rheological properties of foods are determined by a number of weak and strong physical linkages, i.e. hydrophobic, hydrogen bonding, electrostatic and covalent bonds present in a food matrix. The covalent linkages in and between the food biopolymers, i.e. proteins, carbohydrates and lipids, make a major contribution to the textural characteristics of food matrix. Therefore, by altering the number of covalent bonds, either by breaking down the linkages or by producing new linkages in the matrix, the properties of the end-product can be tailored. Enzymes are interesting tools, since they provide a selective and an environmentally friendly alternative to the chemical, thermal or mechanical processing techniques used in the field of food structure engineering. Enzymatic hydrolysis of food biopolymers by carbohydrases, proteases and lipases has widely been used in several food-based applications to improve product characteristics (Sproessler, 1993; Tenkanen et al., 2000; Rastall, 2007). Enzymatic modification of food biopolymers through crosslinking also enables modification of structural properties of the food matrix (Buchert et al., 2007). In addition to the food industry, enzymatic crosslinking and grafting of specific substances to the biopolymers can be exploited in non-food applications, such as in the textile industry and for generation of novel biomaterials (Chen et al., 2003a; Åberg et al., 2004; Freddi et al., 2006).

1.1 Enzymes catalyzing crosslink formation in food matrices

Different types of enzymes have been identified as being able to catalyze crosslinking of biopolymers with subsequent alterations in the structural properties of a food product (for a review see Buchert et al., 2007). Depending on the catalytic mechanism of the enzymes, crosslinking can be based on formation of a wide variety of covalent linkages as listed in Table 1. Although partly acting on the same substrates, differences in the catalytic mechanism between the enzymes mean that their influence on the properties of the end-products may be divergent. For example, laccase, peroxidase and tyrosinase are oxidative enzymes, which can all oxidize phenolic compounds. However, whereas enzymatic reactions by laccase and peroxidase produce radicals,

tyrosinase-catalyzed reactions result in quinone formation, thus causing differences in the subsequent crosslink formation by these enzymes. Tyrosinases, laccases and peroxidases, and also glucose oxidases indirectly, are proposed to be able to crosslink both proteins and carbohydrates in food matrix (Vemulapalli and Hoseney, 1998; Vemulapalli et al., 1998; Hilhorst et al., 1999; Figueroa-Espinoza et al., 1998 and 1999b; Dunnewind et al., 2002; Halaouli et al., 2005). However, tyrosinases mainly act on proteins, whereas laccases and peroxidases act on both carbohydrates and proteins (Buchert et al., 2007). Transglutaminases and sulfhydryl oxidases, on the other hand, are incapable of acting on carbohydrates, and solely catalyze crosslinking of proteins. (Haarasilta et al., 1991; Autio et al., 2005; Buchert et al., 2007)

The focus of this introduction is on the oxidative enzymes tyrosinases and laccases. The catalytic mechanisms of the enzymes are discussed with a view to crosslinking and applicability. Moreover, the enzymology and structure-function of tyrosinases are covered in detail.

Table 1. Crosslinking enzymes for food biopolymers (modified from Buchert et al., 2007).

Cross-linking type	**Enzyme**	**Reaction mechanism**	**Reactive sites in protein**	**Reactive sites in carbo-hydrates**	**Reactive sites in other biopolymers**
Direct	Tyrosinase EC 1.14.18.1	Oxidation of mono- and diphenols to *ortho*-quinones	Tyrosine	*p*-CA and caffeic acid, not FA	
	Laccase EC 1.10.3.2	Oxidation of aromatic components to radicals	Tyrosine Cysteine	Phenolic acids: FA, etc.	Aromatic compounds of lignin
	Peroxidase EC 1.11.1	Oxidation of aromatic components to radicals	Tyrosine Other aromatic AAs	Phenolic acids: FA, etc.	Aromatic compounds of lignin
	Sulfhydryl oxidase EC 1.8.3.2 Glutathione oxidase EC 1.8.3.3	Oxidation of sulfhydryl groups to disulphides (S-S bonds)	Cysteine (-SH)	-	-
	Transgluta-minase EC 2.3.2.13	Formation of isopeptide linkage through acyl-transfer reactions	Glutamine Lysine	-	-
Indirect	Lipoxygenase EC 1.13.11.12	Production of hydro-peroxide radicals from unsaturated fatty acids	Cysteine (-SH)		Pigments Unsaturated fatty acids
	Glucose oxidase EC 1.1.3.4 Hexose oxidase E.C.1.1.3.5	Production of H_2O_2 in conjunction with glucose oxidation	Cysteine (-SH)	Phenolic acids: FA, etc.	

1.2 Mechanisms and occurrence of tyrosinase and laccase

1.2.1 Catalytic mechanism of tyrosinase and laccase

Tyrosinases and laccases are copper-containing metalloproteins, which are known to have multiple functions in nature, such as storage, transport and uptake of metal ions or dioxygen, electron transfer, and enzymatic catalysis. Copper proteins are typically classified to different classes, based on optical and electron paramagnetic resonance (EPR) spectroscopic features. In copper proteins, metal atoms are coordinated by histidine residues. Binding of dioxygen in the copper proteins includes mononuclear (type 1), dinuclear (type 3) and trinuclear (combination of type 2 and type 3) copper centres (Fig. 1). Type 1 and 3 coppers show absorption maxima at about 600 and 330–345 nm, respectively, whereas type 2 copper has undetectable absorption (Gerdemann et al., 2002; Solomon et al., 1996). Type 1 and 2 coppers show an EPR spectrum, whereas type 3 copper gives no EPR signal due to a pair of copper ions which are antiferromagnetically coupled (Makino et al., 1974; Bento et al., 2006).

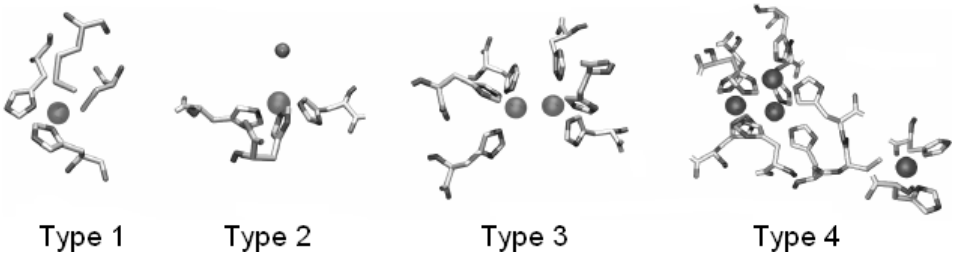

Figure 1. Structures of the copper sites of type 1-4 copper proteins (modified from Tepper, 2005).

Tyrosinases and laccases are dioxygen-binding proteins. Dioxygen-binding proteins play a key role in many biological processes, as they may function in binding of molecular oxygen, activation of the oxygen and oxidation of substrate molecules. Dioxygen-binding proteins comprise enzymes such as mono-oxygenases, dioxygenases and oxidases. The catalytic mechanisms behind oxidation of a substrate typically involve formation of a reactive intermediate by the reaction of a reduced Cu^{+} centre with molecular oxygen, which may also be incorporated to the substrate (Hatcher and Karlin, 2004; Messerscmidt, 1997;

Rosenzweig and Sazinsky, 2006). Tyrosinases belong to the copper-proteins containing a type 3 centre. In addition to tyrosinases, hemocyanins and catechol oxidases are classified as type 3 copper proteins. It should also be noted that in the literature tyrosinases and catechol oxidases together are often referred to as polyphenol oxidases (PPO). Hemocyanins, oxygen carrier proteins, can reversibly bind molecular oxygen, whereas catechol oxidases oxidize diphenols to the corresponding quinones, thus showing diphenolase activity. Tyrosinases can both hydroxylate monophenols and catalyze the oxidation of diphenols to quinones (Fig. 3). Laccases, on the other hand, contain four copper atoms in their active site and are often referred to as blue-colored oxidoreductases. Laccases are the type 4 copper proteins, which are composed of a mononuclear type 1 copper centre and a trinuclear centre, a combination of type 2 and type 3 centres (Fig. 1) (Rosenzweig and Sazinsky, 2006). The molecular mass of mature microbial and plant tyrosinases is around 30–50 kDa (van Gelder et al., 1997; Ramírez et al., 2003; Claus and Decker, 2006; Halaouli et al., 2006a), whereas laccases typically show molecular weights of about 60–80 kDa (Flurkey, 2003; Shleev et al., 2004; Kiiskinen, 2004; Baldrian, 2006).

Crystal structures of tyrosinases and laccases have been established, and examples of the three-dimensional structures of tyrosinase and laccase and the locations of the copper atoms in the protein structures are shown in Fig 2. The central domain of tyrosinase with its active site consists of an α-helical construction, in which a bundle of four α-helices surrounds the catalytic binuclear copper centre. The substrate binding centre, i.e. the dicopper site, is located in the bottom of the large concavity (Cuff et al., 1998; Klabunde et al., 1998; Matoba et al., 2006; Decker et al., 2006). The protein fold of laccases comprises three domains (A, B and C), also called cupredoxin-like domains, which have rather equal size and which are all significant for the catalytic activity of the enzyme (Hakulinen et al., 2002; Ducros et al., 1998; Bertrand et al., 2002; Piontek et al., 2002). A substrate-binding site is located in a cleft between domains B and C; a mononuclear copper centre is located in domain C, whereas a trinuclear copper centre is positioned at the interface between domains A and C (Fig. 2).

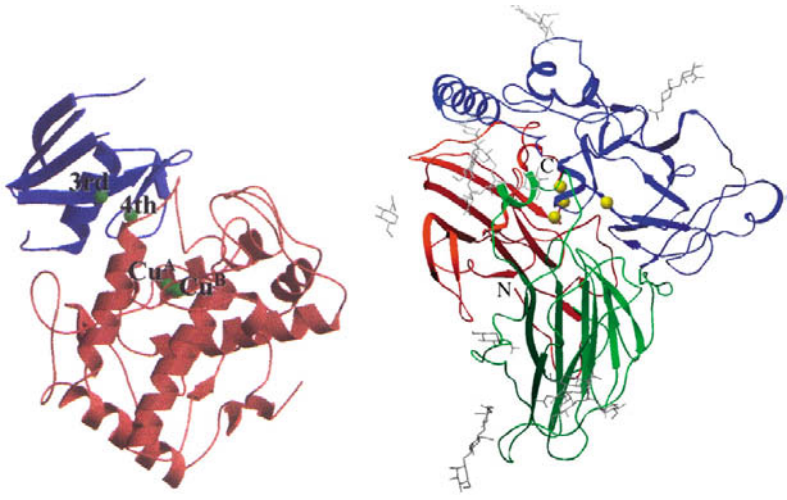

Figure 2. The structures of S. castaneoglobisporus tyrosinase, in which the mature tyrosinase protein is in red and the carrier protein in blue (left) (Matoba et al., 2006), and laccase from M. albomyces, in which domains A, B, and C are in red, green and blue, respectively (right) (Hakulinen et al., 2002). Locations of copper atoms are shown in green and yellow in the tyrosinase and laccase proteins, respectively.

Tyrosinase and laccase catalyze oxidation of substrate using molecular oxygen as a terminal electron acceptor with concomitant reduction of oxygen to water (Fig. 3). Tyrosinases have two type 3 copper atoms shuttling electrons from substrate to oxygen. Tyrosinases are bifunctional enzymes as they catalyze *ortho*-hydroxylation of monophenols (monophenolase activity) and subsequent oxidation of diphenols to *ortho*-quinones (diphenolase activity). Quinones are highly susceptible to non-enzymatic reactions, which may lead to formation of mixed melanins and heterogeneous polymers (Lerch, 1983; Robb, 1984). Whereas tyrosinases oxidize their substrates by removing a pair of electrons from the substrate, the type 4 copper-proteins, laccases, oxidize their substrates with a single electron removal mechanism. Electrons are transferred from substrate molecules through the type 1 copper to the trinuclear type 2/3 centre. After the transfer of four electrons, the dioxygen in the trinuclear centre is reduced to two molecules of water (Bento et al., 2006; Bertrand et al., 2002) (Fig. 3). Although laccases and tyrosinases show overlapping substrate specificity (Käärik, 1965), laccases exhibit no monophenol hydroxylase activity, and the primary oxidation products in laccase-catalyzed reactions are reactive radicals, which

can react further and lead to polymerization, hydration, and disproportionation (Thurston, 1994).

Laccases typically have rather wide substrate specificity. In addition to mono- and polyphenols, laccases have been found to be capable of oxidizing various aromatic compounds, such as substituted phenols, diamines, aromatic amines and thiols, and even some inorganic compounds such as iodine (Thurston, 1994; Flurkey, 2003; Claus, 2003; Baldrian, 2006). Syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine), for example, is typically referred to as a specific substrate for laccase, although in the presence of hydrogen peroxide, peroxidase can also oxidize syringaldazine (Claus, 2003). Moreover, whereas laccases are known to readily oxidize both *para*-, *meta*- and *ortho*-diphenols (hydroquinone/1,4-benzenediol, resorcinol/1,3-benzenediol, and pyrocatechol/1,2-benzenediol, respectively), tyrosinases can only oxidize the *ortho*-diphenols (Messerschmidt, 1997). Furthermore, the substrate specificity of laccases can be broadened by small molecular weight compounds, called mediators. Mediators are compounds that are easily oxidized by laccases, and usually result in the formation of radicals, which can further oxidize more complex substrates (Bourbonnais and Paice, 1990; Call and Mücke, 1997; Riva, 2006).

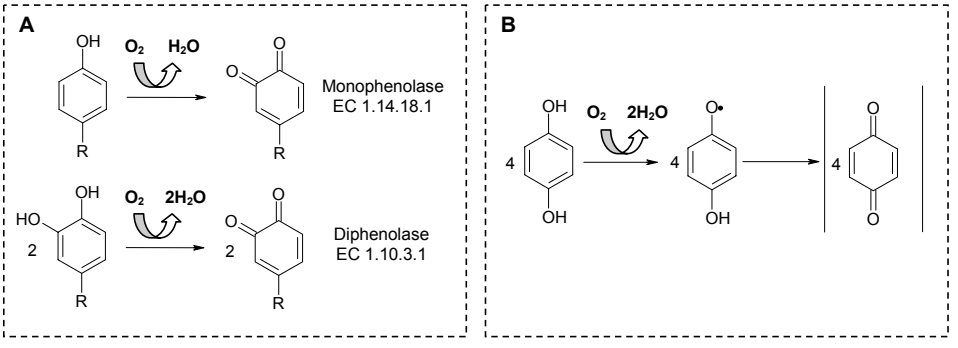

Figure 3. Reactions on phenolic compounds catalyzed by tyrosinases (A) and laccases (B).

Tyrosinase and laccase can also be distinguished on the basis of selective inhibition. For example, specific inhibition of tyrosinase has been reported using tropolone, salicyl hydroxamic acid, 4-hexyl recorcinol, cinnamic acids, naphthalene diol and phenyl hydrazine (Lerner et al., 1971; Walker and McCallion, 1980;

Allan and Walker, 1988; Dawley and Flurkey, 1993). Moreover, laccase is believed to be less sensitive to carbon monoxide than tyrosinase (Arnon, 1949; Messerschmidt, 1997). On the other hand, cetyltrimethyl ammonium bromide and N-hydroxyl glycine have been suggested to specifically inhibit laccase activity (Walker and McCallion, 1980; Lee and Whitaker, 1995).

1.2.2 Occurrence of tyrosinase and laccase and their role in nature

1.2.2.1 Occurrence in nature

Tyrosinases and laccases are widely distributed enzymes in nature. Tyrosinases are found in prokaryotic and eukaryotic microbes, and in mammals, invertebrates and plants. The genes encoding tyrosinases have been characterized in detail from various sources, including bacteria, fungi, plants and mammals. The most extensively investigated tyrosinases are, however, from mammals (Kwon et al., 1987, 1988; Spritz et al., 1997; Kong et al., 2000b; Olivares et al., 2002). The fungal tyrosinases from *Neurospora crassa* (Lerch, 1983) and *Agaricus bisporus* (Wichers et al., 1996) have also both been characterized in detail from the structural and functional points of view. *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin (Della-Cioppa et al., 1998a and 1998b; Matoba et al., 2006). Bacterial tyrosinases were recently reviewed by Claus and Decker (2006), and plant and fungal tyrosinases by van Gelder et al. (1997), Halaouli et al. (2006a), Marusek et al. (2006) and Mayer (2006).

Laccases are also found widely in plants and fungi as well as in some bacteria and insects (Mayer and Staples, 2002). However, contrary to tyrosinases, laccases are not produced by mammalian hosts. Although the first laccase reported in 1883 was from *Rhus vernicifera* (Yoshida, 1883), the Japanese lacquer tree, the majority of laccases hitherto characterized have been derived from fungi. Laccases of plant origin are mainly found in xylem, although laccase activity has also been detected in wounded leaves, where they presumably oxidize monolignols in the early stages of lignification (Bao et al., 1993; Whetten and Sederoff 1995; Mayer and Staples 2002). White-rot fungi, which are efficient lignin degraders, are widely recognised laccase-producers, and it has even been postulated that no white-rot fungi exist without laccase activity (Mayer and Staples, 2002; Baldrian, 2006). However, the white-rot fungus

Phanerochaete chrysosporium, which is reported to produce a range of isoenzymes of lignin peroxidase and manganese peroxidase, does not produce laccase (Hatakka, 1994; Martínez et al., 2004). The origins and characteristics of fungal-derived laccases were recently reviewed by Baldrian (2006).

Most of the reported tyrosinases are intracellular enzymes, whereas the characterized laccases are mostly secreted, extracellular enzymes. The characterized plant and fungal tyrosinases have been intracellular enzymes, possibly bound to organelles or membrane structures. Bacterial *Streptomyces* tyrosinases, however, are found to be secreted (Claus and Decker, 2006). Most of the reported laccases are extracellular, although intracellular laccases have also been characterized (Froehner and Eriksson, 1974; Palmieri et al., 2000; Nagai et al., 2003; Langfelder et al., 2003; McMahon et al., 2007).

1.2.2.2 Role in nature

Tyrosinases are ubiquitous in nature and are involved in several biological functions. They are considered as fundamental enzymes in diverse defence systems, especially in melanogenesis. The term melanogenesis refers to the biosynthesis of melanin pigments (i.e. pigmentation), which are heterogeneous polyphenolic polymers occuring in all living organisms. In the reaction catalyzed by tyrosinase, tyrosine is first oxidized to dopaquinone, which either cyclises to give a dihydroxyindole precursor of black or brownish eumelanins, or reacts with cysteine to give a precursor of reddish brown pheomelanin (Raper, 1928; Mason, 1948) (Fig. 4). Intracellular laccases are also suggested to have a role in pigmentation processes (Nagai et al., 2003; Langfelder et al., 2003).

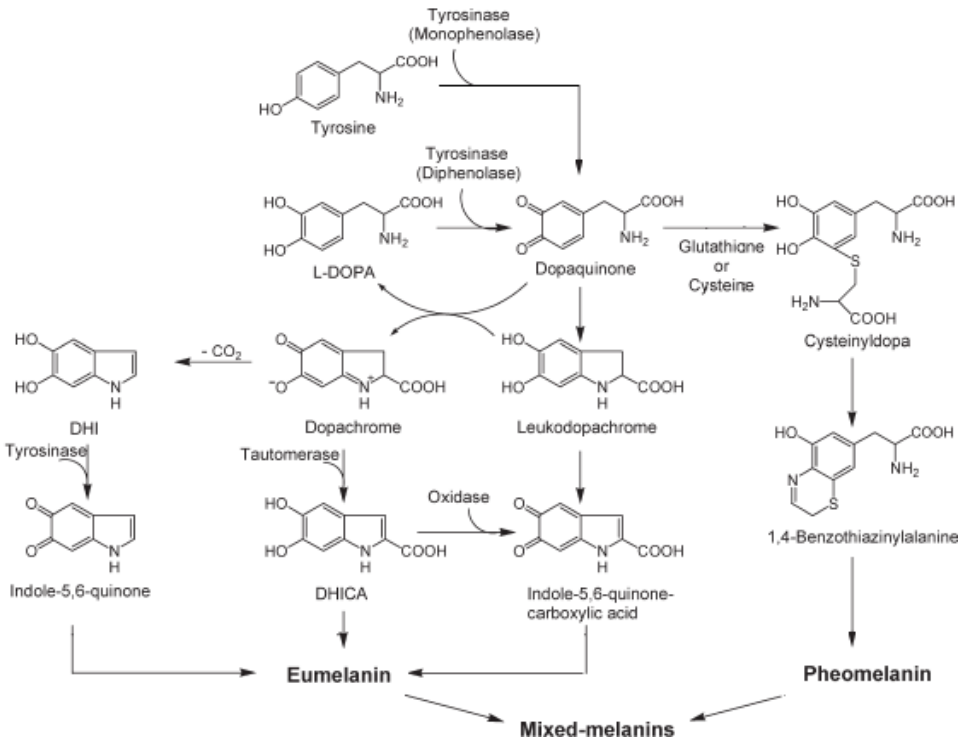

Figure 4. Biosynthetic pathway of melanin catalyzed by tyrosinase. The figure is modified from Kim and Uyama (2005).

In mammals, tyrosinase-related melanogenesis is responsible for pigmentation in skin, eye, and hair. Pigmentation contributes an essential part of the protective function of the skin by absorption of UV radiation (Hearing and Tsukamoto, 1991; del Marmol and Beermann, 1996). Hyperpigmentation and albinism in mammals are caused by abnormal increase or decrease in tyrosinase activity, respectively (Passmore et al., 1999; Solano et al., 2006; Ray et al., 2007). In invertebrates, tyrosinase activity has a role in defence reactions and sclerotization (Sugumaran, 2002). The role of the tyrosinases in microbes is not well understood hitherto. However, it has been proposed that melanin has a role in the formation of reproductive organs and spores and in cell wall protection after physical damage (Lerch, 1983). Tyrosinases also play an important role in regulation of oxidation-reduction potential, and in wound healing systems in plants (Mayer, 1987; Walker and Ferrar, 1998). The defence mechanisms of tyrosinases in plants are suggested to be related to the non-enzymatic reactions of quinones. It has been proposed that the reactive quinones may, for example, create a toxic environment for the

attacker, act by reducing the bioavailability of proteins by alkylation reactions, or contribute to the formation of barriers by polymerizing reactions (Bi and Felton, 1995; Felton et al., 1989 and 1992; Melo et al., 2006).

The role of laccases in nature is related to synthesis and degradation of lignin. The plant origin laccases are proposed to have an important role in lignin biosynthesis via oxidizing monolignols in the early stages of lignification (De Marco and Roublelakis-Angelakis, 1997; Thurston, 1994; Mayer and Staples, 2002). With concomitant action of heme-containing lignin peroxidases and manganese peroxidases, fungal laccases are involved in radical-mediated depolymerization of lignin, either directly by oxidizing the phenolic structures of lignin, or indirectly by mediator-catalyzed reactions (Durán and Esposito, 2000; Balakshin et al., 2001; Flurkey, 2003; Sigoillot et al., 2004; Claus, 2003). In addition, laccases and also tyrosinases are suggested to participate in the synthesis and degradation of humus (Chefetz et al., 1998; Temp et al., 1999; Zavarzina and Zavarzin, 2006). Besides degradation of biopolymers, fungal laccases are suggested to contribute to several other functions, such as pigmentation, fruiting body formation, sporulation and pathogenesis (Leatham and Stahmann, 1981; Thurston, 1994; Nagai et al., 2003; Langfelder et al., 2003). Laccases in plant-pathogenic fungi are proposed to detoxify the toxic components generated by the plant defence systems (Adrian et al., 1998; Mayer and Staples, 2002). Although Arakane et al. (2005) reported that laccase activity may have a more significant role in cuticle tanning in certain invertebrates than tyrosinase activity, generally in insects laccase activity is referred to sclerotization (Sugumaran, 2002; Dittmer et al., 2004). Bacterial laccases have also been suggested to have a role in pigmentation (Faure et al., 1994; Martins et al., 2002). Representative differences in the structural and biochemical characteristics of tyrosinases and laccases are summarized in Table 2.

Table 2. Comparison of mechanisms and structural and biochemical properties of tyrosinase and laccase.

Property	**Tyrosinase**	**Laccase**	**References**
Enzyme class (EC)	EC 1.14.18.1	EC 1.10.3.2	
Type of copper enzyme	Type 3	Type 4	Messerscmidt, 1997; Rosenzweig and Sazinsky, 2006
EPR signal	No	Yes	Makino et al., 1974; Bento et al., 2006
UV/Vis absorption maximum	330–345 nm (600 nm)	600 nm (330–345 nm)	Gerdemann et al., 2002; Solomon et al., 1996
Tertiary structure	Monomer: α-helical construction	Monomer: 3 cuperdoxin-like domains	Matoba et al., 2006; Klabunde et al., 1998; Hakulinen et al., 2002; Ducros et al., 1998; Bertrand et al., 2002
Molecular weight of protein	30–50 kDa	60–80 kDa	van Gelder et al., 1997; Ramírez et al., 2003; Claus and Decker, 2006; Halaouli et al., 2006a; Flurkey, 2003; Shleev et al., 2004; Kiiskinen, 2004; Baldrian, 2006
Substrates	*p*-Monophenols and *o*-diphenols	Substituted phenols, diamines, aromatic amines and thiols	Käärik, 1965; Lerch 1983; Robb, 1984; Thurston, 1994, Xu, 1996; Flurkey, 2003
Specific inhibition by	Tropolone, salicyl hydroxamic acid, 4-hexyl recorcinol, cinnamic acids, naphtalene diol, phenyl hydrazine, CO_2	Cetyltrimethyl ammonium bromide and N-hydroxyl glycine	Lerner et al., 1971; Walker and McCallion, 1980; Allan and Walker, 1988; Dawley and Flurkey, 1993

Mole of O_2 required to oxidize 1 mole substrate	Monophenolase: 1 O_2 Diphenolase: 1/2 O_2	1/4 O_2	Solomon et al., 1996; Matoba et al., 2006; Flurkey, 2003
Primary oxidation product	Quinone	Radical	Lerch 1983; Robb, 1984; Thurston, 1994, Flurkey, 2003
Location in cell	Mainly intracellular	Mainly extracellular	van Gelder et al., 1997; Mayer ans Staples, 2002; Baldrian, 2006
Occurrence in nature	Microbes, plants, invertebrates, mammals (ubiquitous)	Microbes, plants, insects	van Gelder et al., 1997; Claus, 2003; Flurkey, 2003
Role in nature	Defence systems (e.g. pigmentation, sporulation, oxidation-reduction potential, wound healing)	Lignin biosynthesis and degradation, defence systems (e.g. pigmentation, sporulation, detoxification)	Lerch, 1983; Mayer, 1987; Hearing and Tsukamoto, 1991; del Marmol and Beermann, 1996; Walker and Ferrar, 1998; Sugumaran, 2002; Leatham and Stahmann, 1981; Thurston, 1994; Durán and Esposito, 2000; Balakshin et al., 2001; Mayer and Staples, 2002; Nagai et al., 2003; Langfelder et al., 2003; Claus, 2003

1.3 Characteristics of tyrosinases

1.3.1 Modular structure of tyrosinases

On the basis of biological source, the monomeric structures of type 3 copper proteins are recognized to consist of two or three domains with different folding motifs: an N-terminal domain, a central domain with an active site, and a C-terminal domain (Fig. 5). Tyrosinases from higher eukaryotes and plants have all the three domains. Fungal tyrosinases have the central and the C-terminal domain, whereas in bacterial tyrosinases an exogenous protein acts as a C-terminal-like domain, associating with the central domain.

