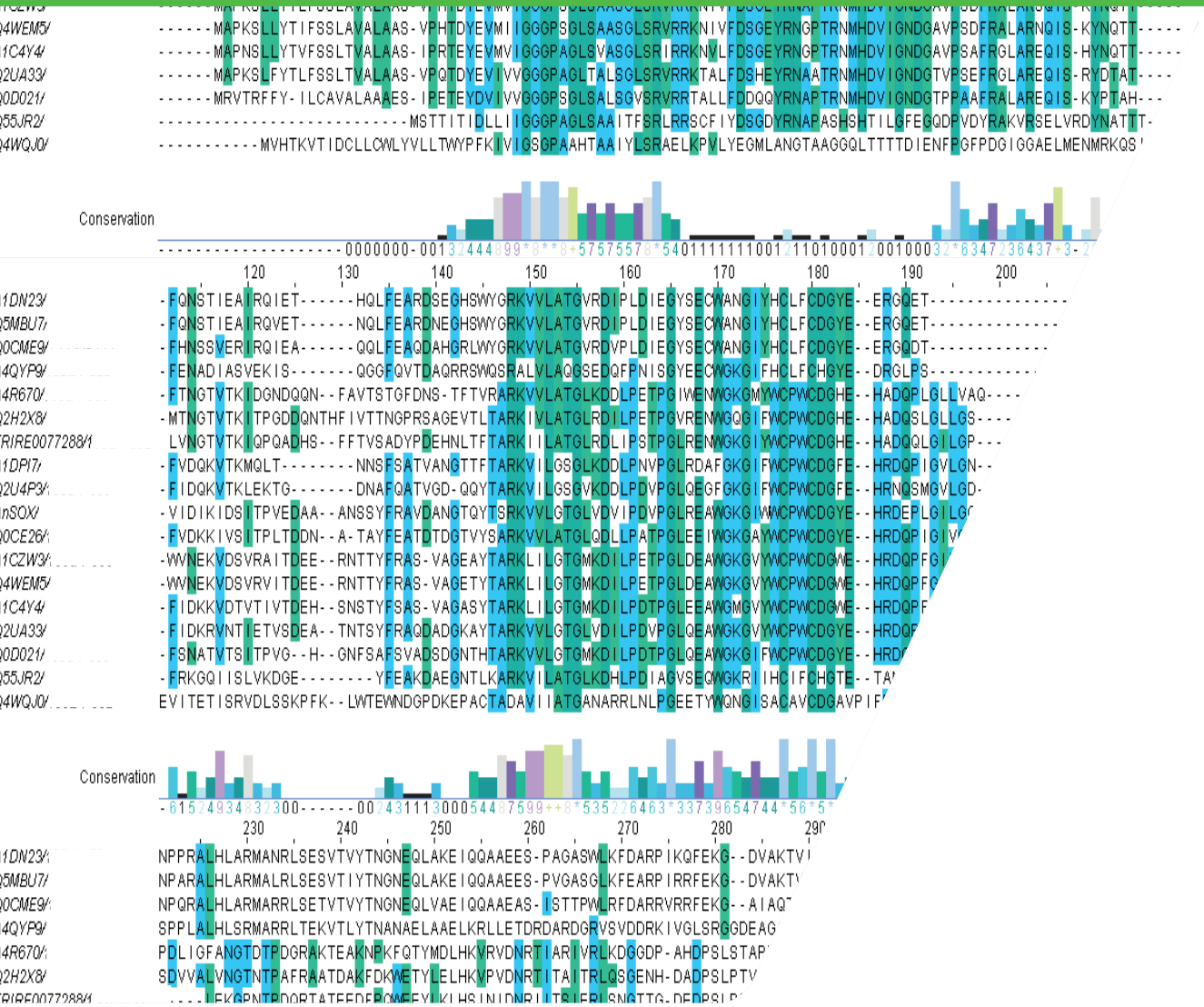

Greta Faccio

Discovery of oxidative enzymes for food engineering

| Tyrosinase and sulfhydryl oxidase

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Discovery of oxidative enzymes for food engineering

Tyrosinase and sulfhydryl oxidase

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Department of Biosciences – Division of Genetics

ACADEMIC DISSERTATION

University of Helsinki
Helsinki, Finland

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Abstract

Enzymes offer many advantages in industrial processes, such as high specificity, mild treatment conditions and low energy requirements. Therefore, the industry has exploited them in many sectors including food processing. Enzymes can modify food properties by acting on small molecules or on polymers such as carbohydrates or proteins. Crosslinking enzymes such as tyrosinases and sulfhydryl oxidases catalyse the formation of novel covalent bonds between specific residues in proteins and/or peptides, thus forming or modifying the protein network of food.

In this study, novel secreted fungal proteins with sequence features typical of tyrosinases and sulfhydryl oxidases were identified through a genome mining study. Representatives of both of these enzyme families were selected for heterologous production in the filamentous fungus *Trichoderma reesei* and biochemical characterisation.

Firstly, a novel family of putative tyrosinases carrying a shorter sequence than the previously characterised tyrosinases was discovered. These proteins lacked the whole linker and C-terminal domain that possibly play a role in cofactor incorporation, folding or protein activity. One of these proteins, AoCO4 from *Aspergillus oryzae,* was produced in *T. reesei* with a production level of about 1.5 g/l. The enzyme AoCO4 was correctly folded and bound the copper cofactors with a type-3 copper centre. However, the enzyme had only a low level of activity with the phenolic substrates tested. Highest activity was obtained with 4-*tert*-butylcatechol. Since tyrosine was not a substrate for AoCO4, the enzyme was classified as catechol oxidase.

Secondly, the genome analysis for secreted proteins with sequence features typical of flavin-dependent sulfhydryl oxidases pinpointed two previously uncharacterised proteins AoSOX1 and AoSOX2 from *A. oryzae*. These two novel sulfhydryl oxidases were produced in *T. reesei* with production levels of 70 and 180 mg/l, respectively, in shake flask cultivations. AoSOX1 and AoSOX2 were

FAD-dependent enzymes with a dimeric tertiary structure and they both showed activity on small sulfhydryl compounds such as glutathione and dithiothreitol, and were drastically inhibited by zinc sulphate. AoSOX2 showed good stability to thermal and chemical denaturation, being superior to AoSOX1 in this respect.
Thirdly, the suitability of AoSOX1 as a possible baking improver was elucidated. The effect of AoSOX1, alone and in combination with the widely used improver ascorbic acid was tested on yeasted wheat dough, both fresh and frozen, and on fresh water-flour dough. In all cases, AoSOX1 had no effect on the fermentation properties of fresh yeasted dough. AoSOX1 negatively affected the fermentation properties of frozen doughs and accelerated the damaging effects of the frozen storage, i.e. giving a softer dough with poorer gas retention abilities than the control. In combination with ascorbic acid, AoSOX1 gave harder doughs. In accordance, rheological studies in yeast-free dough showed that the presence of only AoSOX1 resulted in weaker and more extensible dough whereas a dough with opposite properties was obtained if ascorbic acid was also used. Doughs containing ascorbic acid and increasing amounts of AoSOX1 were harder in a dose-dependent manner. Sulfhydryl oxidase AoSOX1 had an enhancing effect on the dough hardening mechanism of ascorbic acid. This was ascribed mainly to the production of hydrogen peroxide in the SOX reaction which is able to convert the ascorbic acid to the actual improver dehydroascorbic acid. In addition, AoSOX1 could possibly oxidise the free glutathione in the dough and thus prevent the loss of dough strength caused by the spontaneous reduction of the disulfide bonds constituting the dough protein network. Sulfhydryl oxidase AoSOX1 is therefore able to enhance the action of ascorbic acid in wheat dough and could potentially be applied in wheat dough baking.

Preface

This study was carried out at VTT Technical Research Centre of Finland in the protein production team from August 2006 to May 2011. The study was conducted for the first three years with financial support of the Marie Curie mobility actions as part of the EU project “Enzymatic tailoring of polymer interactions in food matrix” (MEST-CT-2005-020924) and subsequently with the financial support of the Finnish Cultural Foundation.

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Contents

Academic dissertation

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List of publications

I. Gasparetti, C.[*], Faccio, G.[*], Arvas, M., Buchert, J., Saloheimo, M. & Kruus, K. Discovery of a new tyrosinase-like enzyme family lacking a C-terminally processed domain: production and characterization of an *Aspergillus oryzae* catechol oxidase. Applied Microbiology and Biotechnology 2010; 86(1):213–26. doi: 10.1007/s00253-009-2258-3. [*]These authors equally contributed to the work.

II. Faccio, G., Kruus, K., Buchert, J. & Saloheimo, M. Secreted fungal sulfhydryl oxidases: sequence analysis and characterisation of a representative flavin-dependent enzyme from *Aspergillus oryzae*. BMC Biochemistry 2010; 11(1):31. doi:10.1186/1471-2091-11-31.

III. Faccio, G., Kruus, K., Buchert, J. & Saloheimo, M. Production and characterisation of AoSOX2 from *Aspergillus oryzae*, a novel flavin-dependent sulfhydryl oxidase with good pH and temperature stability. Applied Microbiology and Biotechnology 2011, 90(3): 941–949. doi: 10.1007/s00253-011-3129-2.

IV. Faccio, G., Flander, L., Buchert, J., Saloheimo, M. & Nordlund, E. Sulfhydryl oxidase enhances the effects of ascorbic acid in wheat dough, Journal of Cereal Science (submitted manuscript).

Author’s contribution

I. The author was responsible for the genome mining study, interpretation of the results and heterologous expression of the novel catechol oxidase that was performed under the supervision of Doc. Markku Saloheimo. The phylogenetic analysis was performed by Mikko Arvas. Chiara Gasparetti was responsible for the purification and biochemical characterisation of the enzyme. The author and Chiara Gasparetti co-drafted the manuscript that was finalised with the contribution of all the authors.

II. The author was responsible for the genome mining study, heterologous expression and purification of the novel sulfhydryl oxidase. The biochemical characterisation was performed by the author under the supervision of Prof. Kristiina Kruus. The author had the main responsibility in writing the publication

III. The author was responsible for the cloning and heterologous expression of the novel sulfhydryl oxidase. The purification and biochemical characterisation of the enzyme was performed by the author under the supervision of Prof. Kristiina Kruus. The author had the main responsibility in writing the publication.

IV. The author was responsible for the experimental work and data interpretation. The author planned the work and interpreted the results together with Dr. Emilia Nordlund and Dr. Laura Flander. The author had the main responsibility in writing the publication that was finalised with the contribution of all the authors.

List of symbols and abbreviations

aa	Amino acids
AA	Ascorbic Acid
AoCO4	Catechol oxidase Q2UNF9 from *Aspergillus oryzae*
AoSOX1	Sulfhydryl oxidase Q2UA33 from *Aspergillus oryzae*
AoSOX2	Sulfhydryl oxidase Q2U4P3 from *Aspergillus oryzae*
Cm	Melting concentration
CO	Catechol oxidase (1,2-benzenediol:oxygen oxidoreductase, EC 1.10.3.1)
CuA, CuB	Copper coordination sites A and B of tyrosinases
dhAA	Dehydroascorbic acid
DNTB	Elman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EC number	Enzyme Commission numbers assigned by IUPAC-IUBMB (www.chem.qmul.ac.uk/iupac/jcbn)
ER	Endoplasmic reticulum
Erv	Protein Essential for Respiration and Viability

GRAS	Generally Regarded as Safe
GSH	Reduced glutathione (tripeptide ECG)
GSSG	Oxidised glutathione (disulfide)
LACC	Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2)
L-DOPA	L-3,4-dihydroxyphenylalanine
MW	Molecular weight
ORF	Open reading frame
PPO	Polyphenol oxidase (monophenol monooxygenase EC 1.14.18.1)
P-SH	Free protein-associated thiol group
P-SS-P'	Inter-protein disulfide bond
QSOX	Flavin-dependant quiescin-sulfhydryl oxidase
ROS	Reactive Oxygen Species
SOX	Sulfhydryl oxidase (glutathione:oxygen oxidoreductase, EC 1.8.3.3)
TBC	4-*tert*-butylcatechol
TYR	Tyrosinase (monophenol, *o*-diphenol/oxygen oxidoreductase, EC 1.14.18.1)
Tm	Melting temperature
Trx	Thioredoxin domain

1. Introduction

1.1 Industrial enzymes

Enzymes are protein molecules responsible for the catalysis of the majority of the reactions occurring in living organisms. The wide variety of enzymes available in nature provides a rich reservoir of reactions that can be potentially exploited for industrial purposes. A large number of enzymes are known and each catalyses efficiently a specific reaction. Enzymes offer a wide range of advantages in industrial applications (Figure 1).