Plants have multiple genes encoding tyrosinase or catechol oxidase, suggesting their differential expression in specific parts of the plant and at different development stages. Plant tyrosinases and catechol oxidases are nuclear-encoded proteins, which contain an N-terminal two-domain transit peptide that post-translationally directs the proteins to the chloroplast thylakoid lumen for subsequent transport and processing to the mature protein (Haruta et al., 1998; Sommer et al., 1994). In the lumen of the chloroplast thylakoid, the enzymes are soluble or loosely associated with the thylakoid membrane (Sommer et al., 1994; Mayer, 2006). In studies with tomato polyphenol oxidase, the precursor protein was found to contain a transit peptide, which was cleaved out by a stromal peptidase in order to reach the correct location in the chloroplast (Koussevitzky et al., 1998). The maturation of the plant tyrosinases and catechol oxidases in turn is proposed to take place by a thylakoid processing peptidase (Sommer et al., 1994; Koussevitzky et al., 1998). In wheat, a genome is reported to contain six genes encoding tyrosinase or catechol oxidase, and the purified protein from wheat bran appeared to be the mature form of enzyme lacking the transit peptide (Anderson and Morris, 2003). In contrast to plant tyrosinases and catechol oxidases, the tyrosinases from fungi and bacteria are known lack the N-terminal transit peptide (Fig. 5). Fungal tyrosinases are generally presumed to be cytoplasmic, possibly bound to organelles or membrane structures (Rast et al., 2003). However, *N. crassa* is known to produce both secreted and intracellular tyrosinases, but the enzyme characterized at the protein level is intracellular (Kupper et al., 1989).

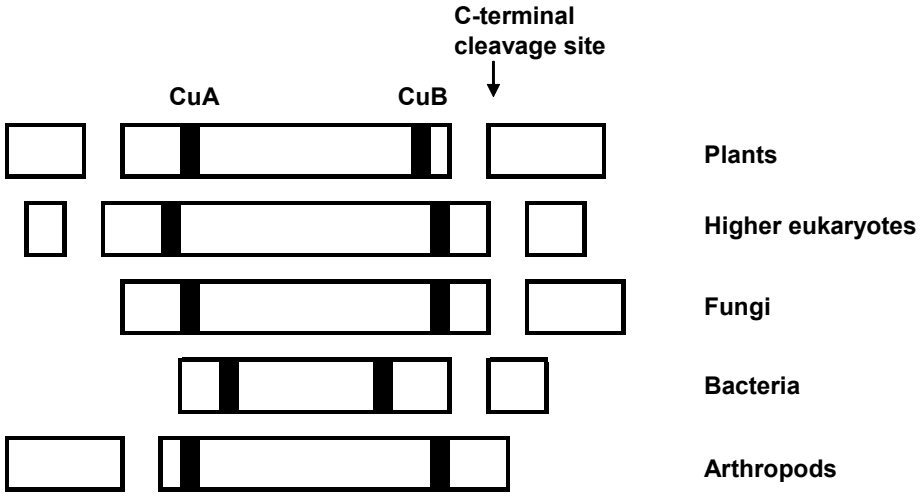

Figure 5. Domains of tyrosinases of different origin. Copper-binding regions shown in black (Modified from van Gelder et al., 1997; Lang and van Holde, 1991).

Fungal and plant tyrosinases may undergo proteolytic cleavage from the C-terminal end. Cleavage of a 15–20 kDa peptide from the C-terminus of a latent tyrosinase protein results in the formation of an active form of tyrosinase (van Gelder et al., 1997). Klabunde et al. (1998) reported that a C-terminal domain covers the active site in the catechol oxidase from the sweet potato *Ipomoea batatas*, and that the enzyme is activated by dissociation of the C-terminal domain. It has also been postulated that the C-terminal domain in the tyrosinases from *N. crassa* (Kupper et al., 1989; van Gelder et al., 1997), *Agaricus* (Espín et al., 1999a) and *Pycnoporus* species (Halaouli et al., 2006b) is proteolytically released from the catalytic domain in order to activate the enzyme. In nature, tyrosinases and catechol oxidases are believed to exist in their latent states until cell disruption, resulting from injury to the organism. The region connecting the central and C-terminal domains, cited also as a linker region, is proposed to be a critical structural element determining the conditions under which the enzyme is activated (van Gelder et al., 1997; Marusek et al., 2006).

The tyrosinases of bacterial origin are often reported to be extracellular enzymes, involved in melanin production (Claus and Decker, 2006). However, the extracellular bacterial tyrosinases do not have signal sequences, but their secretion is proposed to be assisted by a second protein having a signal sequence

(Leu et al., 1992; Tsai and Lee 1998). Compared to plant and fungal tyrosinases, the bacterial tyrosinases also have a shorter sequence, typically encoding a mature protein of 30 kDa. In *Streptomyces* species, the tyrosinase gene is part of the melC operon, containing an additional open reading frame (ORF) called melC1, which in turn is essential for the correct expression of the enzyme (Ikeda et al., 1996). The crystal structure of *S. castaneoglobisporus* tyrosinase was established as a complex with the caddie protein ORF378, which consists of a six-stranded β-sheet and a single α-helix (Matoba et al., 2006). ORF378 is suggested to act as C-terminal domain, as in catechol oxidase, shielding the active site. After dissociation of the caddie protein, the active site becomes accessible to substrates (Matoba et al., 2006; Decker et al., 2006).

The molluscan and arthropod tyrosinases and hemocyanins have profound differences in their quaternary structure, although the oxygen-binding sites are similar (van Holde et al., 2001; Jaenicke and Decker, 2003; Aspan et al., 1995). Two different types of proteins can be distinguished relating to molluscan or arthropod hemocyanins (Lang and van Holde, 1991; van Holde et al., 2001). Comparison of arthropod and molluscan hemocyanins reveals that the function of C-terminal domain of molluscan hemocyanin corresponds to that of N-terminal domain of arthropod hemocyanin (van Holde et al., 2001; Decker et al., 2007). In molluscan hemocyanin, access to the active site is blocked by the C-terminal domain, whereas in arthropodan hemocyanin the active site is shielded by N-terminal domain (Jaenicke and Decker, 2004). The tyrosinases and catechol oxidases from insects and crustaceans are more related to arthropodan hemocyanins, and they are activated by a cleavage of the N-terminal domain (Aspán et al., 1995; Jaenicke and Decker, 2003; Terwilliger and Ryan, 2006). Since in hemocyanins substrates cannot reach the active site, the physiological role the proteins is oxygen binding and transport (Decker and Tuczek, 2000). However, some hemocyanins from arthropods are reported to perform enzymatic catalysis via *in vitro* or *in vivo* activation (Jaenicke and Decker, 2003; Terwilliger and Ryan, 2006).

Mammalian tyrosinases are melanosomal transmembrane proteins with a carboxyl tail oriented to the cytoplasm and a single membrane-spanning helix located in the C-terminal part of the proteins (Jiménez et al., 1988; Wang and Herbert, 2006). The mature protein is located inside the melanosome, and the N-terminal putative signal peptide of 18 amino acids is thought to be involved in

the transfer of the enzyme into the melanosome in the case of human and mouse tyrosinase (Wang and Herbert, 2006).

1.3.2 Tertiary structure and catalytic mechanism of tyrosinase

Establishment of the crystal structure of proteins is essential for understanding biological processes at the molecular level. Among the type 3 copper proteins, the three-dimensional structures of tyrosinase from *S. castaneoglobisporus* (Matoba et al., 2006), catechol oxidase from *Ipomoea batatas* (Klabunde et al., 1998) and molluscan hemocyanin from *Octopus dofleini* (Cuff et al., 1998) have been established, and the typical characteristics of the proteins with respect to their structure and functions have been shown to be different. Based on the accomplished structural data, dioxygen is recognized to bind to all the type 3 copper proteins in the same way. It has been suggested that activation of the proteins and enzymatic catalysis have been preserved during evolution (Decker et al., 2006 and 2007). However, although the folding of type 3 proteins is also observed to be rather similar, comparison of amino acid sequences of tyrosinases reveals high heterogeneity concerning the length and overall identity (Decker et al., 2006). The only highly conserved regions among all tyrosinases can be found in the active site area (van Gelder et al., 1997; Halaouli et al., 2006a; Marusek et al., 2006).

Assessment of the tertiary structures of tyrosinase, catechol oxidase and molluscan hemocyanin shows that the central domain with the active site consists of the α-helical structure, in which a bundle of four α-helices surrounds the catalytic binuclear copper centre (Fig. 6). The binuclear copper site is located in the bottom of the large concavity, serving as a putative substrate binding centre (Cuff et al., 1998; Klabunde et al., 1998; Matoba et al., 2006 Decker et al., 2006). Although there are uniformities in the tertiary folding of type 3 proteins, the quaternary structures of hemocyanins, catechol oxidases and tyrosinases show notable dissimilarities. For example, microbial and plant tyrosinases occur typically as monomers and the corresponding mammalian enzymes are active as dimers (Kwon et al., 1987 and 1988). Tyrosinases from arthropods show structures from monomers to pentamers, and the corresponding hemocyanins consist of hexamers and their multiforms (Jaenicke and Decker, 2003; Terwilliger and Ryan, 2006; Decker et al., 2007). The hemocyanins from molluscs form decamers or didecamers (Cuff et al., 1998).

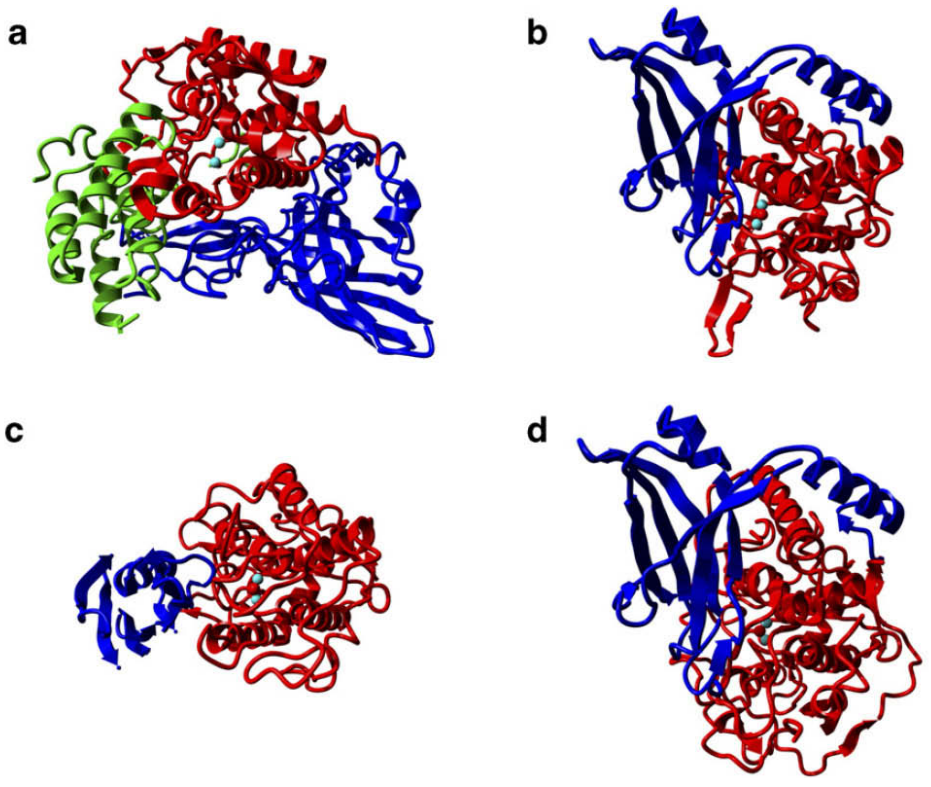

Figure 6. Subunit structures of different type 3 copper proteins. Limulus polyphemus hemocyanin (a), Octopus dofleini hemocyanin (b), Streptomyces castaneoglobisporus tyrosinase (c), Ipomoea batatas catecholoxidase (d) (Decker et al., 2007).

In active sites of all of the type 3 copper proteins, the two closely spaced copper ions (CuA and CuB) are coordinated by three histidine residues (Fig. 7) (Cuff et al., 1998; Klabunde et al., 1998; Matoba et al., 2006). The binuclear active site of tyrosinases is known to exist in three states: *oxy*-tyrosinase, *met*-tyrosinase and *deoxy*-tyrosinase, depending on the bridging substances between the copper atoms, which have different abilities to catalyze the oxidation of mono- and diphenolic compounds (Jolley et al., 1972; Makino and Mason, 1973; Jolley et al., 1974; Matoba et al., 2006). In the *met*- and *oxy*-form of the enzyme, hydroxyl ion or molecular oxygen is bound to the active site, respectively. The resting state of the enzyme is the *met*-form, in which the coppers are in oxidized form (Cu^{2+}-Cu^{2+}) and bridged by a hydroxide ligan. In the *oxy*-form, oxygen is bridged as peroxide between the copper atoms (Matoba et al., 2006; Decker et

al., 2006). The *deoxy*-form of tyrosinase is the reduced and instable state of the enzyme, which immediately binds molecular oxygen to give the *oxy*-form.

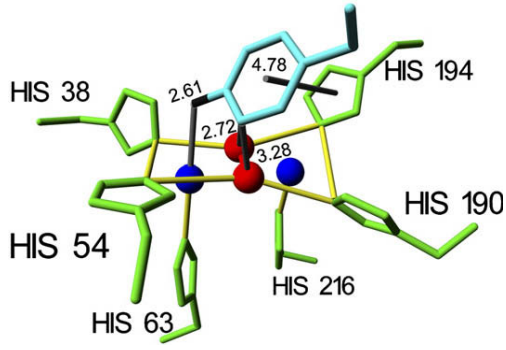

Figure 7. Dioxygen binding and orientation of tyrosine at the active site of oxy-form of Streptomyces tyrosinase. Coppers: blue, histidines: green, dioxygen molecule: red, monophenolic substrate: cyan (its oxygen black), equatorial coordination of CuA and CuB: yellow frame, axial coordination of CuA and CuB: yellow lines. (Decker et al., 2007).

The establishment of the first crystal structure of a tyrosinase by Matoba et al. (2006) has evoked a comprehensive investigation of the catalytic mechanism of tyrosinase (Decker et al., 2006; Rosenzweig and Sazinsky, 2006). It has been shown that in the *met*-form the CuA centre exhibits a tetragonal-pyramidal geometry, with two bridging hydroxide ligands and two histidine residues His38 and His54 in equatorial positions, and His63 in an axial position (Fig. 7) (Decker et al., 2006 and 2007). The much longer His N-CuA bond length of His63 compared to the other two histidines is postulated to indicate this residue to exist in the axial position of CuA (Decker et al., 2006). The position *trans* to His63 is freely accessible from the substrate binding pocket. If the substrate binds to CuA, it would thus bind *trans*-axially to His63. The CuB centre of *met*- and *oxy*-form also exhibits a tetragonal-pyramidal geometry. In the *met*- and *oxy*-forms the His216 N-CuB bond is the longest, indicating His216 to locate in the axial position. Thereby, the position *trans* to His216 is also accessible from the substrate binding pocket, enabling a phenolic substrate to bind to CuB. The role of CuB for substrate binding in tyrosinase is also suggested to contribute to the orientation of the phenyl ring of the substrate through a hydrophobic interaction with His194 (Decker et al., 2006).

In the *oxy*-form of tyrosinase, a substrate is proposed to be oriented through a hydrophobic interaction with His194 on CuB. The C-O bond of the substrate is towards CuA, and the substrate shifts towards CuA to bind in the position *trans* to His63 (Fig. 7). When the O-O axis of the peroxo ligand rotates towards the phenolic ring, an electrophilic attack of the peroxo group on the aromatic ring becomes enabled, with a concomitant cleavage of the O-O bond and a hydroxylation of the aromatic ring. The *ortho*-quinone is formed and released via a diphenolic intermediate that binds bidentately, with subsequent regeneration of the *deoxy*-form of the enzyme (Matoba et al., 2006; Decker et al., 2006).

In nature the resting state of the enzyme usually consists of 85–90 % of a *met*-form, whereas 10–15 % is in an *oxy*-form (Solomon et al., 1996). Both *met*- and *oxy*-states of tyrosinases can catalyze the two-electron oxidation of *ortho*-diphenols to *ortho*-quinones, whereas the hydroxylation reaction that is involved in the monophenolase reaction cycle requires the *oxy*-form of the active site (Fig. 8) (Solomon et al., 1996; Matoba et al., 2006). However, monophenols can also bind to the *met*-form of the enzyme, although they are not oxidized.

When monophenols are oxidized by tyrosinase, a lag period is usually detected, which relates to the state of the active site of the enzymes. During the lag period, the *oxy*-form of tyrosinase is generated from the *met*-form and the rate of oxidation accelerates to reach the maximum, since the *oxy*-form can perform catalysis with both monophenols and diphenols (Cooksey et al., 1997; Land et al., 2004). It has been shown that during oxidation of tyrosine, diphenolic dopa accumulates in the reaction. This dopa is formed due to the non-enzymatic reactions in the catalysis. By oxidizing the dopa, tyrosinase is transformed from the *met*- to the *oxy*-form within the lag period (Cooksey et al., 1997; Land et al., 2004).

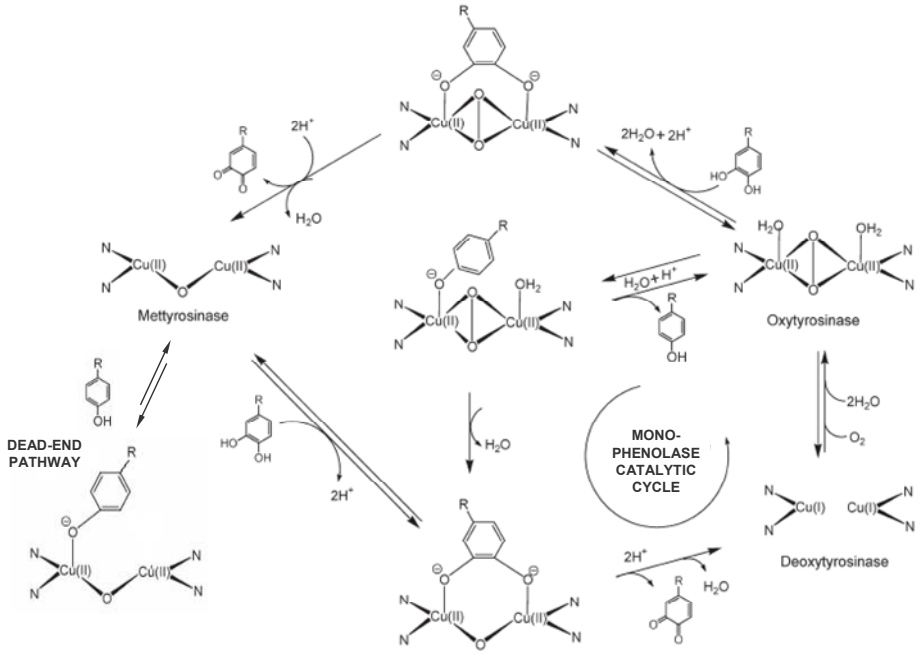

Figure 8. Catalytic cycle of tyrosinase for oxidation of monophenols and diphenols (Modified from Kim and Uyama, 2005).

The difference between catechol oxidase (diphenolase) and tyrosinase (monophenolase and diphenolase) in their capability to oxidize mono- and diphenolic compounds has been proposed to be due to certain structural features. It has been postulated that the size of the active site cavity influences the coordination of the substrate to the copper centre. In catechol oxidase from *I. batatas*, the residue Phe261 partly blocks the substrate binding site, whereas in the analogous position in *S. castaneoglobisporus* tyrosinase there is a glycine residue, making the approach to the copper site more open. Moreover, one of the copper ligands of *S. castaneoglobisporus* tyrosinase, His54, was found to be flexible during the catalysis. As the corresponding residue in catechol oxidase (His109) is constrained by a covalent linkage to a nearby cysteine, Matoba et al. (2006) hypothesized that the restricted position of His109 impedes the correct orientation of monophenols to the copper site. However, some tyrosinases also have the covalent cysteinyl bridge between the proposed CuA histidine, suggesting that the differences in the structure-function of catechol oxidase and tyrosinase are more complex (Decker et al., 2006). Furthermore, Decker et al. (2006) hypothesized the possible role of hydrogen-bonding residues in the

mechanism of substrate binding. The authors postulated that access to a tyrosinase Glu182 residue, which rests just above the dicopper centre, is less restricted than access to the equivalent polar residues in catechol oxidase and hemocyanin. The Glu182 residue could recruit and position phenolic substrates in such a way that the *ortho* carbon is oriented towards the reactive oxygen species (Decker et al., 2006).

1.3.3 Biochemical characteristics of tyrosinases

Many microbial tyrosinases have their pH optima in the neutral and slightly acidic pH range, e.g. *N. crassa* and *A. flavipes* at pH 6.0–7.0 (Horowitz and Shen, 1952; Gukasyan, 1999) and *P. sanguineus* at pH 6.5–7 (Halaouli et al., 2005). However, optimum activity in the alkaline pH range has been reported for *Thermomicrobium roseum* (pH 9.5) (Kong et al., 2000a) and pine needle tyrosinase (9–9.5) (Kong et al., 1998). Tyrosinases are in general reported to not to be very thermostable enzymes. Even short incubation at 70–90 °C is reported to inactivate tyrosinases completely (Kong et al., 1998; 2000b). *P. sanguineus* tyrosinase has reported to show a half-life of 2 h at 50 °C, whereas at 60 °C the enzyme was inactivated completely within 20 min (Halaouli et al., 2005). Inactivation of *A. flavipes* (Gukasyan, 1999) and *N. crassa* (Fling et al., 1963; Horowitz et al., 1970) tyrosinases was also reported at relatively low temperatures. Different isoforms of tyrosinases can also show significantly different biochemical characteristics, as shown in the case of different fungal tyrosinases in Table 3.

Table 3. Biochemical characteristics of fungal tyrosinases.

Origin of enzyme	pH optimum	pI	Temperature stability	K_m (mM) on L-dopa	References
Agaricus bisporus	6.5–7.5	4.7–5.3	up to 60 ºC	0.17–0.26 [a], 0.76 [b], 0.8 [c], 1.5 [d]	Kumar and Flurkey, 1991 [a];Espín et al., 1997, 1998, 1999b and 2000; Munjala and Sawhney, 2002; Wichers et al., 2003; Khan et al., 2005
Pycnoporus sanguineus	6.5–7.0	4.5–5.0	up to 50–60 ºC (20 min)	0.9	Halaouli et al., 2005
Aspergillus oryzae	5.0–6.0	nd [e]	stable up to 60 ºC	nd	Ichishima et al., 1984
Aspergillus flavipes	6.0–6.8	4.6	up to 50 ºC	5	Gukasyan, 1999
Neurospora crassa	nd	8.3	$t_{1/2}$ at 59 ºC 65 min or 5 min [f]	0.88–0.95	Lerch 1983; Fling et al., 1963 [d]
Lentinula edodes [d]	6–6.5	4.3–4.7	nd	0.074–22.01	Kanda et al., 1996

[a] Determined by Kumar and Flurkey, 1991
[b] Determined by Espín et al., 1997
[c] Determined by Espín et al., 1998 and 2000
[d] Determined by Espín et al., 1999b
[e] Not determined
[f] Different values depending on the isoform

1.4 Applications of tyrosinases and laccases

Oxidoreductases are of great interest for many applications in biotechnology, food processing, medicine, and the textile and pulp and paper industry, especially due to their ability to polymerize compounds (Åberg et al., 2004; Halaouli et al., 2005; Freddi et al., 2006; Lantto et al., 2007a and 2007b). The ability to oxidize various small molecular weight phenolic compounds in biopolymers, and the high reactivity of the primary oxidation products, also provide a basis for the wide application potential of tyrosinases and laccases.

Tyrosinases produce quinones, which are prone to react non-enzymatically with nucleophilic moieties, such as lysyl, tyrosyl, cysteinyl and histidinyl residues of proteins (Ito and Prota, 1976; Ito et al., 1984; Marumo and Waite, 1986; Takasaki and Kawakishi, 1997; Xu et al., 1997; McDowell et al., 1999; Burzio, 2000; Land et al., 2004; Zhao and Waite, 2005; Bittner, 2006). Quinones are particularly susceptible to nucleophilic attack by free sulphydryl and amino groups of amino acid side-chains, resulting in formation of tyrosine-cysteine and tyrosine-lysine cross-links in the protein structures (Ito and Prota, 1976; Ito et al., 1984; Marumo and Waite, 1986; Takasaki and Kawakishi, 1997). Quinones can also couple to phenolic compounds to form dimeric phenolic conjugates (Burzio, 2000; Jee et al., 2000; Bertazzo et al., 1999a and 1999b). In fruits and vegetables, tyrosinase activity is also related to undesired browning reactions (Martínez and Whitaker, 1995; Ramirez et al., 2003), and therefore methods for controlling tyrosinase activity are constantly being searched in the food industry.