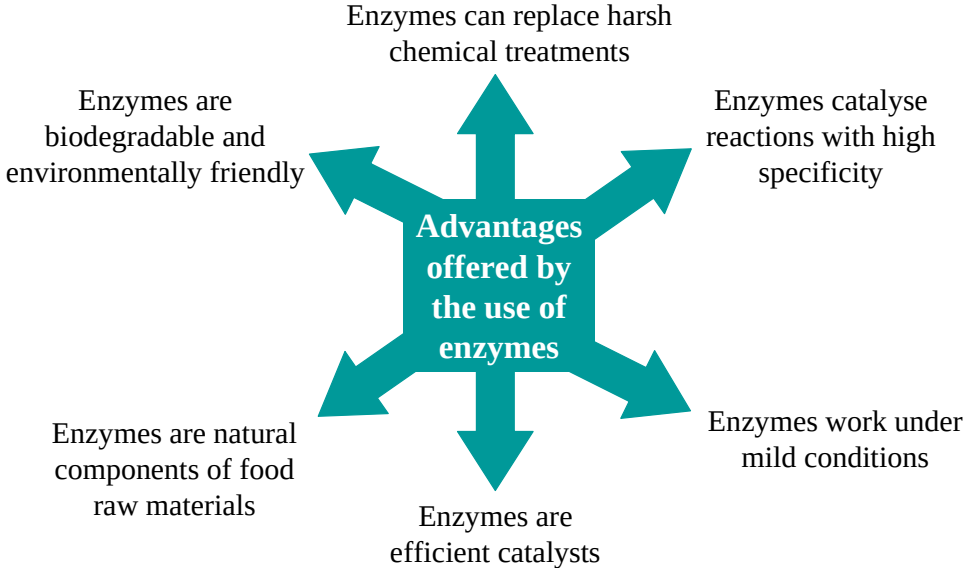

Figure 1. Schematic summary of the advantages offered by the use of enzymes in industrial processes.

The use of enzymes can affect both the economical and environmental aspects of the application. For example in detergents, enzymes provide a faster and improved cleaning effect at lower temperature and with less water required (Olsen, 2004). Additionally, enzymes working in similar conditions, e.g. pH and temperature, but catalysing different transformations can be utilised simultaneously (Olsen, 2004). The first enzyme commercialised for cleaning purposes was trypsin in 1913, although with limited success (Aunstrup & Andresen, 1972). Enzymes caught on in the detergent industry only in the 1960s when a more efficient and alkaline tolerant protease was isolated from *Bacillus* (Aunstrup & Andresen, 1972). Various classes of enzymes are nowadays included in detergents, including proteases, lipases, amylases and cellulases.

Due to their high specificity and rate of catalysis, enzymes are not needed in large amounts and their action can easily be controlled by changing the process conditions, e.g. temperature and pH. The decreased need for chemicals and the lower costs associated with energy consumption and waste treatment can be the main economical reasons for using enzymes. Finally, the production of enzymes in recombinant form, the isolation of more robust enzymes (Zamost et al., 1991) or their optimisation for the process by protein engineering have made them available at an economically feasible cost.

It is noteworthy that some enzymes can also work in organic solvents and in a wide range of pH and temperatures, for example the production of the antibiotics ampicillin and cephalosporin involves the use of an acylase in the presence of organic cosolvents (Illanes et al., 2009, Illanes et al., 2004). Enzymes have also found application in industrial organic synthesis, in which their regio- and stereospecificity, ensures the resolution of racemic solutions, e.g. production of the L-isomer of the amino acids serine and valine, without undesired secondary products (Iborra et al., 1992, Chibata et al., 1976).

1.1.1 Industrial enzymes in food applications

The use of enzymes in food applications dates back to more than 7000 years ago, when the first cheese was produced using the gastric chymosin solution of calves. The advantages offered by the use of enzymes have long been exploited by the food industry in many fields such as the production of cheese and other dairy products, starch processing, brewing, and fruit and wine processing (Table 1).

Nowadays enzymes are applied to different stages of food production in order to modify the raw material, facilitate the processing steps or improve the quality

of the final product with respect to colour, aroma, texture or stability (Finkelstein & Christopher, 1992). Enzymes can be added directly to the product, as in the case of rennet in cheese production (Kumar et al., 2010), or indirectly, by using suitable enzyme-producing microbial strains, as in the case of fungi of the *Penicillium* genus in cheese production, e.g. *P. roqueforti*.

Table 1. Some examples of enzymes of commercial importance in food applications and their main features.

Enzyme	**Classification**	**Organism**	**Mode of action**	**Application**	**Reference**
glucose oxidase	EC 1.1.3.4	*Aspergillus niger*, *Penicillium spp.*	production of hydrogen peroxide and gluconic acid from glucose and oxygen	baking, egg processing	(Bonet et al., 2006, Sisak et al., 2006)
trans-glutaminase	EC 2.3.2.13	*Streptomyces spp.*, *Bacillus subtilis*	incorporation of amines in proteins, crosslinking, deamidation	meat, fish, dairy, wheat and soybean products	(Motoki & Seguro, 1998)
fructosyl-transferase	EC 2.4.1.9	*Aspergillus oryzae*, *Bacillus spp.*	fructosyl group transfer	production of sweeteners	(Nam et al., 2000, Sangeetha et al., 2005)
lipase	EC 3.1.1.3	*Candida rugosa*, *Aspergillus spp.*, *Rhizopus niveus*	hydrolysis of triglycerides and transesterification of lipids	olive oil, aromas, cholesterol-lowering additives	(Meyer, 2010, Contesini et al., 2010, Weber et al., 2002, Liu et al., 2009)
cellulase	endoglucanase EC 3.2.1.4	*Trichoderma reesei*, *Aspergillus oryzae*	hydrolysis of cellulose	fruit juices coffee	(Kapasakalidis et al., 2009, Delgado et al., 2008, Szakacs-Dobozi et al., 1988, Ghorai et al., 2009)

Enzyme	Classification	Organism	Mode of action	Application	Reference
xylanase	EC 3.2.1.8	*Trichoderma spp.*, *Aspergillus spp.*	hydrolysis of xylan	baking juice and wine	(Haros et al., 2002, Polizeli et al., 2005, Marron et al., 2001, Hilhorst et al., 2002)
β-galactosidase	EC 3.2.1.23	*Streptococcus thermophilus*, *Kluyveromyces spp*, *Aspergillus spp.*	hydrolysis of lactose	dairy, digestive supplement	(Rhimi et al., 2010, O'Connell & Walsh, 2007, O'Connell & Walsh, 2008, Husain, 2010)
amylolytic enzymes	α-amylase EC 3.2.1.1, glucoamylase EC 3.2.1.33	*Aspergillus oryzae*, *Aspergillus awamori*, *Aspergillus niger*, *Rhizopus spp.*	degradation of starch	baking, brewing	(Bamforth, 2009, Mondal & Datta, 2008)
protease	endopeptidases EC 3.4 21-99, exopeptidases EC 3.4.11-19	*Aspergillus oryzae*, *Rhizomucor spp.*	hydrolysis of proteins and peptides	cheese, beer, cookies	(Kirk, 2002, Kennedy & Pike, 1981, Kara et al., 2005)
pectinolytic enzymes	polygalacturonase EC 3.2.1.15, pectate lyase EC 4.2.2.2, pectin lyase EC 4.2.2.10	*Aspergillus spp*, *Rhizopus spp.*	degradation of pectin from plant biomass	fruit juice, coffee and tea	(Whitaker, et al., 2002, Whitaker, 1984, Hoondal et al., 2002)
glucose isomerase	EC 5.3.1.5	*Streptomyces spp.*, *Bacillus spp.*, *Aspergillus oryzae*	isomerization of D-glucose to D-fructose and D-xylose to D-xylulose	high fructose corn syrup	(Bhosale et al., 1996, Bennett & Yeager, 2010, Asboth & Naray-Szabo, 2000)

Enzymes from almost all EC-classes have found potential application in the food industry (Table 1). Oxidoreductases (EC 1) such as hexose oxidases and glucose oxidase are used in baking as dough improvers. Members of the transferase class

(EC 2) such as fructosyltransferase can be employed in the production of sweeteners, and transglutaminase is utilised in the preparation of fish and meat products. The class of hydrolases (EC 3) includes proteases, α-amylases and glucoamylases that are used in bread and beer production in order to increase the amount of fermentable sugars and peptides and boost yeast fermentation (Table 1). Proteases and pectinases are also applied in brewing to clear the cloudiness of chilled beer and remove the haze or to improve the yield in juice making, respectively. The class of isomerases (EC 5) is represented by glucose isomerase that is used for the production of D-fructose, a sweetener suitable for people with diabetes (Asboth & Naray-Szabo, 2000). Recently, L-arabinose isomerase has been suggested for application in the production of the sweetener D-tagatose (Rhimi et al., In press).

The addition of enzymes to food raw materials can aim at decreasing the degree of polymerization of the substrates present, e.g. polypeptides and polysaccharides, or at modifying the food components, as in the case of crosslinking enzymes (see next section) or to make a specific conversion, e.g. glucose isomerase.

1.1.2 Enzymes with crosslinking activity in food applications

The use of crosslinking enzymes represents a novel approach to the improvement of the structure and texture of food by increasing the number of covalent bonds between its polymeric components, i.e. carbohydrates or proteins (Table 2).

Crosslinking enzymes such as transglutaminase, tyrosinase, laccase, peroxidase and sulfhydryl oxidase have been investigated in cereal, dairy, meat and fish processing (Table 2, for a review see Buchert et al., 2010). The enzyme glucose oxidase has also been reported to have crosslinking activity on wheat proteins, although not acting directly on proteins but through the production of hydrogen peroxide (Rasiah et al., 2005).

The modification of food proteins via crosslinking affects not only the texture of food but also their digestibility (Monogioudi et al., 2011). Crosslinking has also been reported to decrease the allergenicity of certain proteins (Tantoush et al., 2011, Chung et al., 2004, Stanic et al., 2010, Monogioudi et al., 2011, Gerrard & Sutton, 2005, Tan et al., 2011).

Table 2. Enzymes with reported protein crosslinking activity and examples of their application.

Enzyme	**Classification**	**Organism**	**Mode of action (target aa)**	**Application**	**References**
sulfhydryl oxidase	EC 1.8.3.3	*Aspergillus niger, Penicillium sp. K-6*	formation of disulfide bonds (C)	baking	(Kusakabe et al., 1982, Haarasilta et al., 1991, Haarasilta & Vaisanen, 1989)
laccase	EC 1.10.3.2	*Trametes hirsuta, Polyporus pinsitus*	oxidation of aromatic compounds and cysteine (W, Y, C)	baking, juice and brewing, dairy, reduction of allergenicity	(Selinheimo et al., 2008, Whitehurst & Van Oort, 2009, Faergemand et al., 1998, Ercili Cura et al., 2009, Tantoush et al., 2011)
peroxidase	EC 1.11.1.7	*Coprinus cinereus, Cochlearia armoracia* (horseradish)	oxidation of aromatic compounds (Y)	baking, dairy, reduction of allergenicity	(Faergemand et al., 1998, Takasaki et al., 2005, Matheis & Whitaker, 1984, Chung et al., 2004)
tyrosinase	EC 1.14.18.1	*Agaricus bisporus, Neurospora crassa* and *Trichoderma reesei*	oxidation of phenolic compounds (Y)	dairy, baking, meat, reduction of allergenicity	(Lantto et al., 2007, Onwulata & Tomasula, 2008; 2010, Selinheimo et al., 2007, Stanic et al., 2010, Selinheimo, 2008)
trans-glutaminase	EC 2.3.2.13	*Streptomyces spp.* and *Bacillus subtilis*	formation of isopeptide bonds (Q, K)	dairy, meat and cereal products	(Yokoyama et al., 2004, Santos & Torne, 2009, Hamada, 1994)

1.1.3 Enzymes for the production of bakery products

The bakery industry has taken advantage of enzymes to improve the properties of the dough and of the final baked product, i.e. dough handling properties, bread volume, crumb structure, and shelf life. The most common raw material for bakery products is wheat flour, the major components of which are starch (70–75%), proteins involved in the formation of the gluten structure (10–15%), non-starch polysaccharides (2–3%) and lipids (1.5–2.5%) (Goesaert et al., 2005). The quality of wheat-based products is highly dependent on the behaviour of these components of flour during the dough preparation and the baking phase (Goesaert et al., 2005).

Exogenous enzymes can be added to modify the flour components and thus the rheological properties of dough and bread. The most used enzymes in baking belong to the family of hydrolases (EC 3) and are active on the starch, the proteins or the cell wall polysaccharides. For example, polysaccharide-degrading enzymes such as α-amylase and pentosanases have been shown to significantly improve the volume and the firmness of bread, and have an anti-staling effect (Goesaert et al., 2005, Lagrain et al., 2008, Caballero et al., 2007).

The use of a single enzyme is rarely able to bring about the desired effect on bread and therefore a combination of different enzymes is generally used (Caballero et al., 2007, Caballero et al., 2006). The actions of the different types of enzymes used in the preparation of bakery products are summarized in Table 3.

Table 3. Enzymes with potential or existing applications in breadmaking, their mode of action and some of their effects on dough.