In laccase-catalyzed oxidation reactions, high reactivity of the produced radicals can lead to polymerization of the substrates. Laccases have widely been reported to crosslink arabinoxylan (AX) and pectin via the ferulic acids that are esterified to the biopolymers (Figueroa-Espinoza and Rouau, 1998; Figueroa-Espinoza et al., 1998 and 1999a; Labat et al., 2000). Laccase has also been reported to catalyze polymerization of certain peptides and proteins (Mattinen et al., 2005; Færgemand et al., 1998; Lantto et al., 2005b; Mattinen et al., 2006; Shotaro, 1999; Si and Sørensen, 1993; Yamaguchi, 2000). Although laccase has been shown to directly oxidize both tyrosine and cysteine (Mattinen et al., 2005), it has been proposed that the linkages in proteins are mostly formed between tyrosine residues (Mattinen et al., 2006). Radical-mediated oxidation of free sulphydryl groups of proteins has been found to be accelerated in laccase-catalyzed reactions in the presence of exogenous phenolic acid (Figueroa-Espinoza et al., 1999a). Furthermore, laccase has been shown to form crosslinks between phenolic acids and tyrosine-containing tri-peptides and protein, as shown by Mattinen et al. (2005). The broad substrate specificity of laccases, i.e. the ability to oxidize a wide variety of phenolic as well as non-phenolic compounds, makes them attractive enzymes for applications in various biotechnological processes.

1.4.1 Food applications

The amount, distribution and accessibility of phenolic substrate moieties in raw materials determine the possibility to use oxidative enzymes in specific applications. The general objective in the food industry is to use oxidative crosslinking enzymes in cereal, dairy and meat applications for improving the textural quality of the end-products. In cereal applications, for example, breadmaking quality of weak flours can be improved by oxidative enzymes, especially when combining oxidative enzymes with hydrolytic enzymes, such as xylanases (Primo-Martín et al., 2003). In dairy products, crosslinking can be exploited for prevention of syneresis or to make a soft texture firmer. Alternatively, low-fat fermented milk products with acceptable sensory properties can be produced. In the meat industry, crosslinking enzymes can be exploited in strengthening product texture, particularly in products with low salt and fat contents (Lantto 2005b, 2007a and 2007b). Furthermore, hetero-crosslinking of cereal, milk and meat biopolymers can provide a potential tool to create novel food products with specific functionalities and characteristics. However, despite the active research and promising results as summarized in Tables 4 and 5, commercial tyrosinases and laccases suitable for application in food processes remain to be launched to the market.

Table 4. Tyrosinase as a crosslinking agent in food applications.

Appli-cation	**Substrate**	**Effects**	**References**
Cereal	Protein tyrosyl residues, phenolic compounds of cell wall structures, but not FA	Protein/AX network strengthening.	Kuninori et al., 1976; Hoseney and Faubion, 1981;
		Increased accessibility of iron in polyphenol-containing products	Matuschek and Svanberg, 2005
Dairy	Milk proteins	Crosslinking of proteins	Thalman and Lötzbeyer, 2002; Halaouli et al., 2005
Meat	Pork homogenate	Increased gel firmness	Lantto et al., 2006
Emulsions	Proteins and carbohydrates	Improved emulsifying properties by conjugation of different types of proteins and polysaccharides	Kato et al., 1993; Kato et al., 1991

Table 5. Laccase as a crosslinking agent in food applications.

Appli-cation	Substrate	Effects	References
Cereal	Phenolic compounds, especially FA	Improved dough handling, loaf volume and crumb structure of bread	Si, 1994; Labat et al., 2000 and 2001
Dairy	Milk proteins	Crosslinking of dairy proteins	Færgemand et al., 1998
Meat	Chicken myofibrillar proteins	Gel formation improved at high salt concentration. Decreased gel formation with high laccase dosages	Lantto et al., 2005b
Beverages	Phenolic compounds	Stabilization of beer, wine and juices: controlling colour, haze and turbidity	Minussi et al., 2002 and 2007
Other	FA residues of sugar beet pectin	Gel formation	Kuuva et al., 2003;
	Oxygen and/or phenolic compounds	Improvement of sensory quality by removal of oxygen or by tailoring the flavour compounds with laccase	Carvajal-Millan et al., 2005b; Minussi et al., 2002

1.4.1.1 Application of tyrosinases and laccases in cereal processing

Wheat flour is composed of carbohydrates 70–80 %, proteins 8–18 % and lipids 1.5–2.5 % (MacRitchie 1984, Meuser and Suckow, 1986). Gliadin and glutenin proteins account for approximately 80 % of the wheat proteins, and their viscoelastic nature is the backbone for the formation of a protein network during dough mixing, determining bread quality with respect to crumb structure and loaf volume (Osborne, 1907; Wrigley and Bietz, 1988). Non-starch polysaccharides, typically 2–3% of wheat flour, are mostly built up of AX, which is known to play an important role in the structure formation of the gluten network and dough (Meuser and Suckow, 1986; Wang, 2003).

Rheological properties of gluten, dough and bread may be influenced by oxidative enzymes since they can form covalent linkages on certain proteins and carbohydrates. Due to the ability of laccase and tyrosinase to catalyze oxidation of phenolic compounds present in cereal proteins and polysaccharides, the mechanisms of the enzymes are complex. For example, the enzymes may induce linkages in or between polysaccharides, in or between proteins and between polysaccharides and proteins. The effect of tyrosinase (extract of mushroom, rich in polyphenol oxidase) on wheat dough was described by Kuniori et al. (1976). Tyrosinase-catalyzed formation of a 2-S-cysteinyl-dopa, 2,5-di-S-cysteinyl-dopa and 6-S-cysteinyl-dopa, 5-S-cysteinyl-3,4-dopa and di-dopa crosslinks have been characterised in gluten proteins (Takasaki and Kawakishi 1997; Takasaki et al., 2001). Laccases are typically reported to crosslink mainly the AX in wheat dough via AX-esterified FA (Figueroa-Espinoza and Rouau, 1998; Figueroa-Espinoza et al., 1998 and 1999a; Labat et al., 2000 and 2001). The use of laccases in baking is reported to result in increased strength, stability and reduced stickiness of dough, and thereby also in improved machinability of dough (Labat et al., 2000; Si, 1994). Increased bread volumes and improved crumb structure of the baked product have also been reported (Labat et al., 2000; Si, 1994). However, AX treated by laccase has also been reported to show poor stability properties, probably due to depolymerization by laccase-generated radicals (Carvajal-Millan et al., 2005a).

1.4.1.2 Application of tyrosinases and laccases in dairy processing

Bovine milk contains approximately 3.5 % of proteins, which are divided into caseins (80%) and whey proteins (20%). Casein proteins are mainly located in micelles, which contain calcium and phosphate (6%) and lower levels of magnesium and citrate for stabilization of the protein micelle structure. Sodium caseinate is a mixture of α_{s1}-, α_{s2}-, β- and κ- casein molecules of which β-casein is the major component. Whey proteins consist mainly of β-lactoglobulin (50%), α-lactalbumin (20%), bovine serum albumin (BSA) and immunoglobulins, which are typically globular proteins (Fox and McSweeney, 1998).

In dairy applications, transglutaminase (EC 1.13.11.12) is the most widely studied crosslinking enzyme for polymerization of milk proteins (Lorenzen et al., 1998 and 2002; Lorenzen and Neve, 2003; Nonaka et al., 1989 and 1992). However, Ito et al. (1984) reported oxidation of the tyrosyl residues of dairy

proteins by tyrosinase, and recently tyrosinase has been reported to crosslink casein proteins (Halaouli et al., 2005). Furthermore, tyrosinase and laccase are reported to induce at least partial crosslinking of whey proteins (Thalmann and Lötzbeyer, 2002; Færgemand et al., 1998). Thalmann and Lötzbeyer (2002) reported that tyrosinase from *A. bisporus* could crosslink α-lactalbumin. However, tyrosinase-aided crosslinking of β-lactoglobulin was possible only in the presence of a low molecular weight phenolic compound, which was proposed to act as a bridging agent between the protein subunits (Thalmann and Lötzbeyer, 2002).

1.4.1.3 Application of tyrosinases and laccases in meat processing

Gel formation ability and textural and binding properties of meat are essential in the manufacturing of meat products. Heat-induced gelation of muscle proteins is critical in meat processing, determining the textural characteristics of the end-product (Tornberg, 2005). The myofibrillar proteins, myosin, actin, troponin and tropomyosin, play an essential role in this process, and myosin has the most important role in gelation (Yasui et al., 1980; Belitz and Grosch, 1999). Protein gels are formed by intermolecular interactions of the protein fibres, and the gelation process involves denaturation, aggregation and formation of a three-dimensional network (Fennema, 1976; Lesiów and Xiong, 2001a and 2001b). Fat and salt notably contribute to both the technological and sensory properties of meat products. Therefore, reduction of fat and salt from meat products can be problematical.

Crosslinking enzymes can be used to tailor gelation properties of meat. In addition to transglutaminase, the oxidative enzymes, tyrosinase and laccase, have recently been tested for processing of pork and chicken proteins (Lantto et al., 2005b, 2006, 2007a, 2007b). Of the poultry meat proteins, myosin and troponin T were found to be the most susceptible and actin proteins the most resistant to enzymatic modification. Lantto et al. (2005b, 2007b) reported tyrosinase to be able to effectively improve the gel formation of a 4% chicken breast myofibrillar protein suspension with 0.35 M NaCl, whereas laccase was found to improve gel formation of the myofibrillar proteins only when 0.60 M NaCl was used, suggesting that completely solubilised protein is needed to act as a substrate for laccase. Furthermore, high dosages of laccase were found to lead to protein fragmentation and decreased gel strength (Lantto et al., 2005b). In the

studies of Lantto et al. (2007a), tyrosinase was observed to improve the firmness of the homogenate gels containing a lowered amount of meat and free of phosphate, and to reduce the weight loss in the homogenates with a low amount of meat and NaCl. Laccase, on the other hand, was not able to decrease the weight loss of the homogenates (Lantto et al., 2007b).

1.4.1.4 Miscellaneous food applications of tyrosinases and laccases

Tyrosinase and laccase have been proposed for functionalization of food products, for example, for tailoring antioxidativity of biopolymers. Gelatine-catechin conjugates, synthesized by the laccase-catalyzed oxidation of catechin in the presence of gelatine, were observed to have increased antioxidative properties as compared to unconjugated catechin (Chung et al., 2003). Desentis-Mendoza et al. (2006) reported improved *in vitro* antioxidativity by polymerization of phenolic compounds with laccase and tyrosinase. On the other hand, Sun et al. (2007) proposed that the decrease in antioxidant activity of asparagus juice was due to the laccase activity. Improved iron accessibility *in vitro* by oxidation of polyphenols via tyrosinase catalysis has been reported (Matuschek and Svanberg, 2005). Laccases have been studied for applications in beverage processes. Especially in wine manufacturing, laccases can be used for stabilization and analysis of wine, and for bioremediation of the waste water (Minussi et al., 2002). For example, laccase-mediated bioremediation of waste waters of the food industry, such as wastes of beer factories (Yague et al., 2000) and olive-mills (Greco et al., 1999), has been widely studied, and reviewed by Minussi et al. (2002).

Oxidative crosslinking enzymes have been used to improve the technological or nutritional properties of phenolic acid -containing carbohydrates such as sugar beet pectins and arabinoxylans. Dimerization of ferulic acids in pectins has been exploited in tailoring the characteristics of pectin-derived foods. In particular, crosslinking with laccases and also with peroxidases has been applied to improve the gelling of sugar beet pectin or arabinoxylan with subsequent formation of a hydrated network (Guillon and Thibault, 1990; Micard and Thibault, 1999; Kuuva et al., 2003; Carvajal-Millan et al., 2005b). There has also been considerable interest to generate protein-carbohydrate conjugates through enzymatic hetero-crosslinking, e.g. for stabilization of foams and emulsions (Ganzevles et al., 2006; Kato et al., 1993; Dickinson, 1997). Peroxidase has been

reported to form covalent protein-carbohydrate conjugates between gluten or β-casein and feruloylated arabinoxylans (Hilhorst et al., 1999; Boeriu et al., 2004).

1.4.2 Non-food applications

Tyrosinases and laccases have widely been studied for non-food applications as listed in Tables 6 and 7. Tailoring properties of polymers, for example, through grafting of silk proteins onto chitosan via tyrosinase reactions has been reported (Åberg et al., 2004; Halaouli et al., 2005; Sampaio et al., 2005; Freddi et al., 2006; Anghileri et al., 2007). Wool fibre proteins have been reported to be modified by tyrosinase (Lantto et al., 2005a), and grafting of a small diphenolic molecule L-dopa to wool fibres has also been successfully carried out (Mattinen et al., 2008). Tyrosinases can also be applied in biosensors and microarrays through immobilization of the enzyme (Busch et al., 2006; Streffer et al., 2001; Ahmed et al., 2006). For example, tyrosinase has been applied for detection of toxic phenolic compounds in olive oil (Busch et al., 2006), glucose dehydrogenase incorporated with tyrosinase has also been used for detecting phenolic compounds (Streffer et al., 2001) and Ahmed et al. (2006) reported using tyrosinases as antibody microarrays. Tyrosinases have also been suggested to be potential tools in treating melanoma. It has been proposed that prodrug therapy for melanoma patients could be implemented successfully via tyrosinase activity (Morrison et al., 1985; Jordan et al., 1999 and 2001; Moridani et al., 2001).

Environmental applications of laccases have been thoroughly investigated. In particular, there has been extensive research on the suitability of laccase treatments in the textile and pulp and paper industries and for waster water bioremediation, as also recently reviewed by Xu (2005), Rodriguez Couto and Toca-Herrera (2006a and 2006b) and Riva (2006). However, although studied in a wide range of applications, there are only a few industrial-scale applications of laccases hitherto. Laccase is used for preparing cork stoppers for wine bottles, as the laccase-catalyzed oxidation reactions can reduce the characteristic cork astringency (Conrad et al., 2000; Xu, 2005). In the textile industry, decolourization of dyes can be performed by laccases (Wong and Yu, 1999; Abadulla et al., 2000; Peralta-Zamora et al., 2003; Takada et al., 2003; Rodrígues Couto and Toca-Herrera, 2006a and b). Bleaching of indigo dyed denim fabrics is a known industrial-scale process utilizing laccase (Setti et al., 1999; Xu, 2005). Laccase

can also be used for synthesis of dyes (Setti et al., 1999; Rodrígues Couto and Toca-Herrera, 2000b), and reports on using laccase in organic synthesis have been presented by Mustafa et al. (2005), Riva (2006), and Rodrígues Couto and Toca-Herrera (2006a). In cosmetics, laccase activity can be used in dyeing of hair (Reyes et al., 1999) and stabilization of emulsions (Littoz and McClements, 2007). Laccases have also been suggested to be applicable to the production of functional biocomposites, as mechanical properties of fibres could be tailored via laccase-catalyzed reactions (Mustafa et al., 2005; Riva, 2006; Rodrígues Couto and Toca-Herrera, 2006a).

Table 6. Non-food applications of tyrosinase.

Application	**Details**	**References**
Biosensors	Detection of toxic phenolic compounds	Busch et al., 2006; Streffer et al., 2001; Yaropolov et al., 1995; Mita et al., 2007
	Probes for biocatalytic transformations	Gill et al., 2006
	Grafting carboxyl groups to chitosan for bioremediation / decolourization	Chao et al., 2004
Antibody microarray	Immobilization of antibody via tyrosinase-catalyzed reaction	Ahmed et al., 2006
Medical (prodrug)	Specific release of drug for melanoma by tyrosinase activity	Morrison et al., 1985; Jordan et al., 1999 and 2001
Immunoassays	Tyrosinase as an antigen for detecting melanoma	Chen et al., 1995
Tailoring biopolymers (emulsions)	Grafting chitosan to silk proteins	Freddi et al., 2006; Anghileri et al., 2007; Sampaio et al., 2005
	Grafting peptides to chitosan	Åberg et al., 2004
	Grafting fluorescent proteins to chitosan	Chen et al., 2003b
	Modification of wool fibres	Lantto et al., 2005a; Mattinen et al., 2008
Nanotechnology	Tyrosinase-aided patterning of nano-scale structures on surfaces	Basnar et al., 2007

Laccase-mediated systems have been developed for paper and pulp processing, especially for biobleaching (Bourbonnais and Paice, 1992; Bourbonnais et al., 1997; Rodrígues Couto and Toca-Herrera, 2006a; Call and Mücke, 1997). Lignin degradation and removal of lipophilic compounds from pulp are also reported to be possible by laccase-catalyzed reactions (Bourbonnais and Paice, 1992; Bourbonnais et al., 1997; Rodrígues Couto and Toca-Herrera, 2006a; Call and Mücke, 1997; Gutiérrez et al., 2007). By using a range of organic and inorganic compounds as mediators, bioremediation of organic pollutants is possible through laccase-catalyzed oxidation (Riva, 2006). Waste water treatment, detoxification of soil, as well as reduction of toxic compounds in fishmeal by laccases have been reported (Collins et al., 1996; Martin et al., 2007; Minussi et al., 2002; Torres et al., 2003; Zille et al., 2005; Baron et al., 2007). Furthermore, in biosensor technology, detection of toxicants can be performed through laccase-mediated detector systems (Yaropolov et al., 1995; Minussi et al., 2002; Rodrigues Couto and Toca-Herrera, 2006a).

Table 7. Non-food applications of laccase.

Application	Details	References
Pulp and paper	Biobleaching	Bourbonnais and Paice, 1992;
	Lignin degradation	Bourbonnais et al., 1997; Call and Mücke, 1997; Poppius-Levlin et al., 1999; Camarero et al., 2007; reviewed by Rodrígues Couto and Toca-Herrera, 2006a
	Removal of lipophilic compounds from pulp (mediators)	Gutiérrez et al., 2007
Bioremediation	Detoxification of soil, waste streams and water by enzymatic catalysis	Martin et al., 2007; Minussi et al., 2002; Torres et al., 2003; Zille et al., 2005; reviewed by Rodrígues Couto and Toca-Herrera, 2006b; Canfora et al., 2008
Decontamination of feed	Decrease of toxic compounds in fishmeal by oxidoreductases	Baron et al., 2007
Biosensors	Detection of phenolics (toxic compounds)	Yaropolov et al., 1995; Minussi et al., 2002; reviewed by Rodrigues Couto and Toca Herrera, 2006a
Biofuel cells	Biofuel cells	Palmore and Kim, 1999; Liu and Dong, 2007; Lim and Palmore, 2007; Smolander et al., 2008
Textiles	Decolourization of dyes	Wong and Yu, 1999; Abadulla et al., 2000; Peralta-Zamora et al., 2003; Reyes et al., 1999; reviewed by Rodrígues Couto and Toca-Herrera, 2006a and b
	Bleaching textiles	Setti et al., 1999; reviewd by Rodrígues Couto and Toca-Herrera, 2006a and b; Pazarlioglu et al., 2005
	Synthesis of dyes, textile fibre dyeing	Setti et al., 1999; reviewed by Rodrígues Couto and Toca-Herrera, 2006a and b
Synthetic chemistry	Organic synthesis	Mustafa et al., 2005; reviewed by Riva, 2006, and by Rodrígues Couto and Toca-Herrera, 2006a
Nanotechnology	Functional biocomposites	Xing et al., 2007; Fei et al., 2007; Kim et al., 2007
Cosmetics	Hair dyes	Takada et al., 2003; Rodrígues Couto and Toca-Herrera, 2006a
	Stabilization of emulsions by laccase	Littoz and McClements, 2007

2. Aims of the work

Modification of biopolymers by enzymatic reactions forming covalent conjugates enables incorporation of novel functionalities to the polymers. The changes in the properties of the biopolymers by the enzymes can be exploited in tailoring the technological characteristics of the end-products. The aim of the present work was to characterize a novel *Trichoderma reesei* tyrosinase, and to assess the suitabilities of two oxidative enzymes, i.e. tyrosinase and laccase, for crosslinking of proteins and carbohydrates. The application of tyrosinase and laccase for modification of the textural properties of wheat dough and bread was also evaluated.

More specifically, the aims were:

1. to characterize the biochemical properties of a novel fungal tyrosinase (Publication I)
2. to compare plant and fungal tyrosinases with respect to their substrate specificity and crosslinking ability (Publication II)
3. to elucidate the suitability of tyrosinase and laccase for hetero-crosslinking of carbohydrates and proteins (Publication III)
4. to analyze the suitability of tyrosinase and laccase for improving wheat breadmaking quality (Publications IV and V).

3. Materials and Methods

Materials and methods used in this study are described in detail in the original publications I–V. A general outline of the methodology and the materials used is given below.

3.1 Raw materials

Raw materials used in the work are listed in Table 8. A range of small molecular weight phenolic compounds were tested for determining the substrate specificities of tyrosinases. Di- and tri-peptides, model proteins and arabinoxylan were used as model biopolymers, and wheat gluten and wheat flour in the application studies of breadmaking.

Table 8. Raw materials used in the work.

Substrate	**Studied in publication**
L-Dopa (3,4-dihydroxy-L-phenylalanine)	I–V
L-Tyrosine (3-(4-Hydroxyphenyl)-L-alanine)	I–III, V
Phenol (Hydroxybenzene)	I, II
4-Mercaptophenol (4-Hydroxythiophenol)	I
p-Cresol (4-Methylphenol)	I, II
4-Hydroxyaniline (4-Aminophenol)	I, II
3-Hydroxyanthranilic acid (2-Amino-3-hydroxybenzoic acid)	I
Tyramine (2-(4-Hydroxyphenyl)ethylamine)	I, II
p-Tyrosol (2-(4-Hydroxyphenyl)ethanol)	I, II
p-Coumaric acid (*trans*-4-Hydroxycinnamic acid)	I–III, V
o-Coumaric acid (*trans*-2-Hydroxycinnamic acid)	I
Ferulic acid (trans-4-Hydroxy-3-methoxycinnamic acid)	I–III, V
Aniline	I, II
(-)-Epicatechin ((−)-cis-3,3′,4′,5,7-Pentahydroxyflavane)	I, II
(+)-Catechin hydrate (trans-3,3′,4′,5,7-Pentahydroxyflavane)	I, II
Caffeic acid (3,4-Dihydroxycinnamic acid)	I, II, V
Pyrocatechol (1,2-Dihydroxybenzene)	I, II

Pyrogallol (1,2,3-Trihydroxybenzene)	I, II
Glycine-Tyrosine (GY)	I, II
Tyrosine-Glycine (YG)	I, II
Glycine-Tyrosine-Glycine (GYG)	I, II
Glycine-Glycine-Tyrosine (GGY)	I, II
Tyrosine-Glycine-Glycine (YGG)	I, II
Glycine-Leucine-Tyrosine (GLY)	I, II
α-Casein protein	II, III
Glutenin proteins	IV, V
Gliadin proteins	IV, V
Oat spelt xylan	III
Wheat flour (Paakari, Finland)	V
Wheat flour (Raisio, Finland)	IV
Wheat gluten (crude)	IV

3.2 Enzymes

The enzymes studied in the work are listed in Table 9. Five different tyrosinases of fungal and plant origin were characterized for their substrate specificity and protein crosslinking ability. Laccase from *Trametes hirsuta* was compared to the tyrosinase from *T. reesei* with regard to crosslinking of different biopolymers and breadmaking. Xylanases were used in the dough and breadmaking studies, and also in the experiments concerning hetero-conjugation of biopolymers.

Table 9. Enzymes studied in the work.

Enzyme	Origin	Reference	Abbre-viation	Studied in publication
Fungal tyrosinases	*Trichoderma reesei*	Publication I	TrT	I, II, III, V
	Agaricus bisporus	from Sigma-Aldrich [a]	AbT	II
	Pycnoporus sanguineus	Halaouli et al., 2005	PsT	II
Plant tyrosinases	Apple (*Malus domestica*)	Ni Eidhin et al., 2006	AT	II
	Potato (*Solanum tuberosum*)	Unpublished results [b]	PT	II
Laccase	*Trametes hirsuta*	Produced and purified at VTT [c]	ThL	III, IV, V
Xylanases	*Bacillus subtilis*	from Danisco A/S [a]	BsX	III, IV
	Thermomyces lanuginosus [d]	from Novozymes [a]	TlX	IV, V

[a] Commerical enzyme
[b] Enzyme was provided by the University of Limerick, Ireland
[d] Heterologously expressed in *Aspergillus oryzae*
[c] Purification performed as described by Rittstieg et al. (2002)

3.3 Methods

3.3.1 Enzyme activity assays (Publications I–V)

Tyrosinase activity was measured by a spectrophotometric method as described by Robb (1984), using 15 mM L-dopa and 2 mM L-tyrosine as substrates. Tyrosinase activity was also measured by monitoring consumption of the co-substrate oxygen with a single channel oxygen meter in a sealed and fully filled sample vial. Laccase activity was determined with a spectrophotometric method, as described by Niku-Paavola et al. (1988), using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) as substrate. Laccase activity was also monitored by oxygen consumption measurement, correspondingly to the tyrosinase assay. Xylanase activity was determined by a reductometric assay as described by Bailey et al. (1992), using birch glucuronoxylan as substrate.

3.3.2 Production and purification of TrT (Publication I)

Based on a homology search against the genome sequence of *T. reesei*, a *tyr2* gene of *T. reesei* and the corresponding cDNA were cloned by PCR and sequenced. To overexpress the *tyr2* gene, an expression construct, in which the protein encoding region of the genomic *tyr2* locates between the *cbh1* promoter and terminator, was made *in vivo* with the Gateway recombination system. The construct pMS190 was transformed into a *T. reesei* strain. Based on shake flask cultures, the best overexpression transformant of *tyr2* was grown in a laboratory fermenter with a volume of 20 L.

For purification of TrT, a concentrated culture supernatant was first desalted on a Sephadex G-25 Coarse column (2.6 × 27 cm) in 10 mM Tris-HCl buffer, pH 7.3. The desalted sample was applied to a HiPrep™ 16/10 CM Sepharose Fast Flow column, connected to an ÄKTA Purifier, in 10 mM Tris-HCl buffer, pH 7.3. Bound proteins were eluted in the equilibration buffer with a linear NaCl gradient (0–180 mM in 6 column volumes). Tyrosinase positive fractions were pooled, concentrated with a Vivaspin concentrator (20 ml, molecular weight cut off 10 000), and further applied to gel filtration in a Sephacryl S-100 HR column (1.6 × 90 cm), which was equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl. To monitor purification, SDS-PAGE was performed as described by Laemmli (1970), using 12% Tris-HCl Ready Gels of Bio-Rad.