Enzyme	Organism	Mode of action	Effect	Reference
amylase	*Aspergillus oryzae, Bacillus spp.*	degradation of starch, production of sugars for yeast	improves bread volume, softness, firmness	(Goesaert et al., 2005, Kim et al., 2006, Cherl-Ho, 2009)
hemicellulase	*Trichoderma spp., Aspergillus spp., Bacillus spp.*	hydrolysis of arabinoxylans	improves bread volume and dough strength	(Dagdelen & Gocmen, 2007, Morita et al., 1998)
lipase	*Aspergillus oryzae, Aspergillus niger*	hydrolysis of triglycerides	increases dough stability, bread volume, texture and shelf-life	(Whitehurst & Van Oort, 2009, Gélinas et al., 1998)
tyrosinase	*Trichodserma reesei, Agaricus bisporus*	oxidation of small phenolic compounds, crosslinking of gluten proteins	strengthens the dough, softens the bread crumb and increases the bread volume	(Selinheimo et al., 2007, Takasaki et al., 2001)
laccase	*Trametes hirsuta*	probable cross-linking of water-extractable arabinoxylans	increases dough strength and firmness of oat bread, reduces dough extensibility	(Selinheimo et al., 2007, Flander et al., 2008)
glucose oxidase	*Aspergillus niger, Aspergillus oryzae*	formation of protein cross-links and oxidative gelation of pentosans	increases dough strength and the bread specific volume, decreases crumb hardness	(Rosell et al., 2003, Bonet et al., 2006, Vemulapalli et al., 1998, Hanft & Koehler, 2006)
lipoxygenase	*Glycine max* (soybean)	not clear, oxidation of proteins by the lipid oxidation products	whitens bread colour, improves dough rheology and bread volume	(Junqueira et al., 2007, Tsen & Hlynka, 1963)
sulfhydryl oxidase	*Aspergillus niger*	oxidation of glutathione, possible formation of crosslinked gluten fractions	increases bread volume and strengthens the dough if combined to glucose oxidase or hemicellulase	(Haarasilta & Vaisanen, 1989, Kaufman & Fennema, 1987, Souppe, 2000)
protease	*Aspergillus oryzae, Aspergillus niger*	hydrolysis of proteins	enhances biscuit flavour and colour, decreases dough-strength	(Kara et al., 2005, Mathewson, 2000)

The baking properties are also affected by the endogenous enzymes of the flour, i.e. α-amylases, β-amylases, proteases, peptidases, hemicellulases and oxidases, even if present at low concentrations (Sproessler, 1993). The endogenous enzymes of the flour also play a key role in the improving effect of exogenous chemical additives, as in the case of ascorbic acid (Every, 1999a, Every, 1999b). Ascorbic acid (vitamin C) is currently used as a dough improver. Potassium bromate was previously widely used but was withdrawn due to its possible carcinogenic effect (Kurokawa et al., 1990). The action of ascorbic acid mainly relies on the enzymatic activity of two endogenous enzymes present in the flour, i.e. ascorbic acid oxidase and glutathione dehydrogenase (Grosch & Wieser, 1999). At first, ascorbic acid is oxidised to dehydroascorbic acid either non-enzymatically, by the action of iron and copper ions, or by the endogenous ascorbic acid oxidase. Subsequently, the enzyme glutathione dehydrogenase has been shown to use the dehydroascorbic acid as electron acceptor in the oxidation of the reduced glutathione present in the flour (Walther & Grosch, 1987). The level of reduced glutathione available to attack the inter-glutenin disulfide bonds and weaken the gluten structure is thus decreased.

Enzymes and additives are generally utilised not alone but in different combinations in order to tailor their improving effect on the characteristics of the flour to be utilised and to guarantee constant quality of the final product (Joye et al., 2009).

1.2 Tyrosinase and catechol oxidase

Tyrosinase (EC 1.14.18.1) and catechol oxidase (EC 1.10.3.1) are structurally similar enzymes belonging to the type-3 copper proteins, a group also including the oxygen carrier protein haemocyanin (Halaouli et al., 2006).

Tyrosinases catalyse the *o*-hydroxylation of monophenolic (monophenolase activity, Figure 2 reaction 1) and diphenolic compounds (diphenolase activity or catechol oxidase activity, Figure 2 reaction 2) to the corresponding *o*-quinones and concomitantly reduce molecular oxygen to water. Enzymes catalysing only the second reaction (Figure 2 reaction 2) are called catechol oxidases and only the catalytic activity allows their distinction from tyrosinases.

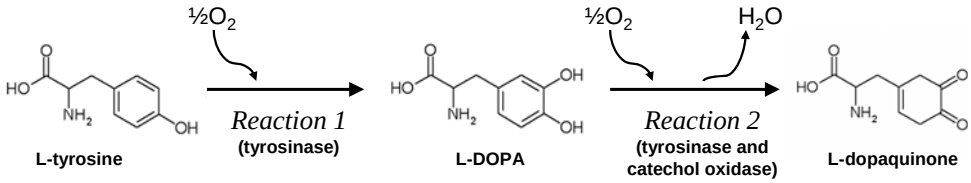

Figure 2. Reactions catalysed by tyrosinase (reactions 1 and 2) and catechol oxidase (reaction 2).

The term ‘polyphenol oxidase’ is sometimes used to designate tyrosinases and catechol oxidases, as well as laccases, without distinction between these enzymes (Marusek et al., 2006, Flurkey et al., 2008, Gerdemann et al., 2002, Flurkey & Inlow, 2008). This is due to the overlap of their substrate specificities. For example, some plant catechol oxidases have a weak monooxygenase activity although they do not accept tyrosine as a substrate (Gerdemann et al., 2002, Mayer & Harel, 1979, Walker & Ferrar, 1998).

Tyrosinases have been investigated in many applications, e.g. in the production of plant-derived food products such as fermented tea leaves, cocoa, and raisins (Seo et al., 2003), in baking (Selinheimo et al., 2008, Lantto et al., 2007), in dairy products (Ercili Cura et al., 2010) and in meat processing (Lantto et al., 2007). Furthermore, tyrosinases have been used for the grafting of silk proteins onto chitosan (Anghileri et al., 2007, Freddi et al., 2006) and for the determination of phenols in wine (Jewell & Ebeler, 2001).

1.2.1 Distribution and physiological role

Tyrosinases and catechol oxidases are widely distributed enzymes and have been isolated from a wide range of organisms from mammals to bacteria (Mayer and Harel, 1979, Mayer, 2006, van Gelder et al., 1997, Lerch, 1983, Halaouli et al., 2006, Kwon et al., 1987, Claus & Decker, 2006). Representative tyrosinases and catechol oxidases identified from various sources are listed in Table 4.

Table 4. Examples of tyrosinases (TYR), catechol oxidases (CO) and polyphenol oxidases (PPO) characterised from different species of various organisms.

Source	**Enzyme**	**Identifier, length (aa)**	**Organism**	**Features**	**References**
bacterium	TYR	Q83WS2, 273	*Streptomyces castaneoglobisporus*	crystal structure in complex with caddie protein ORF378 (PDB: 1WX5)	(Matoba et al., 2006, Kohashi et al., 2004)
bacterium	TYR	NP659960, 609	*Rhizobium etli*	involved in resistance against ROS and phenolic compounds of plant defensive response	(Pinero et al., 2007, Cabrera-Valladares et al., 2006)
bacterium	TYR	ZP_02925214, 518	*Verrucomicrobium spinosum*	the first bacterial tyrosinase with a C-terminal domain	(Fairhead & Thony-Meyer, 2010)
fungus	TYR	CAL90884, 561	*Trichoderma reesei*	the first secreted fungal tyrosinase, C-terminally processed, has crosslinking activity	(Selinheimo et al., 2006, Mattinen et al., 2008)
fungus	TYR	C11562, 556; C59432, 568	*Agaricus bisporus* (button mushroom)	isolated from fruiting bodies, C-terminally processed	(Wichers et al., 1996, Wichers et al., 2003)
plant	TYR	B21677, 593	*Malus x domestica* (apple)	solubilized and proteolyzed during ripening and storage	(Haruta et al., 1998, Murata et al., 1997)
plant	PPO	P93622, 607	*Vitis vinifera* (grapes)	catechol oxidase activity, crystal structure available (PDB: 2P3X1),	(Virador et al., 2010)
plant	CO	Q9ZP19, 496	*Ipomoea batatas* (sweet potato)	involved in wound response, crystal structure available (PDB: 1BT1),	(Klabunde et al., 1998, Eicken et al., 1998, Gerdemann et al., 2001)
animal	TYR	AAB60319, 548	*Homo sapiens*	involved in albinism, vitiligo, melanoma	(Kwon et al., 1987, Jin et al., 2010, Chintamaneni et al., 1991)

Tyrosinases have been reported as both intracellular and secreted enzymes. Examples of intracellular enzymes are those involved in melanogenesis, such as the mammalian (Jimbow et al., 2000), the two fungal enzymes from *Agaricus bisporus* (Wichers et al., 1996, Wichers et al., 2003) and the enzyme from apple that is localised in the plastids (Murata et al., 1997). The bacterial tyrosinases from *Streptomyces* species (Claus & Decker, 2006) and the fungal enzyme from *Trichoderma reesei* (Selinheimo et al., 2006) are secreted (Table 4).

The physiological role of tyrosinases is related to melanin biosynthesis, especially in fungi (Schallreuter et al., 2008, Olivares & Solano, 2009). In fungi, melanins are involved in defence mechanisms against stress factors such as UV or gamma radiation, free radicals, dehydration and extreme temperatures (Halaouli et al., 2006, Riley, 2003, Butler & Day, 1998, Nosanchuk & Casadevall, 2003, Bell & Wheeler, 1986). The stability of fungal spores also benefits from the protective role of melanins (Mayer & Harel, 1979). In addition, tyrosinases are associated with wound healing, with the immune response in plants (van Gelder et al., 1997, Cerenius & Söderhäll, 2004, Muller et al., 2004) and with sclerotization of the cuticle in insects (Terwilliger, 1999, Marmaras et al., 1996). In humans, tyrosinase is involved in the pigmentation in melanocytes (Jin et al., 2010, Schallreuter et al., 2011). Tyrosinase has also been tested as a marker in melanoma patients (Gradilone et al., 2010, Schweikardt et al., 2007) and as a target for the activation of pro-drugs (Jawaid et al., 2009).

1.2.2 Biochemical and molecular properties

Tyrosinases are typically composed of three main domains comprising an N-terminal domain, a central catalytic domain, containing the two copper binding sites (CuA and CuB) and a C-terminal domain connected to the catalytic domain by an unstructured linker region (Figure 3).

Tyrosinases are generally described as monomeric enzymes. The secreted tyrosinases identified from *Streptomyces* species are monomeric (Claus & Decker, 2006), whereas the recently resolved structure of the tyrosinase from *Bacillus megaterium* revealed a dimeric quaternary structure (Sendovski et al., 2011). Evidence for a multimeric structure is available for the tyrosinase from *A. bisporus* that was reported to be a tetramer of 120 kDa, although this has recently been debated (Flurkey & Inlow, 2008, Kim & Uyama, 2005).

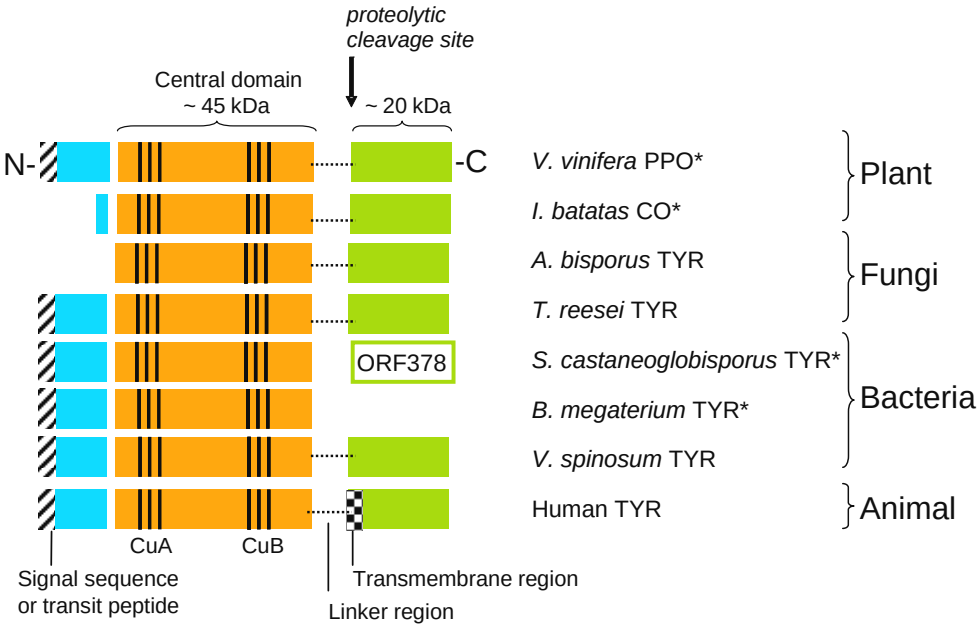

Figure 3. Domain organisation of representative tyrosinases and catechol oxidases from different organisms.The N-terminal domain is in blue and diagonal lines indicate the presence of a signal sequence or transit peptide. The central domain is in orange and vertical lines indicate the CuA and CuB sites. The C-terminal domain is in green and a checkered box indicates the presence of a transmembrane region. An arrow indicates the cleavage site for the release of the C-terminal domain. The caddie protein ORF378 co-crystallised with the TYR from *S. castaneoglobisporus* is boxed. Proteins for which the three-dimensional structure is available are marked by an asterisk (for references see Table 4). The molecular masses are approximate (Flurkey & Inlow, 2008) and the relative sizes are not to scale.