3.3.3 Biochemical characterization of tyrosinases (Publications I and II)

The isoelectric point of TrT was determined by isoelectric focusing on an LKB 2117 Multiphor II Electrophoresis System. Bands containing tyrosinase activity were visualized by staining the gel with 15 mM L-dopa in 0.1 M sodium phosphate buffer (pH 7.0) and proteins by staining with Coomassie Brilliant Blue (Serva Blue R). Determination of the pH optimum of TrT was carried out using 2 mM L-tyrosine as substrate in 50 mM McIlvaine universal buffer (50 mM Na_2HPO_4 – 25 mM citric acid) over a pH range of 3–7, 50 mM Tris-HCl buffer over a pH range of 7–8.5 and 50 mM Glycine-NaOH buffer over a pH range of 8.5–10. Activity was measured by monitoring the consumption of oxygen. The stability of TrT at different pH-values was determined in McIlvaine universal buffer by incubating the enzyme solution in different pH-values at

room temperature for 1 hour and for 1, 2 and 3 days. Temperature stability of TrT was determined at 30, 40 and 50 ºC. In the pH and temperature stability tests, the residual tyrosinase activity was determined by the spectrophotometric activity assay using 15 mM L-dopa as substrate.

Substrate specificity of TrT and the tyrosinases from apple (AT), potato (PT), white rot fungus *P. sanguineus* (PsT) and edible mushroom *A. bisporus* (AbT), and the kinetic constants, K_m, V_{max} and k_{cat}, of mono- and diphenolic compounds for TrT and AbT were determined by measuring the enzymatic activities either by monitoring consumption of oxygen or by using the spectrophotometric activity assay. Enzymatic reactions were carried out at 25 ºC in pH 7. Specific wavelengths for the substrates were determined measuring the UV/visible absorption spectrum of the oxidation products from the particular substrates as a function of reaction time. Inhibition of the plant and fungal tyrosinases by benzoic acid, benzaldehyde, kojic acid, 2-mercaptoethanol, glutathione, ethylenediamine tetraacetic acid, sodium dodecyl sulphate, sodium chloride, sodium azide and hydrogen peroxide was also analyzed. L-dopa (15 mM) and inhibitor compounds (1–100 mM) were dissolved simultaneously in 0.1 M sodium phosphate buffer (pH 7.0), and the inhibition of the activity of the tyrosinases on L-dopa was determined with the spectrophotometric activity assay.

Electron spin resonance (ESR) analysis for detection of semiquinone radicals from tyrosinase-catalyzed reactions was performed on a Bruker EMX X-band ESR spectrometer equipped with an ER4119HS cavity. Microwave power of 20 mW, modulation frequency of 100 kHz, field modulation amplitude of 0.2 gauss, receiver gain of 2×10^3, time constant of 2.56 ms and a conversion time of 2.56 ms were used. The measurement was started by mounting a flat sample cell (0.5 ml) within the ESR cavity. ESR signal appearance and disappearance was monitored as a function of time. The experiment was performed at ambient temperature, and the substrates were dissolved in acetate buffer (pH 6.5) containing 0.05 M Zn^{2+} to stabilize the semiquinones. (Publication II)

3.3.4 Analyses of the reaction products of tyrosinase and laccase (Publications I–V)

The abilities of TrT and ThL to oxidize phenolic model compounds and to crosslink model proteins, α-casein, gliadin and glutenin, and oat spelt xylan were studied. To study the effect of small phenolic molecules on the crosslinking process, L-dopa, ferulic acid and *p*-coumaric acid were alternately added to the reaction mixtures. The enzymatic reactions were monitored by the oxygen consumption method (as described above), and crosslinking of the biopolymers was monitored by SDS PAGE with specific staining procedures and size exclusion chromatography (SEC), as described below. (Publications II, III, V)

SDS-PAGE was performed as described by Laemmli (1970), using 12% Tris-HCl Ready Gels (Bio-Rad), and two specific staining methods were used to mark either proteins or glycoproteins. For protein visualization the gels were stained with Coomassie Brilliant Blue. For detection of the carbohydrate-protein conjugates, the coomassie dye was first removed by destaining the gels with 40 % (v/v) ethanol and 10 % (v/v) acetic acid. After destaining the proteins, the gels were treated with periodic acid and Schiff's reagent to visualize the carbohydrate-protein conjugates (Zacharius et al., 1969). (Publications I–III and V)

Samples from the experiments of the crosslinking of different biopolymers by TrT and ThL were analyzed by size exclusion chromatography (SEC) using ÄKTA Purifier liquid chromatography system. The samples were studied in a Superdex 200 HR 10/30 column, running in 0.1 M sodium phosphate (pH 7), 1 % (w/v) SDS and 0.15 M NaCl, at a flow rate of 0.5 ml/min, in ambient temperature. SDS was added to prevent protein self-aggregation. Eluate was monitored with a UV detector at 280 nm. Fractions of 0.5 ml were collected and further analyzed by fluorescence spectroscopy on 96-well black microtiter plates with a Varioskan^{TM} equipment, using the excitation and emission parameters $\lambda_{ex}/\lambda_{em}$: 274/303 nm, 318/410 nm, 330/430 nm, specific for L-tyrosine (Y), ferulic acid (FA), and *p*-coumaric acid (*p*-CA), respectively. (Publication III)

3.3.5 Characterization of wheat flour and gluten dough (Publications IV and V)

Large deformation rheological tests of TrT- and ThL-treated doughs were performed by uniaxial extension measurements at ambient temperature. Wheat flour dough was prepared with a Mixograph apparatus, by mixing flour with water at a dry matter content of 59 % (or 50 %) for 3.5 min. Gluten dough was made in a Brabender's Farinograph by mixing gluten powder (50 g) and water (80 g) for 5 min. The influence of ThL, TrT and the xylanases, BsX and TlX, on dough rheology was studied by adding the enzymes to flour (or gluten) in the beginning of the mixing. The extensibility and strength of dough samples were characterised using a Kieffer dough and gluten extensibility rig fitted onto a *TA*.X*T*2 Texture Analyzer, equipped with a 5 kg load cell. The rheological tests of doughs were performed as described by Kieffer (1998), with some modifications in dough resting conditions. (Publications IV and V)

For the microscopy of the enzymatically treated doughs, pieces of the doughs were embedded in 1% agar, fixed in 1% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.0), dehydrated with ethanol, and embedded in hydroxyethyl methylacrylate. Sections of 2 μm were cut from the samples, and three different staining procedures were carried out. Using 0.1 % Acid Fuchsin and 0.01 % Calcoflour, cell walls and protein were dyed blue and orange/light grey, respectively. Using Light Green and Lugol's iodine solution (I_2 0.33 %, w/v and KI 0.67 %, w/v) starch was dyed dark blue and protein green. Red staining of proteins with protein-sensitive Xylidine Ponceau (0.2 %) was also performed. The stained dough samples were examined with an Olympus BX-50 microscope connected to a SensiCam PCO CCD camera with an AnalySIS 3.0 image analysis program. (Publication V)

3.3.6 Breadmaking (Publication V)

The impact of TrT and ThL on formation of wheat bread structure was studied by measuring the specific volume and hardness of breads, and observing bread crumb structure (pore size) by visual examination. Wheat flour, baker's yeast, sugar, salt and margarine were mixed with water. The doughs were hand-moulded, and allowed to rest for 20 min at 28 °C in a 75 % water-saturated

atmosphere. Subsequently, the dough was divided into 20 g (or 200 g) pieces, shaped, proofed for 35 (or 55) min at 30 (or 37) °C with 75 (or 80) % water-saturated atmosphere and baked for 13 (or 18) min at 210 °C. Steam was added for 15 (or 20) s during the initial baking phase. The specific volume of the breads was determined by measuring the bread weight and volume. The hardness of the bread crumbs after 2 and 72 hours was analysed by texture profile analysis (TPA) using the *TA*.X*T*2 Texture Analyzer.

4. Results and discussion

4.1 Purification and characterization of TrT (Publication I)

A novel tyrosinase from the filamentous fungus *T. reesei* (TrT) was over-expressed under a strong *cbh1* promoter in its native host. The tyrosinase gene *tyr2* of *T. reesei* was shown to encode a protein with a signal sequence, and the protein was observed to be secreted in a high titer to the culture supernatant in laboratory-scale batch fermentation (20 L). TrT was purified with a three step purification procedure. First a culture filtrate was desalted with gel filtration chromatography. A relatively pure enzyme preparation was achieved by subsequent cation exchange chromatography. TrT was further purified by gel filtration chromatography, which resulted in a pure enzyme preparation (Fig. 9). Based on SDS-PAGE, pure TrT was visualized as a double protein band corresponding to a molecular mass around 43 kDa. Since the encoded amino acid sequence of TrT theoretically gives a molecular mass of 61.151 kDa, the result obtained from SDS PAGE indicated probable processing of the protein. TrT was presumed to be cleaved at the C-terminal end as C-terminal processing of tyrosinases has been previously reported, e.g. for the fungal tyrosinases from *N. crassa* (Kupper et al., 1989; van Gelder et al., 1997), *A. bisporus* (Espín et al., 1999a) and *P. sanguineus* (Halaouli et al., 2006b). When the presumed processing of mature TrT was further investigated by molecular characterization using advanced mass spectrometric analyses, the C-terminal cleavage site of TrT was concluded to locate after Gly410 in the polypeptide, and the molecular weight of the mature protein was defined to be 43.2 kDa.

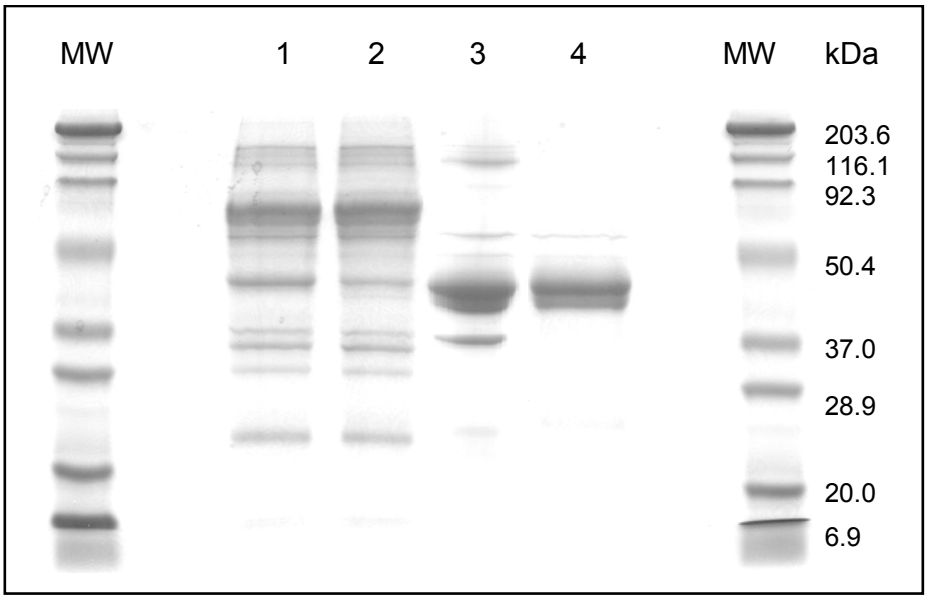

Figure 9. Purification of TrT as analyzed by SDS-PAGE. Lanes on the SDS PAGE gel: MW) molecular weight markers, 1) culture filtrate, 2) desalted culture filtrate, 3) TrT sample after cation exchange, 4) TrT sample after gel filtration. (Publication I)

TrT was able to oxidize Y and L-dopa, thus showing characteristics of a tyrosinase. TrT was also observed to be able to oxidize various *p*-monophenolic and *o*-diphenolic compounds (Table 10). Oxidation rates by TrT were clearly higher for diphenolic compounds than for monophenols, and the phenolic compounds having an amino substitution were oxidized by TrT at a lower rate than the compounds having a carboxyl group in the corresponding position. The kinetic constants K_m and k_{cat} of TrT were determined for diphenolic L-dopa (K_m 3 mM, k_{cat} 22 s^{-1}), and for the monophenols *p*-CA (K_m 1.6 mM, k_{cat} 8 s^{-1}) and *p*-tyrosol (K_m 1.3 mM, k_{cat} 7 s^{-1}). The inability of TrT to oxidize FA was concluded to be due to a methoxy side group adjacent to the phenolic hydroxyl group of FA, preventing hydroxylation of the substrate prior to the oxidation.

Table 10. Characterization of the substrate specificity of TrT. Relative activity (%) on mono- and polyphenols was calculated from oxygen consumption (nmol l^{-1} s^{-1}) according to the stoichiometry that one mono-phenol molecule needs one oxygen molecule and one polyphenol molecule needs half of oxygen molecule in the reaction to form a quinone. (Publication I)

	Activity (%) expressed relative to L-dopa	
SUBSTRATE	**c = 2.5 mM**	**c = 10 mM**
L-Dopa	100	100
L-Tyrosine	11	nd [a]
Phenol	8	8
4-Mercaptophenol	0	0
p-Cresol	12	8
4-Aminophenol	1	nd [a]
3-Hydroxyanthranilic acid	0	0
Tyramine	3	3
p-Tyrosol	23	16
p-Coumaric acid	25	16
o-Coumaric acid	0	0
Ferulic acid	0	0
Aniline	1	0
(-)-Epicatechin	96	89
(+)-Catechin hydrate	142	73
Pyrocatechol	87	72
Pyrogallol	66	52

[a] Not determined due to insolubility of the substrate

TrT was found to be relatively stable at neutral and alkaline pH, whereas in the acidic pH range activity was rapidly lost. TrT showed half-lives of 18 h, 3 h 45 min and 15 min at 30 ºC, 40 ºC and 50 ºC, respectively. Thus, TrT is not a thermostable enzyme. The pI of TrT was observed to be very high, around 9.5, which is rather uncommon among the reported fungal tyrosinases (Table 3). However, some bacterial tyrosinases have been reported to show high pI values (Philipp et al., 1991; Claus and Decker, 2006). High pI was exploited in the purification procedure. Since most hydrolytic enzymes produced by *T. reesei* typically show clearly lower pI values than TrT (Niku-Paavola et al., 1985; Lappalainen, 1988; Lappalainen et al., 2000; Xiong et al., 2004), cation exhange

was successfully used to remove the majority of the contaminating proteins. The determined pH optimum of TrT within the neutral and alkaline pH range, and with a maximal activity at pH 9, also appears rather atypical. Generally a pH optimum in the neutral or slightly acid pH range has been reported for fungal tyrosinases (Gukasyan, 1999; Zhang et al., 1999; Ikehata and Nicell, 2000; Fan and Flurkey, 2004; McMahon et al., 2007; Halaouli et al., 2006a; Ni Eidhin et al., 2006). However, some tyrosinases have been reported to have a pH optimum at alkaline pH (Kong et al., 1998 and 2000a).

Inhibition of TrT was examined using benzoic acid, benzaldehyde, kojic acid, β-mercaptoethanol, glutathione, EDTA, SDS, sodium chloride, sodium azide and hydrogen peroxide as inhibitors. Kojic acid, β-mercaptoethanol and glutathione were the most effective inhibitors for TrT, with close to 100 % inhibition of L-dopa activity even at low concentrations (Publication I, Table 5). Based on the oxygen consumption and spectrophotometric activity assays, kojic acid and β-mercaptoethanol were assumed to have acted by forming a complex with the copper atoms at the active site. On the other hand, glutathione was shown not to inhibit the enzymatic activity of TrT, but to affect the reaction products by inhibiting the formation of dopachrome through a nucleophilic addition reaction.

4.2 Comparison of tyrosinases of plant and fungal origin (Publication II)

The extracellular tyrosinase TrT and the intracellular tyrosinases AT, PT, PsT and AbT were compared for their biochemical characteristics. The enzymes were characterized in terms of substrate and stereo-specificity, semiquinone production, activity on peptides, ability to crosslink α-casein, and inhibition by small molecular weight compounds (Publication II). Furthermore, substrate specificities of TrT and AbT were analyzed in more detailed by determining the kinetic constants K_m and V_{max} and UV/Vis product spectra using small molecular weight phenolic substrates (Unpublished results).

Electron spin resonance spectroscopy (ESR) was used to analyze primary reaction products of the tyrosinase catalysis. On the basis of the ESR experiment, all tyrosinases produced identical semiquinone radicals from the substrates, suggesting a similar reaction mechanism. Moreover, all the enzymes oxidized

monophenolic and diphenolic compounds, thus showing typical characteristics of tyrosinase activity. Although considerable differences in the substrate specificity were noted, the tyrosinases in general showed higher activity on diphenols than on monophenols. The highest monophenolase/diphenolase activity ratio for oxidation of Y and L-dopa was determined for PsT, with a ratio of 0.5. Substitution of phenolic compounds significantly influenced the relative oxidation rates by the enzymes. The presence of an amino group in the substrate molecule decreased the oxidation rate of the substrate, especially by TrT and also to some extent by AT and PT. Conversely, oxidation efficiency of PsT and AbT was observed to be retarded when a carboxylic acid was present in the structure of the substrate.

The suitability of the plant and fungal tyrosinases for crosslinking α-casein was examined. In addition, the capability of the enzymes to oxidize Y bound to different locations of a peptide chain was analyzed with di- and tripeptides. Of the tyrosinases studied, PT, TrT, PsT and AbT were able to oxidize all the tested model peptides, whereas AT did not have detectable activity on the peptides (Table 11). The oxidation rate was found to depend on the peptide length and the location of the tyrosyl residue in the peptide. When compared to the amino acid Y, the di- and tripeptides bound Y was oxidized slower by PsT and faster by PT and TrT.

Table 11. Relative activity of the tyrosinases from apple (AT), potato (PT), P. sanguineus (PsT), T. reesei (TrT) and A. bisporus (AbT) on di- and tripeptides, calculated in relation to L-tyrosine (%). (Publication II)

	Enzyme source				
	AT	**PT**	**PsT**	**TrT**	**AbT**
Substrate (2.5mM)	**Activity in relation to L-tyrosine(%)**				
Y[a]	0	100	100	100	100
YG[b]	0	450	30	130	140
GY	0	520	30	290	115
YGG	0	350	40	110	140
GGY	0	430	60	340	110
GYG	0	480	60	230	110

[a] Y equals tyrosine
[b] G equals glycine

The abilities of PT, AT, TrT, PsT and AbT to crosslink the model protein α-casein were observed to be significantly different. Only TrT and PsT were able to directly crosslink α-casein, whereas AT, PT, and AbT were able to polymerize α-casein only in the presence of a small molecular weight phenolic compound L-dopa (Fig. 10). Since AT and PT had rather poor activity on the amino acid Y, the negligible ability of AT and PT to crosslink α-casein was very probably derived from this property. However, PT oxidized much better the peptide-bound Y, when compared to Y only, but still could not act on protein-bound Y. AbT could also oxidize the peptide-bound Y, but was incapable of direct crosslinking of α-casein. By contrast, PsT could crosslink α-casein, although its activity on the model peptides was relatively low.

Among the tyrosinases studied, TrT showed the best ability to crosslink α-casein. Crosslinking of α-casein by TrT was already detectable within a 2 h reaction time, whereas corresponding protein polymerisation by PsT was observed after 24 h. Unexpectedly, the crosslinking efficiency of TrT decreased when L-dopa was added to the reaction mixture, which was opposite to the effect with the other tyrosinases. This result might relate to the property of TrT to oxidize much more efficiently L-dopa than protein-bound Y. The reactive quinones from the L-dopa oxidation by TrT could have conjugated to the nucleophilic sites of amino acid residues, which can participate in crosslinking, with subsequent blocking of polymerisation of α-casein subunits. Furthermore, if TrT favoured L-dopa over Y, the catalysis would primarily lead to melanogenesis via L-dopa and not protein crosslinking via the Y residues.

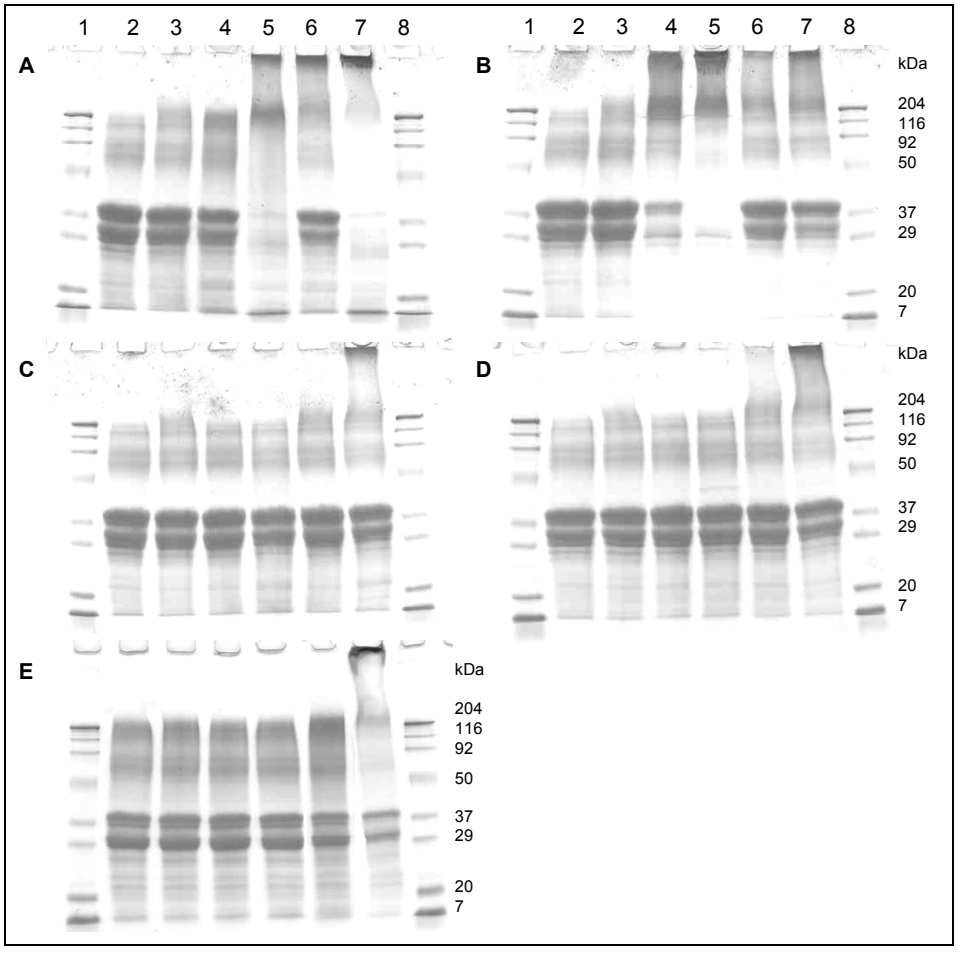

Figure 10. Crosslinking of α-casein proteins by P. sanguineus (gel A) and T. reesei (gel B), apple (gel C), potato (gel D), and A. bisporus (gel E), tyrosinases (TYR) after 24 h incubation. Lanes: 1) Molecular weight marker, 2) α-casein, 3) α-casein + L-Dopa, 4) α-casein + TYR 100 nkat / g protein, 5) α-casein + TYR 1000 nkat / g protein, 6) α-casein + L-Dopa + TYR 100 nkat / g protein, 7) α-casein + L-Dopa + TYR 1000 nkat / g protein, 8) Molecular weight marker. (Publication II)

The reason for the superior protein crosslinking ability of TrT is not obvious, and probably more than one reason lies behind the detected differences between the enzymes. Interestingly, when comparing durations of the lag period, which is characteristic to tyrosinases in the oxidation of monophenols (Cooksey et al.,

1997; Land et al., 2004), TrT was observed to show the shortest lag periods for the monophenols studied. This rapid transformation of TrT to the *oxy*-form, with subsequent readiness for monophenol oxidation could suggest that TrT also had a better ability to oxidize monophenolic protein-bound Y, and thus to crosslink α-casein, when compared to the other tyrosinases. PsT also directly crosslinked casein, but displayed a lag period similar to that of AbT. However, AbT was incapable of direct crosslinking of α-casein. Therefore, in the case of PsT and AbT, there seems to be another explanation besides the lag period for the differences between their crosslinking abilities. Furthermore, AbT and also PT were observed to be able to oxidize peptide-bound Y better than free Y, which is contrary to PsT, which showed a reduced oxidation rate for the peptides. Therefore, the inability of AbT and PT to crosslink α-casein, when compared to PsT, seems rather unexpected.

The differences in the protein crosslinking abilities between the tyrosinases are probably due to the different accessibilities of the peptide-bound Y residues to the active sites of the enzymes. It has been recognized that the active sites of the type 3 copper proteins can be occupied by the amino acid side chains of the copper protein, which may block access of the substrate (Klabunde et al., 1998; Marusek et al., 2006; Matoba et al., 2006; Decker et al., 2007). The entrance to the active site may be covered by certain amino acids, which cover the active site cavity (Marusek et al., 2006; Matoba et al., 2006; Decker et al., 2007). For activation of tyrosinases, the blocking amino acids, i.e. the gate residues, must be removed, and a range of mechanisms have been suggested. For example, the activation of hemocyanins to tyrosinases or catechol oxidases can be performed by a limited proteolysis and/or a conformational shift by detergents (SDS) and other allosteric effectors (i.e. compounds affecting regions other than the active site), such as salts, Zn^{2+} and Mg^{2+} (van Gelder et al., 1997; Espín and Wichers, 1999; Decker and Tuczek, 2000; Jaenicke and Decker, 2003 and 2004; Hristova et al., 2008; Campello et al., 2008; Nillius et al., 2008).