Tyrosinases and catechol oxidases isolated in an active form generally have a molecular weight around 40 kDa, whereas enzymes in the inactive latent form generally have a MW about 60 kDa (Flurkey & Inlow, 2008). The difference in molecular weight has been ascribed to N- or C-terminal proteolytic processing during activation and to the release of the C-terminal domain (Marusek et al., 2006, Flurkey & Inlow, 2008).

The role of the C-terminal domain of tyrosinases and catechol oxidases has long been debated and often supposed to be essential for copper incorporation and correct folding. The first three-dimensional structure of a tyrosinase was that of the enzyme isolated from *S. castaneoglobisporus* (Matoba et al., 2006). This enzyme lacks the C-terminal domain and could be produced in active from only

when co-expressed with a second protein of the same operon that favoured the incorporation of copper (Matoba et al., 2006); a similar role was thus suggested for the C-terminal domain of other tyrosinases. In contrast, the tyrosinases from *B. megaterium* and *Rhizobium etli*, both lacking the C-terminal domain, could be produced in an active form without the assistance of a caddie protein (Kohashi et al., 2004, Cabrera-Valladares et al., 2006, Sendovski et al., 2011). The lack of the C-terminal domain is not common to all bacterial tyrosinases and *Verrucomicrobium spinosum* tyrosinase has been reported to contain the C-terminal domain (Fairhead & Thony-Meyer, 2010).

The type-3 copper protein haemocyanin from *Octopus dofleini* is structurally similar to the catechol oxidase from *Ipomoea batatas*, except for the presence of a C-terminal domain that is absent in the active crystallised form of the catechol oxidase (Gerdemann et al., 2002). In haemocyanins such as that from *O. dofleini*, the C-terminal domain covers the active site, preventing the binding of substrate molecules and any catalytic activity but allowing the binding of molecular oxygen (Cuff et al., 1998). Haemocyanins have been reported to acquire polyphenol oxidase activity after proteolytic treatment (Decker & Tuczek, 2000).

Some tyrosinases have been isolated in an inactive form that can undergo activation upon loosening of their structure by controlled denaturation, e.g. by temperature (Gest & Horowitz, 1958), sodium dodecyl sulphate or proteases (Wan et al., 2009, Wittenberg & Triplett, 1985, Cabanes et al., 2007, Gandia-Herrero et al., 2005b, Gandia-Herrero et al., 2005a, Gandia-Herrero et al., 2004, Lai et al., 2005, Laveda et al., 2001). Tyrosinases characterised both in the latent and active form include those from *A. bisporus* (Flurkey & Inlow, 2008), *Vicia faba* (Robinson & Dry, 1992, Flurkey, 1989) and *Vitis vinifera* (Rathjen & Robinson, 1992).

Tyrosinases and catechol oxidases are active on a wide range of phenolic substrates (Table 5). Tyrosinases and catechol oxidases oxidise diphenolic compounds such as D/L-DOPA, catechol, dopamine, caffeic acid and *ortho*-diphenols, whereas monophenolic compounds such as D/L-tyrosine, phenol, guaiacol, p-coumaric acid and tyramine, can be substrates only for tyrosinases (Table 5). The reaction products are *ortho*-quinones that may further react non-enzymatically towards the formation of melanins (Prota, 1988).

Table 5. Biochemical and molecular properties of some tyrosinases and catechol oxidases from different organisms.

Source	**Enzyme**	**Organism**	**Substrate**	**References**
bacterium	TYR	*Streptomyces castaneoglobisporus*	Monophenols: L-tyrosine. Diphenols: L-DOPA. Others: NR.	(Matoba et al., 2006, Kohashi et al., 2004, Ikeda et al., 1996)
bacterium	TYR	*Rhizobium etli*	Monophenols: L-tyrosine, n-acetyl-L-tyrosine. Diphenols: L-DOPA, catechol, caffeic acid. Others: NR.	(Cabrera-Valladares et al., 2006)
bacterium	TYR	*Verrucomicrobium spinosum*	Monophenols: L-tyrosine. Diphenols: L-DOPA. Others: NR.	(Fairhead, Thony-Meyer, 2010)
fungus	TYR	*Trichoderma reesei*	Monophenols : tyramine, phenol, L-tyrosine, *p*-cresol, *p*-tyrosol, *p*-coumaric acid. Diphenols: L-DOPA, caffeic acid, catechol. Others: pyrogallol, caseins, (−)-epicatechin, (+)-catechin, gliadins, peptides.	(Selinheimo et al., 2009, Selinheimo et al., 2006)
fungus	TYR	*Agaricus bisporus* (button mushroom)	Monophenols L-tyrosine, phenol, *p*-cresol, tyramine, *p*-tyrosol. Diphenols: D/L-DOPA, caffeic acid, catechol. Others: ellagic acid, (−)-epicatechin, (+)-catechin, pyrogallol, peptides	(Selinheimo, 2008, Selinheimo et al., 2009, Selinheimo et al., 2007)
plant	TYR	*Malus x domestica* (apple)	Monophenols: L-tyrosine, *p*-cresol, tyramine, *p*-tyrosol. Diphenols: L-DOPA, catechol, methylcatechol, caffeic acid. Others: chlorogenic acid, (+)-catechin, (−)-epicatechin, pyrogallol.	(Selinheimo et al., 2009, Janovitz-Klapp et al., 1989, Selinheimo et al., 2007)
plant	PPO	*Vitis vinifera* (grapes)	Monophenols: NR. Diphenols: catechol, 4-*tert*-butylcatechol, 4-methylcatechol. Others: NR.	(Virador et al., 2010, Sanchez-Ferrer et al., 1988)
plant	CO	*Ipomoea batatas* (sweet potato)	Monophenols: NR. Diphenols: catechol, caffeic acid, 4-methylcatechol, L-DOPA. Others: NR.	(Eicken et al., 1998)
animal	TYR	*Homo sapiens*	Monophenols :L-tyrosine. Diphenols: L-DOPA.Others: NR.	(Takara et al., 2008, Okombi et al., 2006)

Abbreviations: NR, not reported

The tyrosinases from *T. reesei* and *A. bisporus* are also active on tyrosine-containing peptides and moreover the former enzyme is able to polymerize random coil proteins such as α- and β-caseins from milk and gliadin from wheat (Selinheimo, 2008, Mattinen et al., 2008, Monogioudi et al., 2009).