Among the type 3 copper proteins from arthropods and plants, the amino acid residue blocking the access of a substrate to the active site is suggested to be phenylalanine (Klabunde et al., 1998). Correspondingly, the bulky amino acid phenylalanine, which was presumed to act as a gate residue in the studied plant tyrosinases, AT and PT, may have led to blocking of access of the Y residues of α-casein to the active site of the enzymes. In fungal and bacterial tyrosinases the

corresponding gate residue is usually either leucine or proline, which enables more open access of the substrate to the active site cavity (Matoba et al., 2006; Marusek et al., 2006). Interestingly, the amino acid sequence of TrT around the active site cavity, i.e. the gate residue area, was observed to be different from those of the fungal tyrosinases AbT and PsT (Fig. 11), and also from the various other reported tyrosinases (Marusek et al., 2006). For example, the methionine highly conserved in both plant and fungal tyrosinases (Halaouli, et al., 2006a; Marusek et al., 2006) is phenylalanine (Phe283) in TrT, which could partly account for the superior crosslinking property of TrT compared to the other studied tyrosinases.

```
                        Cu-A SITE

PsT     (AAX44240)    IGGIHGLPYVAWSDAGADDPAEP----SGYCTHGSVLFPTWHRP          98
AbPPO1  (CAA59432)    VAGVHGYPLIPFDDAVGPTEFSPFDQWTGYCTHGSTLFPTWHRP          96
AbPPO2  (CAA61562)    LAGIHGLPFTEWAKERPSMNLYK----AGYCTHGQVLFPTWHRT          92
TrT     (CAL90884)    IAGIHGAPYIEYNKAG----AKSGDGWLGYCPHGEDLFISWHRP          116

AppPPO  (AAA69902)    QAAVHCAYCDGAYDQVGFPEL-------ELQIHNSWLFFPFHRY          217
PotPPO  (AAA85122)    QANIHCAYCNGAYIIGGK----------ELQVHNSWLFFPFHRW          213


                        Cu-B SITE

PsT     (AAX44240)    LEAIHDHIHDSVGG--------------GGQMGDPSVAGFDPIFFLHHCQ    289
AbPPO1  (CAA59432)    IEAIHDNIHVLVGG--------------NGHMSDPSVAPFDPIFFLHHAN    288
AbPPO2  (CAA61562)    LESVHDDIHVMVGYGKI-----------EGHMDHPFFAAFDPIFWLHHTN    284
TrT     (CAL90884)    VEQIHNAIHWDGAC--------------GSQFLAPDYSGFDPLFFMHHAQ    302

AppPPO  (AAA69902)    EGTPHAPVHLWTG---DNTQPN------FEDMGNFYSAGRDPIFFAHHSN    363
PotPPO  (AAA85122)    ENIPHIPVHIWAGTVRGSTFPNGDTSY-GEDMGNFYSAGLDPVFYCHHGN    376
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Figure 11. Amino acid sequence alignments of the studied fungal tyrosinases at conserved CuA and CuB sites. T. reesei (TrT) (CAL90884, Publication I); P. sanguineus (PsT) (AAX44240, Halaouli et al., 2005); and A. bisporus (AbPPO1 amd AbPPO2) (CAA59432 and CAA61562, Wichers et al., 2003). Examples of the sequences of polyphenoloxidases from potato Solanum tuberosum (PotPPO, AAA85122) (Thygesen et al., 1995); and apple Malus domestica (AppPPO, AAA69902) (Boss et al., 1995) are also presented. (Publication II)

The size and charges of the substrate binding area of an enzyme also determine the accessibility and orientation of the substrates to the active site of a tyrosinase. The active site of the *Streptomyces* tyrosinase, for example, is recognized to locate at a bottom of concavity as a presumed substrate-binding pocket, formed by hydrophobic amino acid residues, and appears to be rather

large (Matoba et al., 2006). Therefore, differences in the substrate binding area influence the ability of tyrosinases to bind and oxidize protein-bound substrate. Due to the low homology in the amino acid sequences of tyrosinases, the gate and orientating residues can originate from different parts of the enzymes, depending on the protein folding. Hence, resolving tertiary structures of tyrosinases is important for definition of the essential residues for enzymatic activity and their ability to crosslink proteins. Capabilities of the studied tyrosinases to crosslink protein are summarized in Table 12.

Table 12. Summary of the efficiency of crosslinking of α-casein by tyrosinases from apple (AT), potato (PT), P. sanguineus (PsT), T. reesei (TrT) and A. bisporus (AbT) in the presence or absence of L-dopa. Efficacy is shown as (+, ++, +++), (-) and (--) for induction, no effect and reduction of crosslinking, respectively, when compared to the control reaction without enzymes.

	Substrate	
Enzyme	**α-Casein**	**α-Casein in presence of L-dopa**
AT	-	+
PT	-	+
PsT	+	++
AbT	-	++
TrT	+++	--

The substrate specificities of extracellular TrT and intracellular AbT were further examined using various mono- and diphenolic compounds as substrates and spectrophotometric and oxygen consumption measurements as analytical methods (Unpublished results). First, specific wavelengths for the product formation from substrates were determined by monitoring UV/visible absorption spectra of the oxidation products from the particular substrates (Table 13). Unexpectedly, the oxidation reactions of catechol (Fig. 12) and also hydrocaffeic acid by TrT and AbT showed different product patterns between the enzymes. This observation is interesting, because the primary products from the tyrosinase-catalyzed oxidation reactions are thought to be identical. Although no clear explanation for the result can be concluded, it could be hypothesized that there was a difference in the affinity of the enzymes to the quinone-derived

reaction products. Since the reaction products from the non-enzymatic reactions of quinones might also act as substrates for tyrosinases, TrT could have favoured the oxidation of these products over catechol and hydrocaffeic acid. Hence, the primary oxidation product would not have accumulated in the TrT-catalyzed reactions. Conversely, AbT might not have been able to act so efficiently on the secondary products, but mostly oxidized the primary substrate producing the known oxidation product, for example, from catechol a product with absorbtion maximum at 400 nm (Mason, 1949). The amino acids and their surroundings, which assist the coordination of the substrates to the active site of enzyme, might differ between the tyrosinases. For instance, there might be variations in the balance of acidity and alkalinity in the active site of the enzymes. Therefore, different docking of the substrates with varying polarity and acidity could relate to the observed differences between AbT and TrT.

The determined K_m values of AbT were found to be ten times lower than those of TrT (Table 13). Moreover, these two tyrosinases had notable differences in their affinity between the substrates studied. The K_m values for AbT were observed to be the highest for the substrates in which a carboxyl group was present in the structure. Of the diphenols, AbT showed lowest affinity to caffeic and hydrocaffeic acid, and monophenolic *p*-coumaric acid was not oxidized by AbT. By contrast, an amino group in the substrate molecule appeared to decrease the affinity by TrT. This was also notable among the determined K_m values for the tripeptides, since the highest K_m value and lowest catalytic efficacy (k_{cat}) of TrT was on YGG, in which the tyrosyl residue is in the amino-terminal end of the peptide. Furthermore, whereas AbT was not able to oxidize *p*-coumaric acid and showed lowest affinity on caffeic acid, TrT showed the second and the third highest affinity for caffeic acid and *p*-coumaric acid, respectively. However, despite the observed differences in kinetic constants between TrT and AbT, both enzymes showed highest affinity for monophenolic *p*-tyrosol.

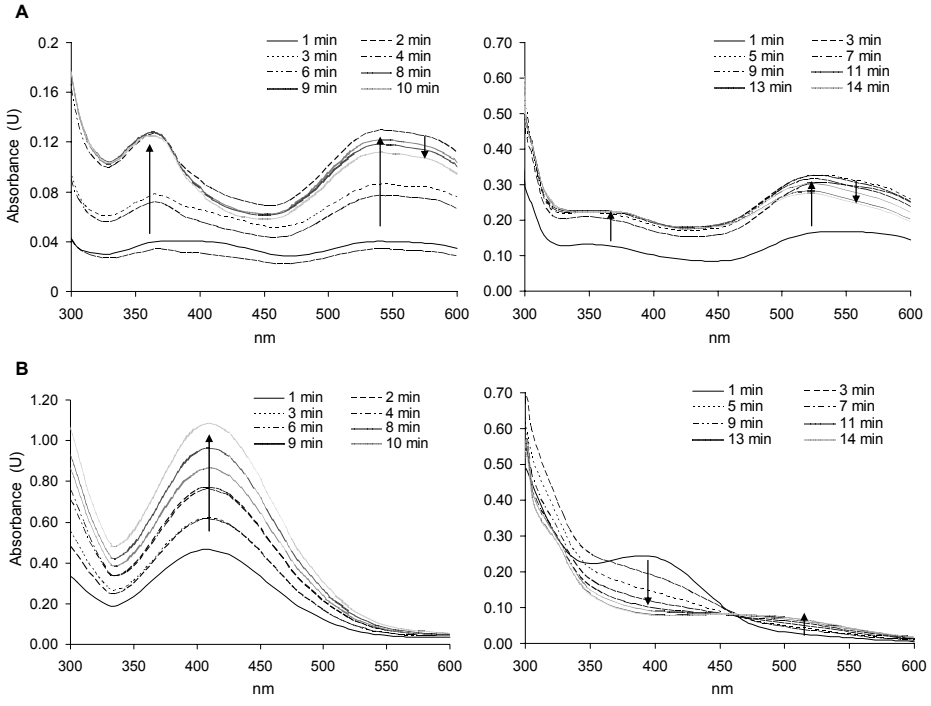

Figure 12. UV/Vis absorption product spectra of the oxidation of catechol (left side) and hydrocaffeic acid (right side) by TrT (A) and AbT (B). Absorption spectra shown as a function of reaction time. (Unpublished results)

When the lag periods for monophenol oxidation by TrT and AbT were followed, durations of the lag periods were longer in the AbT-catalyzed reactions with a few exceptions (Table 13). With AbT the lag increased as a function of substrate concentration in the presence of *p*-tyrosol, L-tyrosine and phenol, but with tyramine and phloretic acid the situation was the reverse. With TrT the lag period was invariably shortened with increasing substrate concentration. This could relate to the high K_m values measured for TrT. The lower substrate concentration was notably below V_{max}, and therefore there was not enough substrate to saturate the active site of TrT. Hence, by increasing the substrate concentration, transformation of the enzyme from *met*-form to the *oxy*-form would be accelerated and the lag period decreased.

Table 13. K_m (mM) and V_{max} (Abs/s) values of tyrosinases from T. reesei (TrT) and A. bisporus (AbT) for mono- and diphenolic compounds, determined by spectrophotometric assay with substrate-specific wavelengths λ (nm). (Unpublished results).

	λ (nm)	K_m (mM)		V_{max} (Abs/s)		Lag (s) with [S] 15mM		Lag with (s) [S] 1mM	
Substrate		**TrT**	**AbT**	**TrT**	**AbT**	**TrT**	**AbT**	**TrT**	**AbT**
Phenol	390	3.8	0.33	0.15	0.24	200	400	500	300
Catechol	400	3.3	0.25	2.3	25				
Catechol	540 [a]	2.5	-	2.3	-				
Tyramine	475	4.5	0.75	0.06	0.26	0	0	400	100–150
Dopamine	475	11	0.84	2	5				
L-Tyrosine	475	-	0.20	0.1 [b]	0.21	0 [c]	200 [c]	0–100 [d]	50–100 [d]
L-Dopa	475	7.5	0.17	3.5	1				
p-Coumaric acid	360	1.5	-	0.7–1	0	100–150	-	300	-
Caffeic acid	480	0.9	1.69	1	5				
Phloretic acid	400	1.4	0.64	0.91	1.29	25–50	0–25	100–150	50–100
Hydrocaffeic acid	400	2.5	0.91	3.3	14				
Hydrocaffeic acid	530 [a]	3.0	-	7–8	-				
Tyrosol	395	0.8	0.06	0.34	0.21	0	200–300	200	100–200

[a] This product was detected only with TrT
[b] This is the reaction rate with a tyrosine concentration of 2.5 mM (not the V_{max} value)
[c] Substrate concentration 2.5 mM
[d] Substrate concentration 0.25 mM

4.3 Crosslinking of proteins and carbohydrates by tyrosinase and laccase (Publication III)

Enzymatic crosslinking can have potential for altering structural or nutritional properties of biopolymers. Hetero-crosslinking of different biopolymers is specifically an attractive tool to tailor the functional properties of the polymeric structures. Therefore, applicability of tyrosinase (TrT) and laccase (ThL) for crosslinking of proteins and carbohydrates substituted with phenolic compounds was examined. The aim was to conduct enzymatic crosslinking between tyrosine side-chains of α-casein and FA (140 µg/ g hOSX) and *p*-CA (100 µg/g hOSX) of enzymatically hydrolyzed oat spelt xylan (hOSX). Reactivity of TrT and ThL on the different substrate mixtures was first studied by measuring consumption of oxygen, after which the reaction products were monitored by SEC combined with fluorescence spectroscopy and by an SDS-PAGE analysis with specific staining methods for proteins and protein-carbohydrate conjugates.

On the basis of the oxygen consumption measurements, it was observed that ThL could oxidize both α-casein and hOSX, thus showing ability to oxidize both protein- and carbohydrate-bound phenolic compounds (Fig. 13). On the other hand, TrT could readily oxidize α-casein, but it was not able to oxidize hOSX (Fig. 11). The inability of TrT to oxidize hOSX was unexpected, as TrT is known to be able to oxidize *p*-CA (Table 10), which is also present in hOSX. It is possible that when *p*-CA is covalently bound to hOSX, the oxidation reaction is hindered due to a steric hindrance in the substrate binding area of TrT. Oxidation of *p*-CA could also be hindered by FA present in hOSX, since FA is a potential substrate analogue of TrT (Cheng et al., 2002). The oxygen consumption rate by ThL significantly increased when FA and *p*-CA were included in the reaction mixture. This result was predictable, since phenolic acids are well-known substrates of laccases. As expected TrT could not oxidize FA (Publications I and II), and although the presence of *p*-CA in the α-casein solution was observed to slightly increase the maximum reaction rate by TrT, a clear lag period prior to the oxidation was observed (Fig. 13). This lag period is proposed to relate to a high affinity of *p*-CA to the active site on tyrosinase, with subsequent temporary retardation of oxidation reaction. Espín et al. (1998) hypothesized that if tyrosinase has high affinity to the monophenolic substrate, the monophenol-inactive *met*-form of tyrosinase becomes saturated by the substrate. Therefore, the transformation from *met*- to *oxy*-form by indirect diphenol oxidation, and thus also the maximum oxidation efficiency of tyrosinase would be delayed.

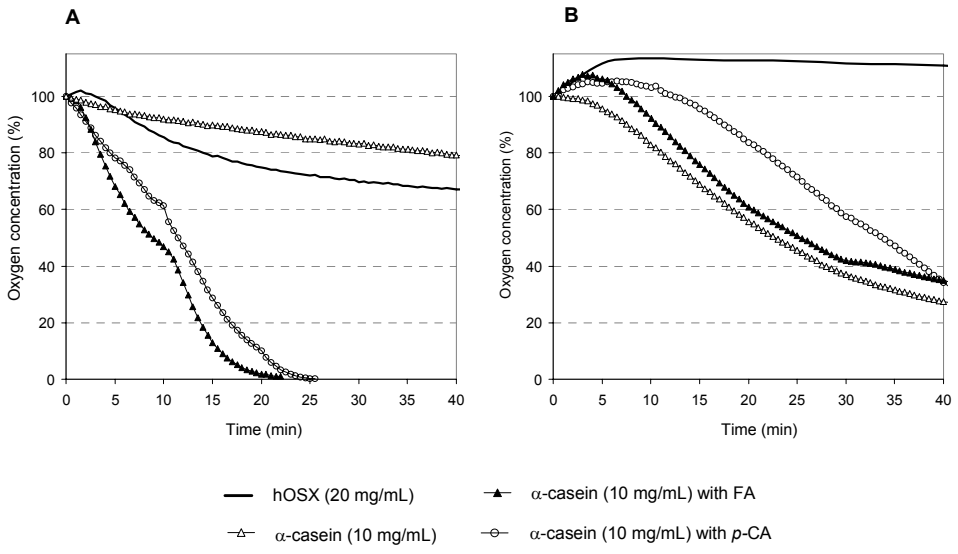

Figure 13. Consumption of oxygen as a function of time by ThL (A) and TrT (B). Reactions in the presence of α-casein, hOSX, α-casein with FA and α-casein with p-CA. FA and p-CA were added at four 10 min intervals to reach a final concentration of 1 mg ml^{-1}. Dosing of enzymes: ThL 15 nkat mg^{-1}, TrT 0.5 nkat mg^{-1}. (Publication III)

The abilities of TrT and ThL to crosslink α-casein with or without FA, *p*-CA and hOSX were monitored by SDS PAGE, SEC and fluorescence spectroscopy. The results obtained corresponded to the oxygen consumption measurements. Compared to ThL, TrT showed much better ability to catalyze direct crosslinking of α-casein (Fig. 14), which most probably resulted from the more efficient oxidation of protein-bound Y by TrT. However, the amount of native Y side chains of α-casein was clearly reduced by both enzymes as analyzed by fluorescence spectroscopy, suggesting that both enzymes could oxidize protein-bound Y.

An addition of FA and *p*-CA to the reaction mixture caused reduction in the TrT-mediated protein crosslinking (Fig. 14). Similarly an addition of diphenolic L-dopa was found to specifically hinder α-casein crosslinking by TrT, whereas enhanced crosslinking was observed by the other tyrosinases studied (Fig. 10). Moreover, Thalmann and Lötzbeyer (2002) reported that lysozyme, α-lactalbumin and β-lactoglobulin proteins were crosslinked by *A. bisporus* tyrosinase most efficiently in the presence of additional low molecular weight phenolic compounds. Therefore, TrT showed unique characteristics in its mechanisms for protein crosslinking. The inhibition of protein crosslinking by the small molecular weight phenolic compounds could be assumed to relate to different affinity properties of the phenolic compounds to the active site of TrT. FA and *p*-CA may have had

higher affinity to the active site of enzyme than the Y residues of α-casein. For example, it was determined that TrT showed higher affinity to *p*-CA (K_m 1.5 mM) than to tri-peptide-bound Y (K_m 3.1–6.0 mM) (Unpublished results). On the other hand, the hydroxyl groups of FA and *p*-CA could also have acted as nucleophiles (Sayre and Nadkarni, 1994), with subsequent grafting of the phenolic acids to the Y-derived quinones in protein, resulting in blocking of crosslinking of α-casein subunits. A similar hypothesis was made concerning the role of L-dopa in the α-casein crosslinking (Publication II).

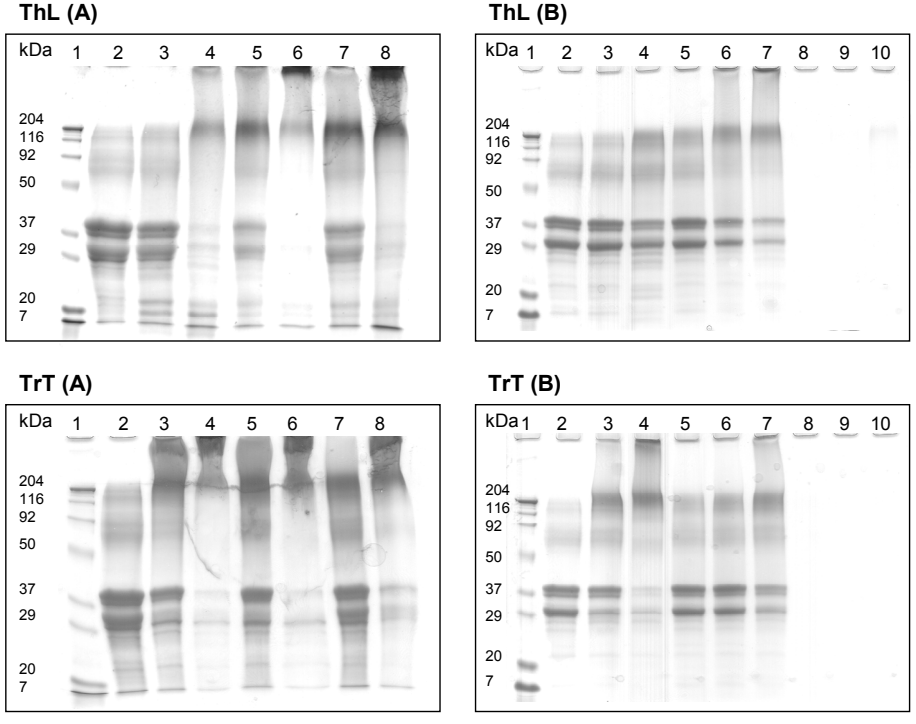

Figure 14. SDS-PAGE gels showing crosslinking of α-casein by ThL and TrT with FA and p-CA (A), and hOSX (B). Lanes, gel (A): 1. Molecular weight marker, 2. α-casein, 3. α-casein + ThL (1.5 nkat) or TrT (0.05 nkat), 4. α-casein + ThL (15 nkat) or TrT (0.5 nkat mg^{-1}), 5. α-casein + FA + ThL (1.5 nkat) or TrT (0.05 nkat), 6. α-casein + FA + ThL (15 nkat) or TrT (0.5 nkat), 7. α-casein + p-CA + ThL (1.5 nkat) or TrT (0.05 nkat), 8. α-casein + p-CA + ThL (15 nkat) or TrT (0.5 nkat). Lanes, gel (B): 1. molecular weight marker; 2. α-casein; 3. α-casein + ThL (1.5 nkat) or TrT (0.05 nkat); 4. α-casein + ThL (15 nkat) or TrT (0.5 nkat); 5. α-casein and hOSX; 6. α-casein and hOSX + ThL (1.5 nkat) or TrT (0.05 nkat); 7. α-casein and hOSX + ThL (15 nkat) or TrT (0.5 nkat); 8. hOSX; 9. hOSX + ThL (1.5 nkat) or TrT (0.05 nkat); 10. hOSX ThL (15 nkat) or TrT (0.5 nkat). Enzyme dosing as nkat per mg α-casein. (Details in Publication III Fig. 3 and 6)

The mechanisms by which *p*-CA inhibited α-casein crosslinking by TrT might also relate to the delayed transformation of the enzyme to the *oxy*-form. Based on the observations of the oxygen consumption assay (Fig. 13), protein-bound Y appeared to accelerate the conversion of the enzyme from the *met*-form to the *oxy*-form, whereas *p*-CA retarded this transformation. The favoured oxidation of *p*-CA could also have hindered the oxidation of the Y residues of protein and led primarily to the *p*-CA-related catalysis instead of polymerization of α-casein, as was proposed for the mode of action of added L-dopa on protein crosslinking (Publication II). The reactive quinones resulting from oxidation of *p*-CA could also have reacted non-enzymatically with the nucleophilic side groups (Bittner, 2006; McDowell et al., 1999; Ito et al., 1984; Burzio, 2000; Takasaki and Kawakishi, 1997) in α-casein, thus blocking the sites for conjugation of α-casein subunits. However, when higher enzyme dosage was used *p*-CA was also found to enhance protein crosslinking (Fig. 14). Hence, it can be hypothesized that *p*-CA also acted as a bridging agent in α-casein crosslinking. In agreement with Thalmann and Lötzbeyer (2002), it could be suggested that *p*-CA was first conjugated to protein, after which the compound was again available for enzymatic oxidation and was capable of crosslinking of protein subunits.

In the case of ThL, the presence of FA and *p*-CA enhanced significantly the crosslinking of α-casein (Fig. 14). Based on the fluorescence spectroscopy measurements with the $\lambda_{ex}/\lambda_{em}$ parameters specific for Y, FA and *p*-CA, a decrease in the amount of Y residues of α-casein and grafting of FA and *p*-CA to α-casein by ThL was observed. This suggests that the phenolic acids worked as bridging agents through the Y residues of α-casein, leading to the protein polymerization. Previously, Mattinen et al. (2005) have shown that the ThL-catalyzed reactions with FA and the tripeptide Gly-Leu-Tyr can lead to the formation of FA-Y conjugates, further supporting the theory of hetero-conjugation of α-casein and phenolic acids through the Y side chains of protein. However, due to the complexity of radical-mediated reactions and taking into account the reports that ThL is also able to oxidize other amino acids than Y (Mattinen et al., 2006), conjugation of the phenolic acids to other side chains of α-casein than Y cannot be excluded.

Indications of hetero-crosslink formation between α-casein and hOSX by both oxidative enzymes could be detected as visualized by the glycoprotein specific staining method in the SDS PAGE analysis (Publication III, Fig. 6). Comparable observations referring to the hetero-conjugation of α-casein and hOSX were also made using the SEC analysis, since a distinctive product, which was present only in the enzyme-treated mixtures of hOSX and α-casein, was detected in the chromatogram (Publication III, Fig. 1). ThL could catalyze the formation of α-casein-hOSX conjugates more efficiently than TrT, and

although hetero-crosslink formation by TrT was detected, blocking of α-casein crosslinking in the presence of hOSX was also observed in the TrT-catalyzed reactions (Fig. 14). The mechanism by which the hetero-conjugation took place in the TrT-mediated crosslinking reactions is not clear. One possibility is that the phenolic acids of hOSX functioned as nucleophiles and coupled to the Y-derived quinones of α-casein, resulting in the hetero-conjugation. In contrast to TrT, and similarly to the impacts of FA and *p*-CA, the presence of hOSX clearly enhanced protein crosslinking by ThL (Fig. 14). Presumably the α-casein-hOSX crosslinking by ThL could take place via a similar radical-mediated mechanism to that proposed for the crosslinking reactions of α-casein in presence of FA and *p*-CA. Phenolic acids of hOSX could have coupled to the Y residues of α-casein and also acted as bridging agents for protein crosslinking. A number of possible covalent linkages between the phenolic acids and biopolymers formed by TrT and ThL are presented in Fig 15. Furthermore, the capability of TrT and ThL to crosslink biopolymers in the presence or absence of additional phenolic compounds is summarized in Table 14.

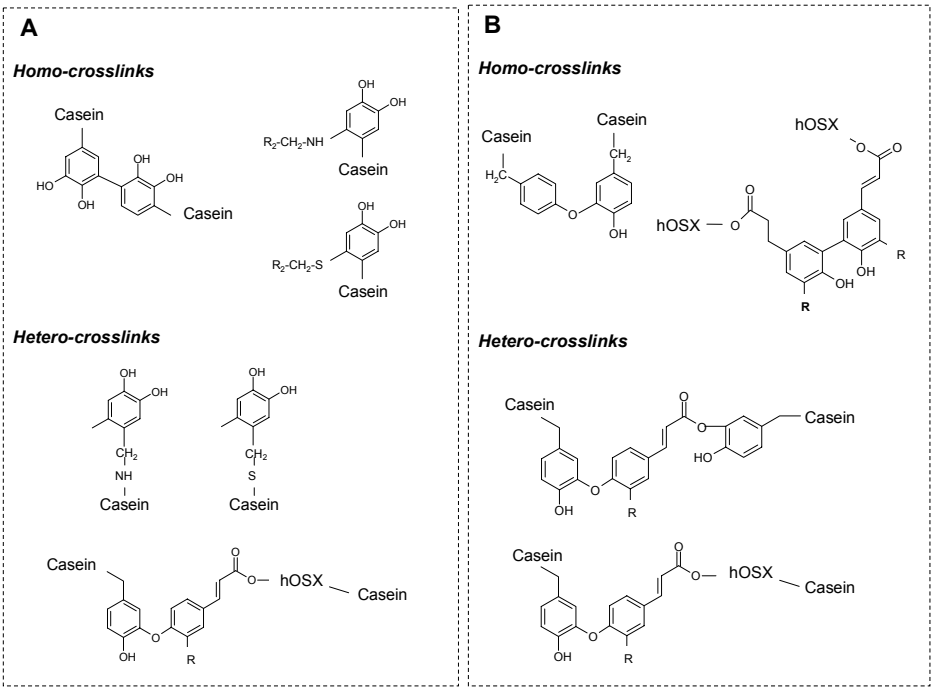

Figure 15. Schematic presentation of possible crosslinks formed in α-casein (homo-crosslinks), between α-casein and p-CA or FA (hetero-crosslinks), and between α-casein and hOSX (hetero-crosslinks) by TrT (A) and ThL (B). R in the aromatic ring structure signifies hydrogen or a methoxy group (Publication III).

Table 14. Efficiency of crosslinking of α-casein and hOSX by tyrosinase (TrT) and laccase (ThL) with or without additional phenolic compounds. Efficacy shown as (+, ++, +++) for induction or (-, --) for reduction of crosslinking, when compared to the control reaction without enzymes.

	Crosslinking of biopolymers			**Efficacy of crosslinking of α-caseinthe presence of additional compounds**		
Enzyme	α-Casein	hOSX	α-Casein-hOSX	p-CA	FA	hOSX
TrT	+++	-	+	-/+ [a]	-	--
ThL	+	++	++	++	+++	++

[a] based on the high or low enzyme dosage, either induction or reduction of crosslinking, respectively.

4.4 Impact of tyrosinase and laccase on wheat dough and bread (Publications IV and V)

Effects of ThL and xylanases from *T. lanuginosus* (TlX) and *B. subtilis* (BsX) on white wheat flour and wheat gluten dough were investigated. The impact of ThL on the structure formation of wheat flour dough and bread was further compared to that of TrT. Effects of ThL and TrT were examined using isolated gluten proteins for crosslinking tests, and performing dough- and bread-making experiments. The effects of TrT and ThL on dough and bread were also studied in combination with xylanase TlX. SDS PAGE was used for monitoring the changes in the model proteins, and dough rheology was studied by the Kieffer dough and gluten extensibility rig. The Kieffer analysis determines the extensibility (E_x) of dough and the resistance to stretching (R_{max}), and enables detection of the breakdown and formation of covalent bonds in dough (Kieffer et al., 1998).

4.4.1 Impact of ThL on rheology of wheat flour and gluten dough

A ThL addition during mixing of wheat flour and gluten dough resulted in hardening of dough as a function of ThL dosage (5–50 nkat/g flour), observed as an increase in R_{max} and a decrease in E_x at R_{max} of dough (Fig. 16). The effect of

ThL was greater in wheat flour than in gluten dough. This is most probably due to the efficient crosslinking of arabinoxylans (AX) of wheat flour by the ThL. Laccase-mediated crosslinking of AX through coupling of FA residues has previously been reported by Figueroa-Espinoza and Rouau (1998) and Figueroa-Espinoza et al. (1998, 1999a and 1999b). Interestingly, when isolated gluten was treated with ThL, hardening of gluten dough was also observed (Publication IV, Fig. 6). It is proposed that ThL could affect the gluten structure formation, first by producing FA-derived radicals in the AX present in the crude gluten, but also by acting directly on the gluten proteins, with subsequent protein crosslinking. Since the content of AX in gluten was relatively low, the hardening of gluten network by ThL might have resulted partly from crosslinking of the gluten proteins. When the influence of ThL was studied in combination with xylanase, the effect of the laccase appeared to dominate at low xylanase dosage. However, when xylanase was added at a high concentration, the hardening effect of ThL on dough was decreased. The softening of dough by xylanase is expected to be a consequence of the redistribution of water from AX to gluten and also of the removal of the physical barrier of gluten formation, as proposed by Courtin and Delcour (2001 and 2002) and Frederix et al. (2003). Based on the observations that the impact of ThL was weaker in gluten than in flour doughs, and that with a high xylanase dosage the effect of ThL was clearly reduced, it could be concluded that the hardening of ThL-treated wheat flour doughs was mainly due to the crosslinking of AX rather than the gluten proteins.

When the dosage of ThL was increased to 100 and 200 nkat/g flour, the strength of wheat flour dough significantly decreased (Fig. 16), and a typical viscoelastic dough structure was not formed. This could be due to uneven distribution of water in dough or to disturbance of disulphide bridge formation in gluten by substantial action of ThL (Rouau et al., 1994; Wang, 2003; Létang et al., 1999). It was also observed that the laccase-treated flour doughs became softer in prolonged incubations (Fig. 16). This effect was even strengthened as a function of ThL dosage. The softening phenomenon was presumably related to the laccase-mediated fragmentation of the crosslinked AX network, as also proposed by Carvajal-Millan et al. (2005a). Carvajal-Millan et al. (2005a) concluded that the degradation of AX and the subsequent weakening of the AX gel proceeded through a laccase-mediated radical mechanism. Hence, the secondary reactions of FA-derived radicals could have resulted in breaking of the covalent linkages in AX.

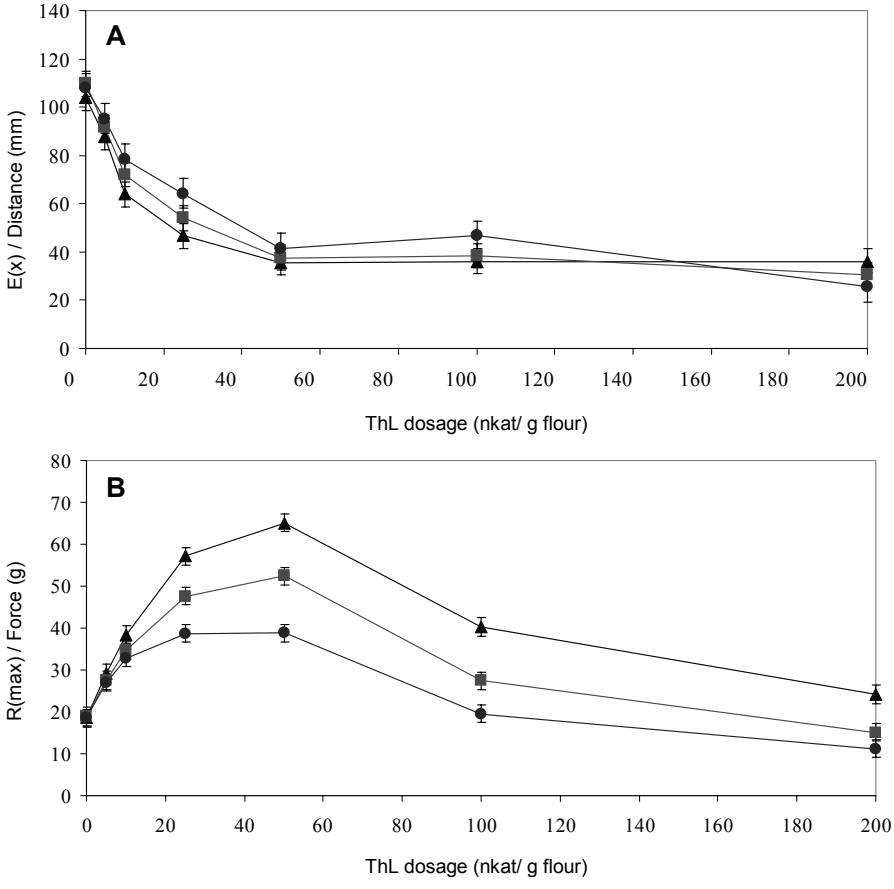

Figure 16. Effect of ThL as a function of dosage on the distance E_x at R_{max} (B) and the force R_{max} (A) parameter of wheat flour dough with different dough resting times: 15 min (▲), 30 min (■) and 45 min (●). (Publication IV)

4.4.2 Enzyme-aided wheat breadmaking

The influence of ThL on the structure formation of wheat flour dough and also on the bread was compared to that of TrT. When the ability of ThL and TrT to crosslink gliadin and glutenin proteins was examined by an SDS-PAGE analysis, TrT was observed to crosslink gliadin proteins effectively, leading to the formation of high molecular weight protein structures (Publication V, Fig. 2). ThL was also found to crosslink gliadins, but only when a high enzyme dosage and prolonged incubation were used. This is the first report to show that wheat gluten proteins can be crosslinked by laccase.

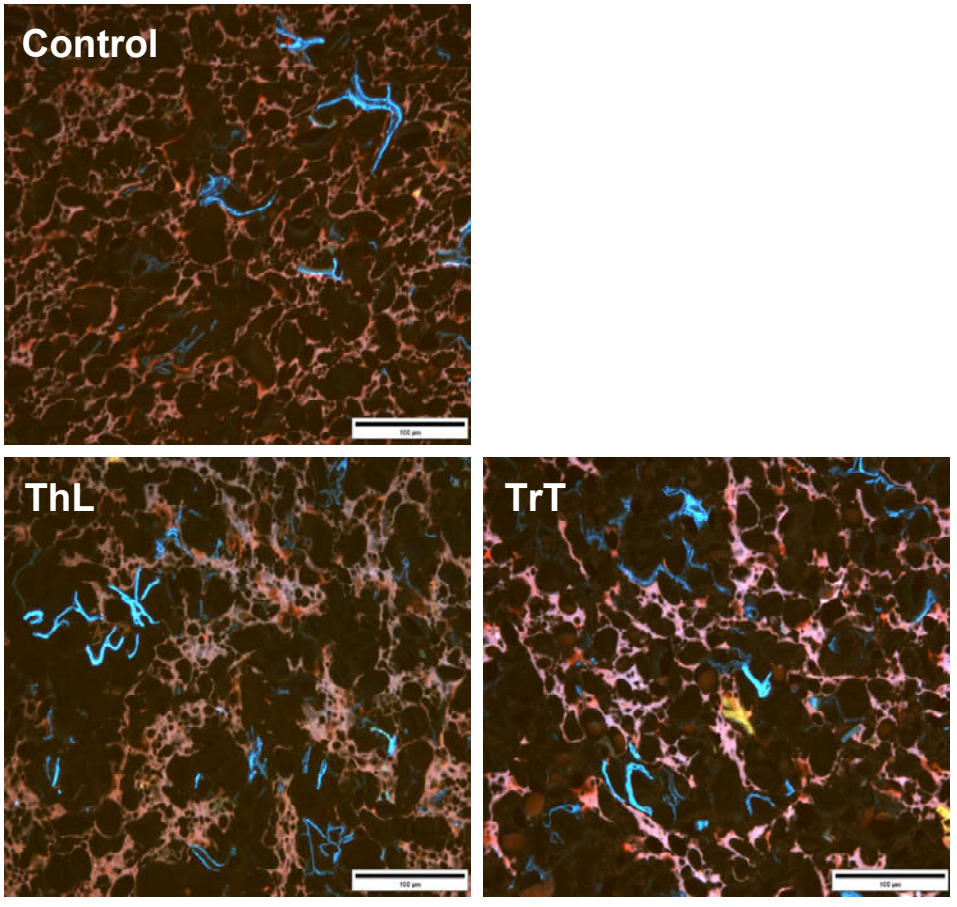

Figure 17. Microscopy of doughs treated with ThL and TrT. Scale bar 100 µm. Staining with 0.1% Acid Fuchsine and 0.01% Calcoflour: cell walls dyed blue and protein orange and/or light gray. Treatments: no enzyme (A), ThL, 30 nkat/g of flour (B), TrTYR2, 30 nkat/g of flour (C).

Examination of large deformation rheology of dough showed that in addition to ThL, TrT also made doughs stiffer and less extensible (Publication V, Fig. 3). The effect increased as a function of enzyme dosage. Whereas the effect of ThL was assumed to be based mainly on the crosslinking of AX via the FA moieties, TrT is believed to strengthen the gluten network and dough through crosslinking of the gluten proteins, as shown by both the SDS PAGE analysis (Publication V, Fig. 2) and by microscopy of doughs (Fig. 17). The proposal is consistent with the results obtained from the combined treatments with TrT and TlX, in which the impact of TlX was clearly weaker than in the combined treatments with ThL

and TlX (Publication V, Fig. 3). Hence, if TrT acted primarily on the gluten proteins, which are the major elements in dough structure formation, hydrolysis of AX by the xylanase would not be so visibly detectable in the rheological properties of the TrT-treated dough. Interestingly, when the laccase-treated doughs were examined at the microscopic level (Fig. 17), the gluten proteins of dough clearly seemed also to be modified by ThL. It is assumed that the crosslinked AX could have acted as a matrix reinforcer of wheat flour dough by increasing the effective concentration of gluten in the ThL-treated dough. Corresponding strengthening of polymeric structures was also suggested by Parovuori et al. (1997) after examining the gel structures of mixtures of amylose and amylopectin.

The results obtained in this work indicate that ThL and TrT may influence the dough matrix through several different mechanisms. First, in addition to crosslinking of AX, formation of FA-derived radicals by laccase is reported to accelerate oxidation of thiol groups of gluten proteins (Labat et al., 2000). Hence, the radicals of AX-bound FA formed by ThL could possibly have disturbed the so-called natural kinetics of disulphide formation in gluten polymers, by increasing the oxidation rate of thiol groups, and subsequent abnormal gluten and dough structure formation. ThL could also have oxidized tyrosine residues of gluten, assisting thereby in the protein aggregation. Furthermore, as ThL was observed to catalyze hetero-conjugation of proteins and carbohydrates (Publication III), corresponding hetero-crosslinking reactions might also have occurred in the wheat dough matrix. In the case of TrT, it is assumed that the impact was mainly due to protein crosslinking. However, TrT could also have influenced the dough via other mechanisms. For example, as TrT is able to oxidize *p*-CA and caffeic acid (Publication V, Table 1) which are both present in wheat flour, coupling of the phenolic moieties to the TrT-oxidized protein might also have occurred.

In the breadmaking studies, both ThL and TrT were found to soften bread crumb structure and to increase bread volume (Fig. 18 and 19). The highest increases in volume and softness of bread crumb were achieved in combination with xylanase. The improvements in the textural properties of bread are proposed to relate to intensification and facilitation of the development of the gluten network by the enzymes. Reinforcement of gluten matrix by ThL and TrT appeared to be caused by crosslinking of AX and proteins, respectively. TlX, on the other hand, solubilised and hydrolyzed AX, subsequently improving the gluten structure formation.

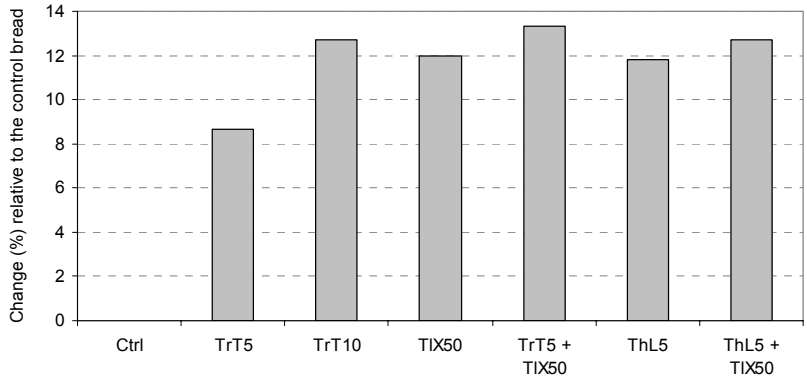

Figure 18. Effect of laccase (ThL), tyrosinase (TrT), and xylanase (TlX) on the specific volume of wheat bread. Enzyme dosages (nkat) are per g flour. Results are shown as a change (%) compared to the control bread.

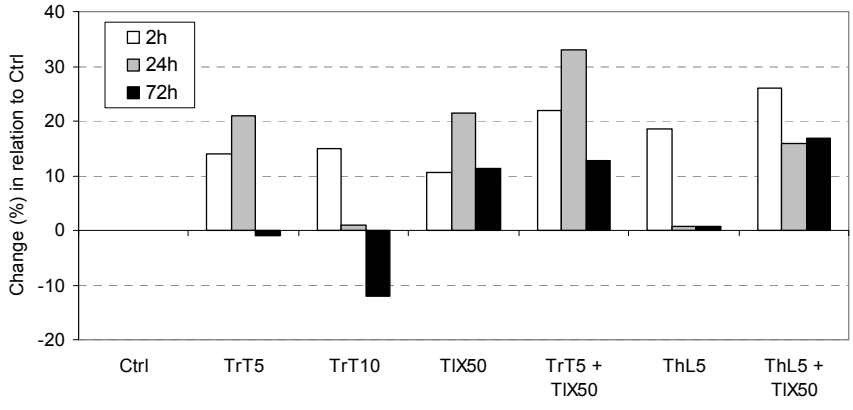

Figure 19. Effect of laccase (ThL), tyrosinase (TrT), and xylanase (TlX) on softness of the bread crumb, measured 2 h, 24 h and 72 h after baking. Enzyme dosages (nkat) are per g flour. Results are shown as a change (%) compared to the control bread.

Interestingly, the pore-size of bread crumbs of 20g-scale made with TrT turned out to be remarkably larger and more irregular when compared to the other breads. Normally this kind of large and irregular pore-size of bread crumb is detected when the formation of the gluten network is disturbed, for example if water-insoluble cell wall material is included in flour (Gan, et al., 1995; Laurikainen et al., 1998). When interpreting the result, the impact of TrT on

bread can be compared to that of transglutaminase, which has also been reported to crosslink the gluten proteins of wheat flour. Transglutaminase is reported to cause a firm bread crumb with a small and regular pore size (Autio et al., 2005), which is opposite to the influence of TrT. Transglutaminase catalyzes the formation of only inter- or intramolecular ε-(γ-glutamyl) lysine isopeptide bonds in proteins. On the other hand, the quinones derived from oxidation of phenolic moieties by tyrosinase can couple to lysyl, tyrosyl, cysteinyl and histidinyl moieties, leading, for example to formation of di-tyrosine, tyrosine-cysteine and tyrosine-lysine cross-links in proteins (Ito and Prota, 1976, Ito et al., 1984; McDowell et al., 1999; Land et al., 2004; Bittner, 2006). In particular, formation of tyrosine-cysteine linkages by TrT could have disturbed the gluten structure formation by hindering the intra- and inter-chain formation of disulphide linkages in the gluten proteins. Hence, the ability of gluten network to retain gas may have been altered, which could partly explain the irregular and uneven pore size of breads caused by TrT. The irregular and large pore size of breads of 20g-scale was not detectable by ThL-treatments, which further indicates clear differences in the crosslinking mechanisms of ThL and TrT.

AX appeared also to have a role in the formation of irregular pore size by TrT, since an addition of TlX in combination with TrT made the pore-size of bread crumb more regular and smaller. It has been reported that xylanase could compensate for the lower gluten aggregation caused by glutenin-associated AX that sterically hinder gluten protein network formation in dough (Gruppen et al., 1993, Rouau et al*.,* 1994; Wang, 2003). It has been hypothesized that AX can affect gluten indirectly by changing the water distribution in dough, but also by having a direct interaction with gluten (Gruppen et al., 1993, Rouau et al*.,* 1994; Wang, 2003). Xylanase-catalyzed hydrolysis of the gluten-associated AX could also have caused improved gluten aggregation and bread structure formation. Hence, if TrT caused hetero-crosslinking of gluten proteins to AX, hydrolysis of AX by TlX might have suppressed the impact of the hetero-crosslinking on bread texture characteristics. The observed changes in the pore-size of bread crumb caused by TlX might also relate to redistribution of water from the hydrolyzed AX to gluten.

Although the mechanisms of action of ThL and TrT in wheat flour dough were different, the influence of the enzymes on the structural properties of the wheat flour dough and bread appeared to be rather similar. As both ThL and TrT were

active in the white wheat flour and improved the textural quality of the breads, the enzymes showed potential for use as process aids in the cereal food industry. The impacts of ThL and TrT on wheat flour dough and bread are summarized in Table 15.

Table 15. Summary of impacts of tyrosinase (TrT) and laccase (ThL) on wheat flour dough and bread.

Enzyme	**Main substrate in wheat flour**	**Other potential substrates in wheat flour**	**Effect on dough structure**	**Effect on bread structure**
TrT	Y residues of wheat proteins	Phenolic acids, such as *p*-CA and caffeic acid	Increased strength and decreased extensibility	Increased volume and softness of fresh bread crumb. Irregular pore size of the crumb
ThL	AX-bound FA	Phenolic acids, such as *p*-CA and caffeic acid, and Y residues of wheat proteins	Increased strength and decreased extensibility. Softening as a function of dough resting time	Increased volume and softness of fresh bread crumb. Impacts reduced by xylanase

5. Conclusions

The availability of microbial and plant tyrosinases in sufficient amounts has hampered testing and use of the enzymes in applications. The unavailability of tyrosinases has mostly been due to difficulties in the production and purification of the enzyme. An extracellular tyrosinase from the filamentous fungus *T. reesei* (TrT) was produced, purified and characterized in this work. The enzyme was the first secreted tyrosinase of fungal origin characterized at the protein level. The high production level and the relatively simple purification procedure of TrT allowed detailed characterization of the enzyme and testing in applications. TrT showed a pH optimum in the alkaline pH range and had a pI value around 9.5, which was rather uncharacteristic compared to the reported fungal tyrosinases. The enzyme was able to oxidize a variety of mono- and diphenolic compounds, including the characteristic substrates of tyrosinase, Y and L-dopa.

TrT was observed to have distinctive substrate specificity when compared with tyrosinases of fungal and plant origin. Generally, substitution of substrate molecules significantly influenced the ability of the tyrosinases to oxidize small molecular weight mono- and di-phenolic compounds and di and tri-peptides containing Y. The enzymes also differed in their capability of protein crosslinking. TrT showed the best ability for direct polymerization of α-casein protein, indicating that it also had the highest potential for oxidation of protein-bound Y. The other tyrosinases crosslinked α-casein mainly in the presence of a small molecular weight phenolic compound that presumably acted as a bridging agent between the protein subunits. There is no definite explanation for the observed differences between the tyrosinases. However, it could be hypothesized that the different cellular locations and roles of the tyrosinases in nature could be related to the detected differences in the biochemical characteristics, especially the substrate specificities and protein crosslinking abilities of the enzymes. TrT is a secreted enzyme, whereas the other studied tyrosinases are intracellular. Therefore, the enzymes most probably have different functions in their hosts. In order to investigate the phenomena behind the observed differences between the tyrosinases, tertiary structures of the enzymes are required.

TrT was noted to be more efficient in crosslinking cereal and milk proteins than a laccase ThL. On the other hand, ThL was more efficient in catalyzing homo-

crosslinking of arabinoxylan and also hetero-conjugation of xylan (hOSX) to α-casein. Moreover, small molecular weight phenolic acids were found to induce and inhibit the α-casein crosslinking ability of ThL and TrT, respectively. TrT and ThL also contributed to the structure formation of wheat flour dough and bread through different mechanisms of crosslinking. Gluten proteins and arabinoxylans of wheat flour were concluded to act as primary substrates for TrT and ThL, respectively, with subsequent crosslinking of the biopolymers. However, the impacts of TrT and ThL on the textural properties of wheat dough and bread were relatively similar. Both enzymes increased the dough strength and improved the baking quality of white wheat flour breads, especially when used in combination with xylanase.

This work elucidated that the novel tyrosinase from *T. reesei* had an exceptional ability to crosslink food proteins compared to the other studied tyrosinases and laccase. Moreover, hetero-conjugation of carbohydrates to proteins by tyrosinase and laccase was conducted. The enzymes showed potential for application in the food industry, as the enzymatic modification of food biopolymers through covalent crosslinking enables altering the technological properties of the biopolymeric structures and introducing specific functions to the matrices. In particular, although health-promoting foods, i.e. low-calorie, low-fat, low-additive products, are becoming more important in the food market, consumers still make purchase decisions based mainly on the palatability of the product, not its healthiness. Hence, the challenge in the food industry is to make healthy food products with excellent sensory properties. It was shown in this work that food biopolymer crosslinking and grafting of specific substances to the biopolymers via tyrosinase- and laccase-catalyzed oxidation reactions can be exploited for improving the textural characteristics of macromolecular structures.

References

Abadulla, E., Tzanov, T., Costa, S., Robra, K.H., Cavaco-Paulo, A., Gübitz, G. (2000) Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 66:3357–3362.

Åberg, C.M., Chen, T., Olumide, A., Raghavan, S.R., Payne, G.F. (2004) Enzymatic grafting of peptides from casein hydrolysate to chitosan. Potential for value-added by-products from food-processing wastes. J Agric Food Chem 52:788–793.

Adrian, M., Rajaei, H., Jeandet, P., Veneau, J., Bessis, R. (1998) Resveratrol oxidation in *Botrytis cinerea conidia*. Phytopathology 88:472–476.

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 Membrane Sci 282:311–321.

Allan, A.C., Walker, J.R. (1988) The selective inhibition of catechol oxidases by salicylhydroxamic acid. Phytochemistry 27:3075–3076.

Anderson, J.V., Morris, C.F. (2003) Purification and analysis of wheat grain polyphenol oxidase protein. Cereal Chemistry 80:135–143.

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 127:508–519.

Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R., Kramer, K.J. (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc Nat Acad Sci 102:11337–11342.

Arnon, D. (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–8.

Aspan, A., Huang, T., Cerenius, L., Soöderhåll, K. (1995) cDNA cloning of prophenol oxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. Proc Natl Acad Sci 92:939–943.

Autio, K., Kruus, K., Knaapila, A., Gerber, N., Flander, L., Buchert, J. (2005) Kinetics of transglutaminase-included cross-linking of wheat proteins in dough. J Agric Food Chem 53:1039–1045.

Bailey, M.J., Biely, P., Poutanen, K. (1992) Interlaboratory testing methods for assay of xylanase activity. J Biotechnol 23:257–270.

Balakshin, M.Y., Evtuguin, D.V., Pascoal Neto, C., Cavaco-Paulo, A. (2001) Polyoxometalates as mediators in the laccase catalyzed delignification. J Mol Catal B Enzym 16:131–140.

Baldrian, P. (2006) Fungal laccases – occurrence and properties. FEMS Microbiol Rev 30(2):215–242.

Bao, W., O'Malley, D.M., Whetten, R., Sederoff, R.R. (1993) A laccase associated with lignification in Loblolly pine xylem. Science 260:672–674.

Baron, C.P., Borresen, T., Jacobsen, C. (2007) Comparison of methods to reduce dioxin and polychlorinated biphenyls contents in fishmeal: extraction and enzymatic treatments. J Agric Food Chem 55(4):1620–1626.

Basnar, B., Xu, J.P., Li, D., Willner, I. (2007) Encoded and enzyme-activated nanolithography of gold and magnetic nanoparticles on silicon. Langmuir 23:2293–2296.

Belitz, H.D., Grosch, W. (1999) Muscle tissue: composition and function. In: Belitz, H.D., Grosch, W. (eds.) Food Chemistry. 2. ed. SpringerVerlag, Berlin Heidelberg, Germany. Pp. 532–550.

Bento, I., Arménia Carrondo, M., Lindley, P.F. (2006) Reduction of dioxygen by enzymes containing copper. J Biol Inorg Chem 11:539–547.

Bertazzo, A., Costa, C.V.L., Allegri, G., Favretto, D., Traldi, P. (1999a) Application of matrix-assisted laser desorption/ionization Mass Spectrometry to the detection of melanins formed from dopa and dopamine. J Mass Spectrom 34:922–929.

Bertazzo, A., Costa, C.V.L., Allegri, G., Schiavolin, M., Favretto, D., Traldi, P. (1999b) Enzymatic oligomerization of tyrosine by tyrosinase and peroxidase studied by matrix-assisted laser desorption/ionozation Mass Spectrometry. Rapid Commun Mass Spectrom 13:542–547.

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 41:7325–7333.

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 21:1511–1530.

Bittner, S. (2006) When quinones meet amino acids: chemical, physical and biological consequences. Amino acids 30:205–224.

Boss, P.K., Gardner, R.C., Janssen, B.J., Ross, G.S. (1995) An apple polyphenol oxidase cDNA is up-regulated in wounded tissues. Plant Mol Biol 27:429–433.

Boeriu, C.G., Oudgenoeg, G., Spekking, W.T., Berendsen, L.B., Vancon, L., Boumans, H., Gruppen, H., van Berkel, W.J., Laane, C., Voragen, A.G. (2004) Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with beta-casein. J Agric Food Chem 52:6633–6639.

Bourbonnais, R.E., 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. Microbiol. Biotechnol. 36:823–827.

Bourbonnais, R., Paice, M.G. (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102.

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 63(12):4627–4632.

Buchert, J., Selinheimo, E., Kruus, K., Mattinen, M.-L., Lantto, R., Autio, K. (2007) Cross-linking enzymes in food processing. In: Rastall, R. (ed). Novel enzyme technology for food applications. Woodhead Publishing Ltd, Cambridge. 336 p.

Burzio, L.A. (2000) Cross-linking in adhesive quinoproteins: studies with model decapeptides. Biochemistry 39:11147–11153.

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 54:4371–4377.

Call, H.P., Mücke, I. (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator systems (LignozymR process). J Biotechnol 53:163–202.

Camarero, S., Ibarra, D., Martínez, A.T., Romero, J., Gutiérrez, A., del Río, J.C. (2007) Paper pulp delignification using laccase and natural mediators. Enzyme Microb Technol 40:1264–1271.

Campello, S., Beltramini, M., Giordano, G., Di Muro, P., Marino, S.M., Bubacco, L. (2008) Role of the tertiary structure in the diphenol oxidase activity of *Octopus vulgaris* hemocyanin. Archives Biochem Biophys 471:159–167.

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 56:1398–1407.

Carvajal-Millan, E., Guigliarelli, B., Belle, V., Rouau, X., Micard, V. (2005a) Storage stability of laccase induced arabinoxylan gels. Carbohydr Polym 59:181–188.

Carvajal-Millan, E., Landillon, V., Morel, M.-H., Rouau, X., Doublier, J.-L., Micard, V. (2005b) Arabinoxylan gels: impact of the feruloylation degree on their structure and properties. Biomacromolecules 6:309–317.

Chao, A.-C., Shyu, S.-S., Lin, Y.-C., Mi, F.-L. (2004) Enzymatic grafting of carboxyl groups on to chitosan – to confer on chitosan the property of a cationic dye adsorbent. Bioresour Technol 91:157–162.

Chefetz, B., Chen, Y., Hadar, Y. (1998) Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl Environ Microbiol 64:3175–3179.

Chen, T., Embree, H.D., Brown, E.M., Taylor, M.M., Payne, G.F. (2003a) Enzyme-catalyzed gel-formation of gelatin and chitosan. Potential for in situ applications. Biomaterials 24:2831–2841.

Chen, T., Small, D.A., Wu, L.-Q., Rubloff, G.W., Ghodssi, R., Vazquez-Duhalt, R., Bentley, W.E., Payne, G.F. (2003b) Nature-inspired creation of protein-polysaccharide conjugate and its subsequent assembly onto a patterned surface. Langmuir 19:9382–9386.

Chen, Y.-T., Stockert, E., Tsang, S., Coplna, K.A., Old, L.J. (1995) Immunophenotyping of melanomas for tyrosinase: implications for vaccine development. Proc Nat Acad Sci USA 92:8125–8129.

Cheng, T., Wenyuan, Z., Yan, L. (2002) Aloin, cinnamic acid and sophorcarpidine are potent inhibitors of tyrosinase. Chin Med J 115:1859–1862.

Chung, J.-E., Kurisawa, M., Ujama, H., Kobayashi, S. (2003) Enzymatic synthesis and antioxidant property of gelatine-catechin conjugates. Biotechnol Lett 25:1993–1997.

Claus, H. (2003) Laccases and their occurrence in prokaryotes. Arch Microbiol 179:145–150.

Claus, H., Decker, H. (2006) Bacterial tyrosinases. System Appl Microbiol 29:3–14.

Collins, P.J., Kotterman, M.J.J., Field, J.A., Dobson, A.D.W. (1996) Oxidation of anthracene and benzo[a]pyrene by laccases from *Trametes versicolor*. Appl Environ Microbiol 62:4563–4567.

Conrad, L.S., Sponholz, W.R., Berker, O. (2000) Treatment of cork with a phenol oxidizing enzyme. US Patent 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 272:26226–26235.

Courtin, C.M., Delcour, J.A. (2002) Arabinoxylans and endoxylanases in wheat flour bread-making, J Cereal Sci 35:225–243.

Courtin, C.M., Delcour, J.A. (2001) Relative activity of endoxylanases towards water-extractable and water-unextractable arabinoxylan. J Cereal Sci 33:301–312.

Cuff, M.E., Miller, K.I., van Holde, K.E., Hendrickson, W.A. (1998) Crystal structure of a functional unit from *Octopus dofleini* hemocyanin. J Mol Biol 278:855–870.

Dawley, R.M., Flurkey, W.H. (1993) Differentiation of tyrosinase and laccase using 4-hexylresorcinol, a tyrosinase inhibitor. Phytochemistry 33:281–284.

Decker, H., Tuczek, F. (2000) Tyrosinase/catecholoxidase activity of hemocyanins: structural basis and molecular mechanism. Trends Biochem Sci 25:392.

Decker, H., Schweikardt, T., Nillius, D., Jaenicke, E., Tuczek, F. (2007) Similar enzyme activation and catalysis in hemocyanins and tyrosinases. Gene 398:183–191.

Decker, H., Schweikardt, T., Tuczek, F. (2006) The first crystal structure of tyrosinase: all questions answered? Angew Chem Int Ed 45:4546–4550.

Della-Cioppa, G., Garger, S.J., Holtz, R.B., McCulloch, M.J., Sverlow, G.G. (1998a) Method for making stable extracellular tyrosinase and synthesis of polyphenolic polymers therefrom. US Patent 5801047.

Della-Cioppa, G., Garger, S.J., Sverlow, G.G., Turpen, T.H., Grill, L.K., Chedekal, M.R. (1998b) Melanin production by Streptomyces. US Patent 5814495.

del Marmol, V., Beermann, F. (1996) Tyrosinase and related proteins in mammalian pigmentation. FEBS Letters 381:165–168.

De Marco, A., Roubelakis-Angelakis, K.A. (1997) Laccase activity could contribute to cell-wall reconstitution in regenerating protoplasts. Phytochemistry 46:421–425.

Desentis-Mendoza, R.M., Hernández-Sánchez, H., Moreno, A., Rojas del, E.C., Chel-Guerrero, L., Tamariz, J., Jaramillo-Flores, M.E. (2006) Enzymatic polymerization of phenolic compounds using laccase and tyrosinase from *Ustilago maydis*. Biomacromolecules 7:1845–1854.

Dickinson, E. (1997) Enzymic crosslinking as a tool for food colloid rheology control and interfacial stabilization. Trends Food Sci Technol 8:334–339.

Dittmer, N.T., Suderman, R.J., Jiang, H., Zhu, Y.C., 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 34:29–41.

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 5:310–316.

Dunnewind, B., van Vliet, T., Orsel, R. (2002) Effect of oxidative enzymes on bulk rheological properties of wheat flour doughs. J Cereal Sci 36:357–366.

Durán, N., Esposito, E. (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl Cat B Environ 28:83–99.

Espín, J.C., Wichers, H.J. (1999) Activation of a latent mushroom (*Agaricus bisporus*) tyrosinase isoform by sodium dodecyl sulfate (SDS). Kinetic Properties of the SDS-Activated Isoform. J Agric Food Chem 47:3518–3525.

Espín, J.C., Fenoll, L.G., Gilabert, M.A., García-Ruiz, P.A., Tudela, J., García-Cánovas, F. (2000) Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase. Eur J Biochem 267:1270–1279.

Espín, J.C., Leeuwen, J., Wichers, H.J. (1999a) Kinetic study of the activation process of a latent mushroom (*Agaricus bisporus*) tyrosinase by serine proteases. J Agric Food Chem 47:3509–3517.

Espín, J.C., Jolivet, S., Wichers, H.J. (1999b) Kinetic Study of the Oxidation of ç-L-Glutaminyl-4-hydroxybenzene Catalyzed by Mushroom (Agaricus bisporus) Tyrosinase. J Agric Food Chem 47:3495–3502.

Espín, J.C., García-Ruiz, P.A., Tudela, J., García-Cánovas, F. (1998) Study of stereospecificity in mushroom tyrosinase. Biochem J 331:547–551.

Espín, J.C., Morales, M., García-Ruiz, P.A., Tudela, J., García-Cánovas, F. (1997) Improvement of a continuous spectrophotometric method for determining the monophenolase and diphenolase activities of mushroom polyphenol oxidase. J Agric Food Chem 45:1084–1090.

Fan, Y., Flurkey, W.H. (2004) Purification and characterization of tyrosinase from gill tissue of Portabella mushrooms. Phytochemistry 65:671–678.

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

Faure, D., Bouillant, M.L., Bally, R. (1994) Isolation of *Azospirillum lipoferum* 4T Tn5 mutants affected in melanization and laccase activity. Appl Environ Microbiol 60:3413–3415.

Fei, J., Song, H.K., Palmore, G.T.R. (2007) A biopolymer composite that catalyzes the reduction of oxygen to water. Chem Mater 19:1565–1570.

Felton, G.W., Donato, K., Del Vecchio, R.J., Duffey, S.S. (1992) Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a *noctuid herbivore*. J Insect Physiol 38:277–285.

Felton, G.W., Donato, K., Del Vecchio, R.J., Duffey, S.S. (1989) Activation of plant polyphenol oxidases by insect feeding damage reduces nutritive quality of foliage for *noctuid herbivores*. J Chem Ecol 15:2667–2694.

Fennema, O.R. (1976) Principles of food science, Part I. In: Food Chemistry. Fennema, O.R. (ed.). Marcel Dekker Inc., New York. 205 p.

Figueroa-Espinoza, M.C., Rouau, X. (1998) Oxidative cross-linking of pentosan by a fungal laccase and horseradish peroxidase: mechanism of linkage between feruloylated arabinoxylans. Cereal Chem 75:259–265.

Figueroa-Espinoza, M.-C., Morel, M.-H., Surget, A., Asther, M., Moukha, S., Sigoillot, J.-C., Rouau, X. (1999a) Attempt to cross-link feruloylated arabinoxylans and proteins with a fungal laccase. Food Hydrocolloids 13:65–71.

Figueroa-Espinoza, M.C., Morel, M.H., Surget, A., Rouau, X. (1999b) Oxidative cross-linking of wheat arabinoxylans by manganese peroxidase. Comparison with laccase and horseradish peroxidase. Effect of cysteine and tyrosine gelation. J Sci Food Agric 79:460–463.

Figueroa-Espinoza, M.C., Morel, M.H., Rouau, X. (1998) Effect of lysine tyrosine, cysteine and glutathione on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. J Agric Food Chem 46:2583–2589.

Fling, M., Horowitz, N.H., Heinemann, S.F. (1963) The isolation and properties of crystalline tyrosinase from *Neurospora*. J Biol Chem 238:2045–2053.

Flurkey, W.H. (2003) Laccase. In: Whitaker, J.R., Voragen, A.G.J., Wong, D.W.S. (eds). Handbook of food enzymology. Marcel Dekker, Inc., New York. Pp. 525–537.

Fox, P.F., McSweeney, P.L.H. (1998) Dairy chemistry and biochemistry. Springer-Verlag. http://www.knovel.com/knovel2/Toc.jsp?BookID=937&VerticalID=0.

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 heterogenous reaction conditions. J Biotechnol 125:281–294.

Frederix, S.A., Courtin, C.M., Delcour, J.A. (2003) Impact of xylanases with different substrate selectivity on gluten–starch separation of wheat flour. J Agric Food Chem 51:7338–7345.

Froehner, S.C., Eriksson, K.E. (1974) Purification and properties of *Neurospora crassa* laccase. J Bacteriol October 120(1):458–465.

Gan, Z., Ellis, P.R., Schofield, J.D. (1995) Mini review: gas cell atabilisation and gas retention in wheat bread dough. J Cereal Sci 21:215–230.

Ganzevles, R.A., Zinoviadou, K., Vliet, T. van Cohen, S.M.A., de Jongh, H.H.J. (2006) Modulating surface rheology by electrostatic protein/polysaccharide interactions. Langmuir 22:10089–10096.

Gerdemann, C., Eicken, C., Krebs, B. (2002) The crystal structure of catechol oxidase: new insight into the function of type-3 copper proteins.

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 128:15376–15377.

Greco, G., Toscano, G., Cioffi, M., Gianfreda, L., Sannino, F. (1999) Dephenolisation of olive mill waste-waters by olive husk. Water Reasearch 33:3046–3050.

Gruppen, H., Kormelink, F.J.M., Voragen, A.G.J. (1993) Water-unextractable cell-wall material from wheat flour. 3. A structural model for arabinoxylans. J Cereal Sci 18:111–128.

Guillon, F., Thibault, J.-F. (1990) Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. Carbohydr Polym 12:353–374.

Gukasyan, G.S. (1999) Purification and some properties of tyrosinase from *Aspergillus flavipes* 56003. Biochemistry (Mosc) 64:417–420.

Gutiérrez, A., Rencoret, J., Ibarra, D., Molina, S., Camarero, S., Romero, J., DelRio, J.C., Martinez, A. T. (2007) Removal of Lipophilic Extractives from Paper Pulp by Laccase and Lignin-Derived Phenols as Natural Mediators. Environ Sci Technol 41:4124–4129.

Haarasilta, S., Pullinen, T., Väisänen, S., Tammersalo-Karsten, I. (1991) Enzyme product and method of improving the properties of dough and the quality of bread. US Patent 4990343.

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. Nature Struct Biol 9:601–605.

Halaouili, S., Asther, M., Sigoillot, J.-C., Hamdi, M., Lomascolo, A. (2006a) Fungal tryrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. J Appl Microbiol 100:219–232.

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 Microbiol Biotechnol 70:580–589.

Halaouli, S., Asther, Mi., 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 98:332–343.

Haruta, M., Murata, M., Hiraide, A., Kadokura, H., Yamasaki, M., Sakuta, M., Shimizy, S., Homma, S. (1998) Cloning genomic DNA encoding for apple polyphenol oxidase and comparison of the gene product in *Escherichia coli* and apple. Biosci Biotechnol Biochem 62:358–362.

Hatakka, A. (1994) Ligning-modifying enzymes from selected white-rot fungi. Production and role in lignin degradation. FEMS Microbiol Rev 13:125–135.

Hatcher, L.Q., Karlin, K.D. (2004) Oxidant types in copper-dioxygen chemistry: the ligand coordination defines the Cu_n-O_2 structure and subsequent reactivity. J Biol Inorg Chem 9:669–683.

Hearing, V.J., Tsukamoto, K. (1991) Enzymatic control of pigmentation in mammals. FASEB J 5:2902–2909.

Hilhorst, R., Dunnewind, B., Orsel, R., Stegeman, P., van Vliet, T., Gruppen, H., Schols, H.A. (1999) Baking performance, rheology, and chemical composition of wheat dough and gluten affected by xylanase and oxidative enzymes. J Food Sci 64:808–813.

Horowitz, N.H., Shen, S.C. (1952) *Neurospora* tyrosinase. J Biol Chem 197:513–520.

Horowitz, N.H., Fling, M., Feldmann, H.M., Pall, M.L., Froehner, S.C. (1970) Derepression of tyrosinase synthesis in *Neurospora* by amino acid analogs. Dev Biol 21:147–156.

Hoseney, R.C., Faubion, J.M. (1981) A mechanism for the oxidative gelation of wheat-lour water-soluble pentosans.Cereal Chem 58:421–424.

Hristova, R., Dolashki, A., Voelter, W., Stevanovic, S., Dolashka-Angelova, P. (2008) o-Diphenol oxidase activity of molluscan hemocyanins. Comp Biochem 3:439–446.

Ichishima, E., Maeba, H., Amikura, T., Sakata, H. (1984) Multiple forms of protyrosinase from *Aspergillus oryzae* and their mode of activation at pH 3.0. Biochim Biophys Acta 786:25–31.

Ikeda, K., Masujima, T., Suzuki, K., Sugiyama, M. (1996) Cloning and sequence analysis of the highly expressed melanin- synthesizing gene operon from *Streptomyces castaneoglobisporus*. Appl Microbiol Biotechnol 45:80–85.

Ikehata, K., Nicell, J.A. (2000) Characterization of tyrosinase for the treatment of aqueous phenols. Bioresour Technol 74:191–199.

Ito, S., Prota, G. (1976) A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase. Experientia 33:1118–1119.

Ito, S., Kato, T., Shinpo, K., Fujita, K. (1984) Oxidation of tyrosine residues in proteins by tyrosinase. Biochem J 222:407–411.

Jaenicke, E., Decker, H. (2004) Functional changes in the family of type 3 copper proteins during evolution. Chembiochem 5:163–169.

Jaenicke, E., Decker, H. (2003) Tyrosinase of crustaceans form hexamers, Biochem J 371:515–523.

Jee, J.-G., Park, S.-J., Kim, H.-J. (2000) Tyrosinase-induced cross-linking of tyrosine-containing peptides investigated by matrix–assisted laser desorption/ ionization time-of–flight mass spectrometry. Rapid Commun Mass Spectrom 14:1563–1567.

Jiménez, M., Kameyama, K., Maloy, W L., Tomita, Y., Hearing, V.J. (1988) Mammalian tyrosinase: biosynthesis, processing, and modulation by melanocyte-stimulating hormone. Proc Natl Acad Sci USA 85(11):3830–3834.

Jolley, R.L., Evans, L.H., Makino, N., Mason, H.S. (1974) Oxytyrosinase. J Biol Chem 249:335–345.

Jolley, R.L., Evans, L.H., Mason, H.S. (1972) Reversible oxygenation of tyrosinase. Biochem Biophys Res Commun 46:878–884.

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 9:1549–1558.

Jordan, A.M., Khan, T.H., Osborn, H.M., Photiou, A., Riley, P.A. (1999) Melanocytedirected enzyme prodrug therapy (MDEPT) development of a targeted treatment for malignant melanoma. Bioorg Med Chem 7:1775–1780.

Kanda, K., Sato, T., Ishii, S., Enei, H., Ejiri, S. (1996) Purification and properties of tyrosinase isozymes from gill of *Lentinus edodes* fruiting bodies. Biosci Biotechnol Biochem 60:1273–1278.

Kato, A., Minaki, K., Kobayashi, K. (1993) Improvement of emulsifying properties of egg white proteins bythe attachment of polysaccharide through maillard reaction in a dry state. J Agric Food Chem 41:540–543.

Kato, A., Wada, T., Kobayashi, K., Seguro, K., Motoki, M. (1991) Ovomucin-food protein conjugates prepared through the transglutaminase reaction. Agric Biol Chem 55:1027–1031.

Khan, A.A., Akhtar, S., Husain, Q. (2005) Simultaneous purification and immobilization of mushroom tyrosinase on an immunoaffinity support. Process Biochemistry 40:2379–2386.

Kieffer, R., Wieser, H., Henderson, M.H., Graveland, A. (1998) Correlations of the breadmaking performance of wheat flour with rheological measurements on a micro-scale. J Cereal Sci 27:53–60.

Kiiskinen, L.-L. (2004) Characterization and heterologous production of a novel laccase from *Melanocarpus albomyces*. Doctoral thesis, VTT Biotechnology, Espoo. 94 p. + app. 42 p.

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 62:1707–1723.

Kim, S.Y., Zille, A., Murkovic, M., Güebitz, G., Cavaco-Paulo, A. (2007) Enzymatic polymerization on the surface of functionalized cellulose fibers. Enzyme Microb Technol 40:1782–1787.

Klabunde, T., Eicken, C., Sacchettini, J.C., Krebs, B. (1998) Crystal structure of a plant catechol oxidase containg a dicopper center. Nat Struct Biol 5:1084–1090.

Kong, K.-H., Hong, M.-P., Choi, S.-S., Kim, Y.-T., Cho, S.-H. (2000a) Purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. Biotechnol Appl Biochem 31:113–118.

Kong, K.H., Park, S.Y., Hong, M.P., Cho, S.H. (2000b) Expression and characterization of human tyrosinase from a bacterial expression system. Comp Biochem Physiol B Biochem Mol Biol 125:563–569.

Kong, K.-H., Lee, J.-L., Park, H.-J., Cho, S.-H. (1998) Purification and characterization of the tyrosinase isozymes of pine needles. Biochem Mol Biol Int 45:717–724.

Koussevitzky, S., Ne'eman, E., Sommer, A., Steffens, J.C., Harel, E. (1998) Purification and properties of a novel chloroplast stromal peptidase. Processing of polyphenol oxidase and other imported precursors. J Biol Chem 273:27064–27069.

Kumar, M., Flurkey, W.H. (1991) Activity, isoenzymes, and purity of mushroom tyrosinase in commercial preparations. Phytochemistry 30:3899–3902.

Kuninori, T., Nishiyama, J., Matsumoto, H. (1976) Effect of mushroom extract upon the physical properties of dough. Cereal Chem 53:420–428.

Kupper, U., Niedermann, D.M., Travaglini, G., Lerch, K. (1989) Isolation and characterization of the tyrosinase gene from *Neurospora crassa*. J Biol Chem 264:17250–17258.

Kuuva, T., Lantto, R., Reinikainen, T., Buchert, J., Autio, K. (2003) Rheological properties of laccase-induced sugar beet pectin gels. Food Hydrocolloids 17:679–684.

Kwon, B.S., Wakulchik, M., Haq, A.K., Halaban, R., Kestler, D. (1988) Sequence analysis of mouse tyrosinase cDNA and the effect of melanotropin on its gene expression. Biochem Biophys Res Commun 153:1301–1309.

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 USA 84:7473–7477.

Käärik, A. (1965) The identification of the mycelia of wood–decay fungi by their oxidation reactions with phenolic compounds. Bibl Mycol 81:1–151.

Labat, E., Morel, M.H., Rouau, X. (2001) Effect of laccase and manganese peroxidase on wheat gluten and pentosans during mixing. Food Hydrocolloids 15:47–52.

Labat, E., Morel, M.H., Rouau, X. (2000) Effect of laccase and ferulic acid on wheat flour dough during mixing. Cereal Chem 77:823–828.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Land, E.J., Ramsden, C.A., Riley, P.A. (2004) Quinone chemistry and melanogenesis. Methods Enzymol 378:88–109.

Lang, W.H., van Holde, K.E. (1991) Cloning and sequencing of *Octopus dofleini* hemocyanin cDNA: Derived sequences of functional units Ode and Odf. Proc Natl Acad Sci USA 88:244–248.

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 38:143–158.

Lantto, R., Puolanne, E., Kruus, K., Buchert, J., Autio, K. (2007a) Tyrosinase-aided protein crosslinking: effects on gel formation of chicken breast myofibrils and texture and water-holding of chicken breast meat homogenate gels. J Agric Food Chem 55:1248–1255.

Lantto, R., Puolanne, E., Katina, K., Niemistö, M., Buchert, J., Autio, K. (2007b) Effect of laccase and transglutaminase on the textural and waterbinding properties of cooked chicken breast meat gels. Eur Food Res Technol 225:75–83.

Lantto, R., Plathin, P., Niemistö, M., Buchert, J., Autio, K. (2006) Effects of transglutaminase and freeze-dried aple pomace powder on gel forming and structure of pork meat. LWT-Food Sci Technol 39:1117–1124.

Lantto, R., Heine, E., Freddi, G., Lappalainen, A., Miettinen-Oinonen, A., Niku-Paavola, M.-L., Buchert, J. (2005a) Enzymatic modification of wool with tyrosinase and peroxidase. J Textile Inst 96:109–116.

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 53:9231–9237.

Lappalainen, A. (1988) Cellulolytic and xylanolytic enzymes of *Trichoderma reesei*. Doctoral thesis. Espoo: VTT, Publications 50. 54 p. + app. 51 p.

Lappalainen, A., Siika-Aho, M., Kalkkinen, N., Fagerström, R., Tenkanen, M. (2000) Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points. Biotechnol Appl Biochem 31:61–68.

Laurikainen, T., Härkönen, H., Autio, K., Poutanen, K. (1998) Effects of enzymes in firbre-enriched baking. J Sci Food Agric 76:239–249.

Leatham, G.F., Stahmann, M.A. (1981) Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J Gen Microbiol 125:147–157.

Lee, C.Y., Whitaker, J.R. (1995) Enzymatic browning and its prevention. American Chemical Society Symposium Series 600. American Chemical Society, Washington, D.C. 338 p.

Lerch, K. (1983) *Neurospora tyrosinase*: structural, spectroscopic and catalytic properties. Mol Cell BioChem 52(2):125–138.

Lerner, H.R., Harel, E., Lehman, E., Mayer, A.M. (1971) Phenylhydrazine, a specific irreversible inhibitor of catechol oxidase. Phytochemistry 10:2637–2640.

Lesiów, T., Xiong, Y. (2001a) Gelation properties of poultry myofibrillar proteins and comminuted poultry meat. Fleischwirtschaft Int 4:39–44.

Lesiów, T., Xiong, Y. (2001b) Mechanism of rheological changes in poultry myofibrillar proteins during gelation. Avian and Poultry Biology Reviews 12:137–149.

Létang, C.L., Piau, M., Verdier, C. (1999) Characterization of wheat flour-water doughs. Part I: Rheometry and microstructure. J Food Eng 41:121–132.

Leu, W., Chen, L., Liaw, L., Lee, Y. (1992) Secretion of the *Streptomyces* tyrosinase is mediated through its trans-activator, MelC. J Biol Chem 267:20108–20113.

Lim, K.G., Palmore, G.T.R. (2007) Microfluidic biofuel cells: the influence of electrode diffusion layer on performance. Biosens Bioelect 22:941–947.

Littoz, F., McClements, D.J. (2007) Bio-mimetic approach to improving emulsion stability: cross-linking adsorbed beet pectin layer using laccase. Food hydrocolloids. *In press*.

Liu, Y., Dong, S. (2007) A biofuel cell with enhanced power output by grape juice. Electrochem Commun 9:1423–1427.

Lorenzen, P.C., Neve, H. (2003) Enzymatic cross-linking of proteins in the manufacture of fermented milk. In: Fermented Milk. Proceedings of the IDF-seminar on Aroma & Texture of Fermented Milk. Kolding, Denmark. Pp. 241–249.

Lorenzen, P.C., Neve, H., Mautner, A., Schlimme, E. (2002) Effect of enzymatic cross-linking of milk proteins on functional properties of set–style yogurt. Int Dairy Technol 55:152–157.

Lorenzen, P.C., Schlimme, E, Roos, N. (1998) Cross-linking of sodium caseinate by a microbial transglutaminase. Nahrung 42:151–154.

MacRitchie, F. (1984) Baking quality of wheat flours. Adv Food Res 29:201–277.

Makino, N., Mason, H.S. (1973) Reactivity of oxytyrosinase toward substrates. J Biol Chem 248:5731–5735.

Makino, N., McMahill, P., Mason, H.S. (1974) The oxidation state of copper in resting tyrosinase. J Biol Chem 249(19):6062–6066.

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 41:5395–5402.

Martínez, D., Larrondo, L.F., Putnam, N., Gelpke, M.D., Huang, K., Chapman, J., Helfenbein, K.G., Ramaiya, P., Detter, J.C., Larimer, F., Coutinho, P.M., Henrissat, B., Berka, R., Cullen, D., Rokhsar, D. (2004) Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium strain RP78*. Nature Biotechnol 22:695–700.

Martínez, M.V., Whitaker, J.R. (1995) The biochemistry and control of enzymatic browning. Trends Food Sci Technol 6:195–200.

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 277:18849–18859.

Marumo, K., Waite, J.H. (1986) Optimization of hydroxylation of tyrosine and tyrosine-containing peptides by mushroom tyrosinase. Biochim Biophys Acta 872:98–103.

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 100:108–123.

Mason, H.S. (1948) The chemistry of melanin. III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. J Biol Chem 172:83–92.

Mason, H.S. (1949) The chemistry of melanin. VI. Mechanism of the oxidation of catechol by tyrosinase. 181:803–812.

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 281:8981–8990.

Mattinen, M–L., Lantto, R., Selinheimo, E., Kruus, K., Buchert, J. (2008) Oxidation of peptides and proteins by *Trichoderma reesei* and *Agaricus bisporus* tyrosinases. J Biotechn 133:395–402.

Mattinen, M.-L., Hellmann, M., Permi, P., Autio, K., Kalkkinen, N., Buchert, J. (2006) Effect of protein structure on laccase-catalyzed protein oligomerization. J Agric Food Chem 54:8883–8890.

Mattinen, M.-L., Kruus, K., Buchert, J., Nielsen, J.H., Andersen, H.J., Steffensen, C.L. (2005) Laccase-catalysed polymerization of tyrosine-containing peptides. FEBS J 272:3640–3650.

Matuschek, E., Svanberg, U. (2005) The effect of fruit extracts with polyphenol oxidase (PPO) activity on the in vitro accessibility of iron in high–tannin sorghum. Food Chem 90:765–771.

Mayer, A.M. (2006) Polyphenol oxidases in plant and fungi: going places? A review. Phytochemistry 67:2318–2331.

Mayer, A.M. (1987) Polyphenol oxidases in plant – Recent progress. Phytochemistry 26:11–20.

Mayer, A.M., Staples, R.C. (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565.

McDowell, L.M., Burzio, L.A., Waite, J.H., Schaefer, J. (1999) Rotational echo double resonance detection of cross-links formed in mussel byssus under high–flow stress. J Biol Chem 274:20293–20295.

McMahon, A.M., Doyle, E.M., Brooks, S.J., OConnor, K.E. (2007) Biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. Enzyme Microbial Technol 40:1435–1441.

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 67:277–285.

Messerschmidt, A. (1997) Multi-copper oxidases. World Scientific, Singapore.

Meuser, F., Suckow, P. (1986) Non-starch polysaccharides. In: Blanshard, J.M.V., Frazier, P.J., Galliard, T. (eds). Chemistry and physics of baking. The Royal Society of Chemistry, London. Pp. 42–61.

Micard, V., Thibault, J.-F. (1999) Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. Carbohydr Polym 39:265–273.

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 Mol Catal B Enzym 45:102–107.

Minussi, R.C., Pastore, G.M., Duran, N. (2002) Potential applications of laccase in the food industry. Trends Food Sci Technol 13:205–216.

Mita, D.G., Attanasio, A., Arduini, F., Diano, N., Grano, V., Bencivenga, U., Rossi, S., Amine, A. (2007) Moscone D. Enzymatic determination of BPA by means of tyrosinase immobilized on different carbon carriers. Biosens Bioelectron 23:60–65.

Moridani, M.Y., Scobie, H., Salehi, P., O'Brien, P.J. (2001) Catechin metabolism: glutathione conjugate formation catalyzed by tyrosinase, peroxidase, and cytochrome p450. Chem Res Toxicol 14:841–848.

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 USA 82:2960–2964.

Munjala, N., Sawhney, S.K. (2002) Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. Enzyme Microb Technol 30:613–619.

Mustafa, R., Muniglia, L., Rovel, B., Girardin, M. (2005) Phenolic colorants obtained by enzymatic synthesis using a fungal laccase in a hydroorganic biphasic system. Food Res Int 38:995–1000.

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 body. Microbiology 149:2455–2462.

Ni Eidhin, D.M., Murphy, E., OBeirne, D. (2006) Polyphenol oxidase from apple (*Malus domestica* Borkh. cv Bramle's seedling) purification strategies and characterization. J Food Sci 71(1):C51–C58.

Niku-Paavola, M.-L., Karhunen, E., Salola, P., Raunio, V. (1988) Ligninolytic enzymes of the white-rot-fungus Phlebia ferulic. Biochem J 254:877–884.

Niku-Paavola, M.-L., Lappalainen, A., Enari, T.M., Nummi, M. (1985) A new appraisal of the endoglucanases of the fungus *Trichoderma reesei*. Biochem J 231(1):75–81.

Nillius, D., Jaenicke, E., Decker, H. (2008) Switch between tyrosinase and catecholoxidase activity of scorpion hemocyanin by allosteric effectors. FEBS Letters 582:749–754.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., Motoki, M. (1992) Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J Food Sci 57:1214–1218.

Nonaka, M., Tanaka, H., Okiyama, A., Motoki, M., Ando, H., Umeda, K., Matsuura, A. (1989) Polymerization of several proteins by Ca^{2+} -independent transglutaminase derived from microorganisms. Agric Biol Chem 53:2619–2623.

Olivares, C., Garcia-Borron, J.C., Solano, F. (2002) Identification of active site residues involved in metal cofactor binding and stereospecific substrate recognition in mammalian tyrosinase. Implications to the catalytic cycle. Biochemistry 41:679–686.

Osborne, T.B. (1907) The proteins of the wheat kernel. Carnegie Inst. of Washington publication, no. 84, Washington.

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 66(3):920–924.

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 464:110–117.

Parovuori, P., Manelius, R., Suortti, T., Bertoft, E., Autio, K. (1997) Effect of enzymically modified amylopectin on the rheological properties of ferulic-amylopectin mixed gels. Food Hydrocolloids 11:471–477.

Passmore, L.A., Kaesmann-Kellner, B., Weber, B.H. (1999) Novel and recurrent mutations in the tyrosinase gene and the P gene in the *German albino* population. Hum Genet 105:200–210.

Pazarlioglu, N.K., Sariişik, M., Telefoncu, A. (2005) Laccase: production by *Trametes versicolor* and application to denim washing. Process Biochem 40:1673–1678.

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 Environ 42:131–144.

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

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 277:37663–37669.

Poppius-Levlin, K., Wang, W., Tamminen, T., Hortling, B., Viikari, L., Niku-Paavola, M.-L. (1999) Effects of laccase/HBT treatment on pulp and lignin structures. J Pulp Paper Sci 25:90–94.

Primo-Martín, C., Valera, R., Martínez-Anaya, M.A. (2003) Effect of pentosanase and oxidases on the characteristics of doughs and the glutenin macropolymer (GMP). J Agric Food Chem 51:4673–4679.

Ramirez, E.C., Whitaker, J.R., Virador, V.M. (2003) Polyphenol oxidase. In: Whitaker, J.R., Voragen, A.G.J., Wong, D.W.S. (eds). Handbook of food enzymology. Marcel Dekker, Inc., New York. Pp. 509–523.

Raper, H.S. (1928) The aerobic oxidases. Physiol Rev 8:245–282.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O. (2003) Cell wall-associated enzymes in fungi. Phytochemistry 64:339–366.

Rastall, R. (2007) Novel enzyme technology for food applications. Woodhead Publishing Ltd, Cambridge. 336 p.

Ray, K., Chaki, M., Sengupta, M. (2007) Tyrosinase and ocular diseases: some novel thoughts on the molecular basis of oculocutaneous albinism type 1. Prog Retin Eye Res 26:323–358.

Reyes, P., Pickard, M.A., Vazquez-Duhalt, R. (1999) Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. Biotechnol Lett 21:875–880.

Rittstieg, K., Suurnäkki, A., Suortti, T., Kruus, K., Guebitz, G., Buchert, J. (2002) Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. Enz Microb Technol 31:403–410.

Riva, S. (2006) Laccases: blue enzymes for green chemistry. Trends Biotechnol 24:219–226.

Robb, D.A. (1984) Tyrosinase. In: Lontie, R. (ed). Copper proteins and copper enzymes. Vol. 2. CRC Press, Inc., Boca Raton, FL. Pp. 207–270.

Rodríguez Couto, S., Toca-Herrera, J.L. (2006a) Industrial and biotechnical applications of laccases: a review. Biotechnology Adv 24:500–513.

Rodríguez Couto, S., Toca-Herrera, J.L. (2006b) Mini review: lacasses in the textile industry. Biotechnol Molec Biol Rev 1:115–120.

Rosenzweig, A.C., Sazinsky, M.H. (2006) Structural insights into dioxygen-activating copper enzymes. Curr Opin Struct Biol 16:729–735.

Rouau, X., Ei-Hayek, M.L., Moreau, D. (1994) Effect of an Enzyme Preparation Containing Pentosanases on the Bread-making Quality of Flours in Relation to Changes in Pentosan Properties. J Cereal Sci 19:259–272.

Sampaio, S., Taddei, P., Monti, P., Buchert, J., Freddi, G. (2005) Enzymatic grafting of chitosan onto *Bombyx mori* silk fibroin: kinetic and IR vibrational studies. J Biotechnol 116:21–33.

Sayre, L.M., Nadkarni, D.V. (1994) Direct conversion of phenols to O-Quinones by copper(I) dioxygen-questions regarding the monophenolase activity of tyrosinase mimics. J Am Chem Soc 116:3157–3158.

Setti, L., Giuliani, S., Spinozzi, G., Pifferi, P.G. (1999) Laccase-catalyzed oxidative coupling of 3-methyl 2-benzothiazolinone hydrazone and methoxyphenols. Enzyme Microb Tech 25:285–289.

Shleev, S.V., Morozova, O.V., Nikitina, O.V., Gorshina, E.S., Rusinova, T.V., Serezhenkov, V.A., Burbaev, D.S., Gazaryan, I.G., Yaropolov, A.I. (2004) Comparison of physico chemical characteristics of four laccases from different basidiomycetes. Biochimie 86:693–703.

Shotaro, Y. (1999) Method for Cross-linking Protein by Using Enzyme. US Patent 6,121,013.

Si, J.Q. (1994) Use of laccase in baking industry. International Patent Application PCT/DK94/00232.

Si, J.Q., Sørensen, L. (1993) Use of laccases in baking. PCT Patent Application PCT/DK94/00232.

Sigoillot, C., Camarero, S., Vidal, T., Recorda, E., Asthera, M., Pérez-Boad, M., Martínez, M.J., Sigoillot, J.-C., Asther, M., Colom, J.F., Martínez, A.T. (2004) Comparison of different fungal enzymes for bleaching. Appl Microb Biotechnol 64:346–352.

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. Enz Microb Technol 43:93–102.

Solano, F., Briganti, S., Picardo, M., Ghanem, G. (2006) Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. Pigment Cell Res 19:550–571.

Solomon, E.I., Sundaram, U.M., Machonkin, T.E. (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2606.

Sommer, A., Neeman, E., Steffens, J.C., Mayer. A.M., Harel, E. (1994) Import, targeting, and processing of a plant polyphenol oxidase. Plant Physiology 105:1301–1311.

Spritz, R.A., Oh, J., Fukai, K., Holmes, S.A., Ho, L., Chitayat, D., France, T.D., Musarella, M.A., Orlow, S.J., Schnur, R.E., Weleber, R.G., Levin, A.V. (1997) Novel mutations of the tyrosinase (TYR) gene in type I oculocutaneous albinism (OCA1). Hum Mutat 10:171–174.

Sproessler, B.G. (1993) Milling and Baking. In: Nagodawithana, T., Reed, G. (eds). Enzymes in food processing. 3rd ed. Academic Press, Inc., San Diego. Pp. 293–320.

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 427:201–210.

Sugumaran, M. (2002) Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 15:2–9.

Sun, T., Powers, J.R., Tang, J. (2007) Enzyme-catalyzed change of antioxidants content and antioxidant activity of Asparagus juice. J Agric Food Chem 55:56–60.

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 52:557–563.

Takasaki, S., Kawakishi, S. (1997) Formation of protein-bound 3,4-dihydroxyphenylalanine and 5-S-cysteinyl-3,4-dihydroxyphenylalanine as new cross-linkers in gluten. J Agric Food Chem 45:3472–4375.

Takasaki, S., Kawakishi, S., Murata, M., Homma, S. (2001) Polymerization of gliadin mediated by mushroom tyrosinase. Lebensm Wiss u Technol 34:507–512.

Temp, U., Meyrahn, H., Eggert, C. (1999) Extracellular phenol oxidase patterns during depolymerization of low-rank coal by three dasidiomycetes. Biotechnol Lett 21:281–287.

Tenkanen, M., Salmenkallio-Marttila, M., Poutanen, K. (2000) Baking with enzymes: what makes it happen. The World of Food Ingredients May/June:38–41.

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

Terwilliger, N.B., Ryan, M.C. (2006) Functional and phylogenetic analyses of phenoloxidases from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana*, *Triops longicaudatus*) crustaceans, Bio. Bull 210:38–50.

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

Thurston, C. (1994) The structure and function of fungal laccases. Microbiology 140:19–26.

Thygesen, P.W., Dry, I.B., Robinson, S.P. (1995) Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns. Plant Physiol 109:525–531.

Tornberg, E. (2005) Effect of heat on meat proteins-Implications on structure and quality of meat products. Meat Sci 70:493–508.

Torres, E., Bustos-Jaimes, I., Le Borgne, S. (2003) Potential use of oxidative enzymes for the detoxification of organic pollutants. Appl Catal B Environ 46:1–15.

Tsai, T.T., Lee, Y.H. (1998) Roles of copper ligands in the activation and secretion of *Streptomyces* tyrosinase. J Biol Chem 273:19243–19250.

van Gelder, C.W., Flurkey, W.H., Wichers, H.J. (1997) Sequence and structural features of plant and fungal tyrosinases. Phytochemistry 45(7):1309–1323.

van Holde, K.E., Miller, K.I., Decker, H. (2001) Hemocyanins and invertebrate evolution. J Biol Chem 276:15563–15566.

Vemulapalli, V., Hoseney, R.C. (1998) Glucose oxidase effects on gluten and water solubles. Cereal Chem 75:859–862.

Vemulapalli, V., Miller, K.A., Hoseney, R.C. (1998) Glucose oxidase in breadmaking systems. Cereal Chem 75:439–452.

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

Walker, J.R.L., McCallion, R.F. (1980) The selective inhibition of orthoand para-diphenol oxidases. Phytochemistry 19:373–377.

Wang, M. (2003) Effect of pentosans on gluten formation and properties. Doctoral thesis, Wageningen University, Department of Agrotechnology and Food Sciences, the Netherlands. P. 190.

Wang, N., Hebert, D.N. (2006) Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. Pigment Cell Research 19(1):3–18.

Wichers, H.J., Gerritse, Y.A., Chapelon, C.G.J. (1996) Tyrosinase isoforms from the fruitbodies of *Agaricus bisporus*. Phytochemistry 43:333–337.

Wichers, H.J., Recourt, K., Hendriks, M., Ebbelaar, C.F.M., Biancone, G., Hoeberichts, F.A., Mooibroek, H., Soler-Rivas, C. (2003) Cloning, expression and characterisation of two tyrosinase cDNAs from *Agaricus bisporus*. Appl Microbiol Biotechnol 61:336–341.

Whetten, R., Sederoff, R. (1995) Lignin Biosynthesis. The Plant Cell 7:1001–1013.

Wong, Y., Yu, J. (1999) Laccase-catalyzed decolorization of synthetic dyes. Water Research 33:3512–3520.

Wrigley, C.W., Bietz, J.A. (1988) Proteins and amino acids. In Pomeranz, Y. (ed). Wheat chemistry and technology. Vol. I. American Association of Cereal Chemists, Inc., St. Paul, Minnesota. Pp. 159–200.

Xing, Q., Eadula, S.R., Lvov, Y.M. (2007) Cellulose fiber-enzyme composites fabricated through layer-by-layer nanoassembly. Biomacromolecules 8:1987–1991.

Xiong, H., von Weymarn, N., Leisola, M., Turunen, O. (2004) Influence of pH on the production of xylanases by *Trichoderma reesei* Rut C–30. Process Biochemistry 39:729–733.

Xu, F. (2005) Applications of oxidoreductases: recent progress. Industrial Biotechnol 1:38–50.

Xu, F. (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. Biochemistry 35:7608–7614.

Xu, R., Xin, H., Hopkins, T.L., Kramer, K.J. (1997) Catecholamine and histidyl protein cross-linked structures in sclerotized insect cuticle. Insect Biochemistry Mol Biol 27:101–108.

Yague, S., Terron, M.C., Gonzalez, T., Zapico, E., Bocchini, P., Galletti, G.C., Gonzalez, A.E. (2000) Biotreatment of tannin-ich beer-factory wastewater with white-rot basidiomycete *Coriolopsis gallica* monitored by pyrolysis/gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 14:905–910.

Yamaguchi, S. (2000) Method for cross-linking protein by using enzyme. US Patent 6121013.

Yaropolov, A., Kharybin, A., Emmeus, 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 308:137–144.

Yasui, T., Ishioroshi, M., Samejima, K. (1980) Heat induced gelation of myosin in the presence of actin. J Food Sci 44:61–78.

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

Zacharius, R.M., Zell, T.E., Morrison, J.H., Woodlock, J.J. (1969) Glycoprotein staining following electrophoresis on acrylamide gel. Anal Biochem 30:148152.

Zavarzina, A.G., Zavarzin, A.A. (2006) Laccase and tyrosinase activities in lichens. Microbiology 75:546–556.

Zhang, X., van Leeuwen, J., Wichers, H.J., Flurkey, W.H. (1999) Characterization of tyrosinase from the cap flesh of portabella mushrooms. J Agric Food Chem 47:374–378.

Zhao, H., Waite, J.H. (2005) Coating proteins: structure and cross-linking in fp-1 from the green shell mussel *Rerna canaliculus*. Biochemistry 44:15915–15923.

Zille, A., Munteanu, F.-D., Gübitz, G.-M. Cavaco-Paulo, A. (2005) Laccase kinetics of degradation and coupling reactions. J Mol Catal B Enzym 33:23–28.

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Title

Tyrosinase and laccase as novel crosslinking tools for food biopolymers

Abstract

Tyrosinases and laccases are oxidoreductases, which catalyze oxidation of mono- and polyphenolic compounds. Oxidation products are prone to react further and may lead to polymerization. Biopolymer-bound phenolic compounds are also susceptible to oxidation by tyrosinase and laccase, which enables crosslinking of the biopolymers.

A novel fungal tyrosinase from *Trichoderma reesei* (TrT) was characterized in this work. Substrate specificity and protein crosslinking ability of TrT was compared to other fungal and plant tyrosinases. TrT and a laccase from *Trametes hirsuta* (ThL) were also examined for hetero-crosslinking of carbohydrates and proteins and for improving bread quality.

TrT was over-expressed in its original host and purified with a three step purification procedure. TrT showed a molecular mass of 43.2 kDa. TrT was processed from the C-terminus by cleavage of a peptide of 20 kDa. TrT had the highest activity and stability in the neutral and alkaline pH range. When apple (AT), potato (PT), *Pycnoporus sanguineus* (PsT) and *Agaricus bisporus* (AbT) tyrosinases were compared to TrT, the enzymes were found to have different features in terms of substrate specificity and protein crosslinking ability. TrT had the highest activity on most of the tested monophenols. TrT also had clearly the best ability to crosslink the model protein α-casein, although PsT could also directly crosslink α-casein. By an addition of a small molecular weight phenolic compound to the reaction mixture, AbT, PT and AT were also able to crosslink α-casein.

The ability of TrT and the ThL laccase, to catalyze hetero-crosslinking of α-casein to hydrolyzed oat spelt xylan (hOSX) was studied. TrT was able to crosslink α-casein more efficiently than ThL, whereas only ThL was able to polymerize hOSX. Both ThL and TrT were observed to be able to catalyze hetero-crosslinking of α-casein and hOSX. Both ThL and TrT also improved wheat bread quality, as indicated by an increase in bread volume and crumb softness. ThL and TrT affected the bread mainly by crosslinking of arabinoxylan and gluten proteins, respectively.

It was shown in this work that tyrosinase and laccase can be applied to generate novel food structures and biopolymers with added functionalities.

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Selinheimo, Emilia

Nimeke

Elintarvikebiopolymeerien ristisilloittaminen tyrosinaasilla ja lakkaasilla

Tiivistelmä

Tyrosinaasit ja lakkaasit ovat entsyymejä, jotka katalysoivat mono- ja polyfenolisten yhdisteiden hapettumisreaktioita. Reaktiotuotteiden jatkoreaktiot voivat johtaa substraattien polymeroitumiseen. Tyrosinaasit ja lakkaasit pystyvät hapettamaan myös biopolymeerirakenteissa esiintyviä fenolisia yhdisteitä, minkä johdosta kyseisten biopolymeerien entsyymiavusteinen ristisilloittaminen on mahdollista.

Tässä työssä karakterisoitiin uutta *Trichoderma reesei* -homeesta peräisin olevaa tyrosinaasia. *T. reesei* -tyrosinaasin substraattispesifisyyttä sekä kykyä ristisilloittaa maitoproteiini α-kaseiinia verrattiin home- ja kasviperäisiin tyrosinaaseihin. *T. reesei* -tyrosinaasin kykyä muodostaa heterokonjugaatteja proteiinien ja hiilihydraattien välille sekä entsyymin vaikutuksia vehnäleivonnassa verrattiin *Trametes hirsuta* -lakkaasiin.

T. reesei -tyrosinaasi tuotettiin ylituottomenetelmällä alkuperäisessä tuottoisännässään. Entsyymi puhdistettiin kolmivaiheisella kromatografisella prosessilla. *T.reesei* -tyrosinaasin molekyylipainoksi määritettiin 43,2 kDa, ja polypeptidin C-terminaalisesta päästä todetiin katkenneen noin 20 kDa -kokoinen peptidi. *T. reesei* -tyrosinaasi oli aktiivinen neutraalilla ja emäksisellä pH-alueella. Verrattaessa *T. reesei* -tyrosinaasia omenasta, perunasta, *Pycnoporus sanguineus* sekä *Agaricus bisporus* -homeista peräisin oleviin tyrosinaaseihin havaittiin entsyymeiden substraattispesifisyyden ja kyvyn ristisilloittaa maitoproteiinia eroavan toisistaan huomattavasti. *T. reesei* -tyrosinaasi hapetti monofenolisia yhdisteitä ja ristisilloitti kaseiinia selvästi tehokkaimmin. Myös *P. sanguineus* -tyrosinaasi kykeni polymeroimaan kaseiinia. Kun reaktioseokseen lisättiin pienimolekyylipainoista di-fenolia, kaikki tyrosinaasit kykenivät ristisilloittamaan kaseiinia.

T. reesei -tyrosinaasia ja *T. hirsuta* -lakkaasia verrattiin suhteessa niiden kykyyn katalysoida polymerointireaktioita α-kaseiinin ja ksylaanin fenolisten sivuryhmien kautta. Tyrosinaasi kykeni polymeroimaan α-kaseiinia lakkaasia paremmin. Toisaalta lakkaasi kykeni polymeroimaan ksylaania, mutta tyrosinaasi ei. Huolimatta havaituista eroista sekä lakkaasin että tyrosinaasin osoitettiin muodostavan heteroristisidoksia maitoproteiinin ja ksylaanin välillä. Vehnäleivonnassa molemmat entsyymit lisäsivät leivän ominaistilavuutta sekä pehmeyttä. Lakkaasin vaikutus johtui ilmeisesti pääosin vehnän arabinoksylaanin ristisiloittumisesta ja tyrosinaasin gluteeniproteiinien ristisilloitumisesta.

Saavutettujen tulosten perusteella voidaan todeta, että *T. reesei* -tyrosinaasi ja *T. hirsuta* -lakkaasi soveltuvat elintarvikkeiden rakenne- ja funktionaalisten ominaisuuksien muokkaamiseen.

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