The crosslinking activity of tyrosinases is due to the non-enzymatic reaction of the oxidised products of tyrosine and other substrate phenols with lysyl, tyrosyl, cysteinyl and histidinyl residues in proteins. As a result, di-tyrosine, tyrosine-cysteine and tyrosine-lysine couplings are produced (Bittner, 2006, Ito & Prota, 1977, Ito et al., 1984, Land et al., 2004, McDowell et al., 1999). Tyrosinases can crosslink peptides and proteins in milk, meat and cereals (Lantto et al., 2007, Selinheimo et al., 2007, Ercili Cura et al., 2010, Freddi et al., 2006, Mattinen et al., 2008, Aberg et al., 2004, Halaouli et al., 2005).

Tyrosinases and catechol oxidases with various physico-chemical features have been reported from various organisms. These enzymes generally have a pH optimum in the neutral or slightly acidic range (Figure 4). The tyrosinase from *T. reesei* and the catechol oxidase from *I. batatas* have a basic pH optimua of 9 and 8, respectively (Selinheimo et al., 2006, Eicken et al., 1998).

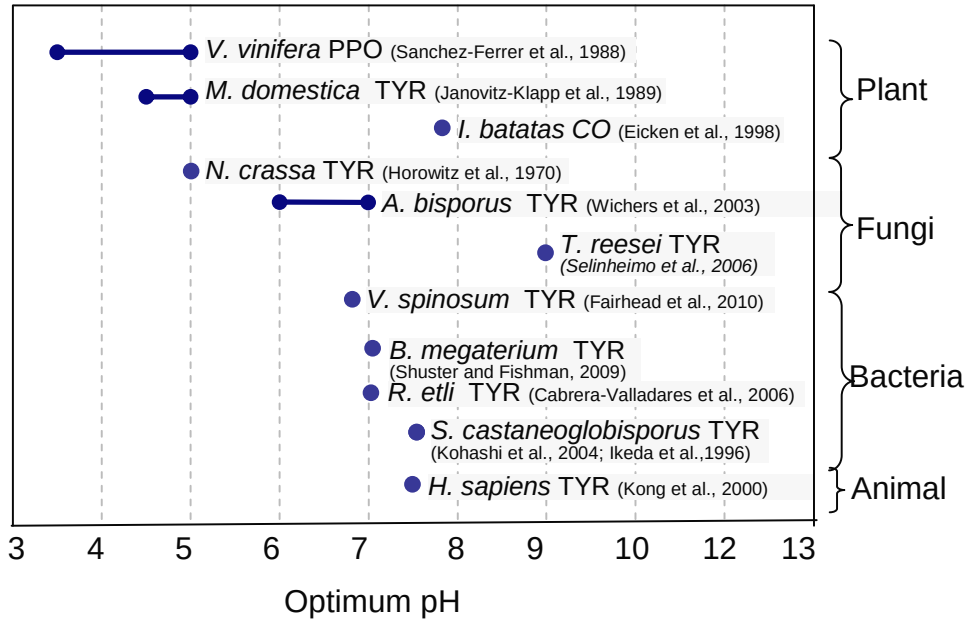

Figure 4. Optimum pH values of catechol oxidases and tyrosinases from different sources.

Generally, tyrosinases are assayed for phenol oxidation activity at a temperature of 25–30°C. However, tyrosinases and catechol oxidases with significantly higher temperature optima have also been reported (Figure 5). For example, tyrosinases with optima at 65°C and 75°C have been isolated from *Pycnoporus sanguineus* and *Bacillus thuringiensis*, respectively.

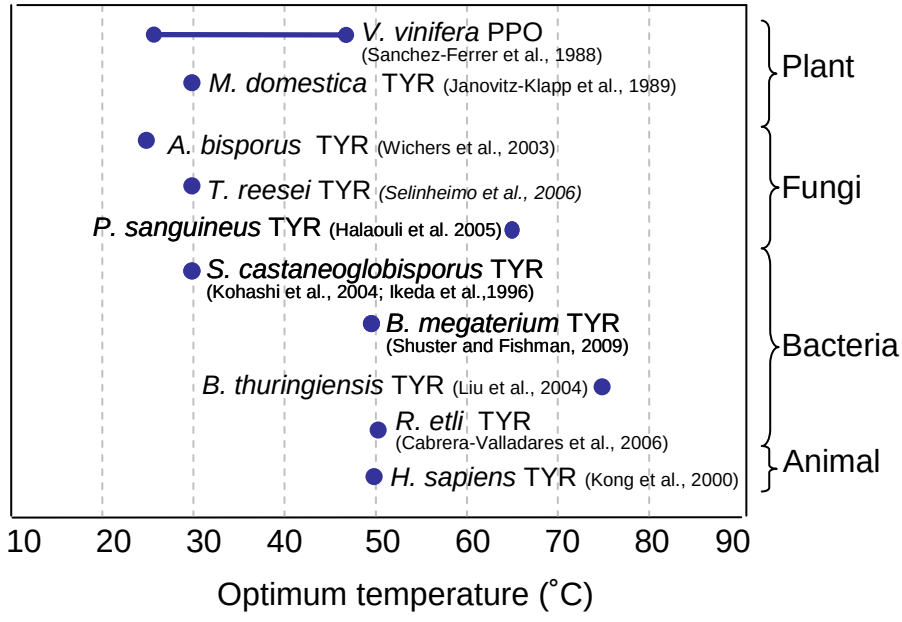

Figure 5. Optimum temperature of representative tyrosinases and catechol oxidases from different sources.

1.2.3 Sequence features

Sequence analysis studies and the available three-dimensional structures of type-3 copper proteins have allowed identification of the key primary structure features necessary for the correct folding and activity of tyrosinases.

A thioether bridge found in the proximity of the cofactor binding site has been proposed to confer rigidity to the structure (Matoba et al., 2006, Decker et al., 2006). This link has been detected between a cysteine residue (underlined in Table 6 and shown in Figure 6) and the second histidine residue of the CuA site in the haemocyanin from *Octopus dofloeini*, the tyrosinase from *Neurospora crassa* and the catechol oxidase from *Ipomoea batatas* (Klabunde et al., 1998, Cuff et al., 1998, Merkel et al., 2005, Lerch, 1982).

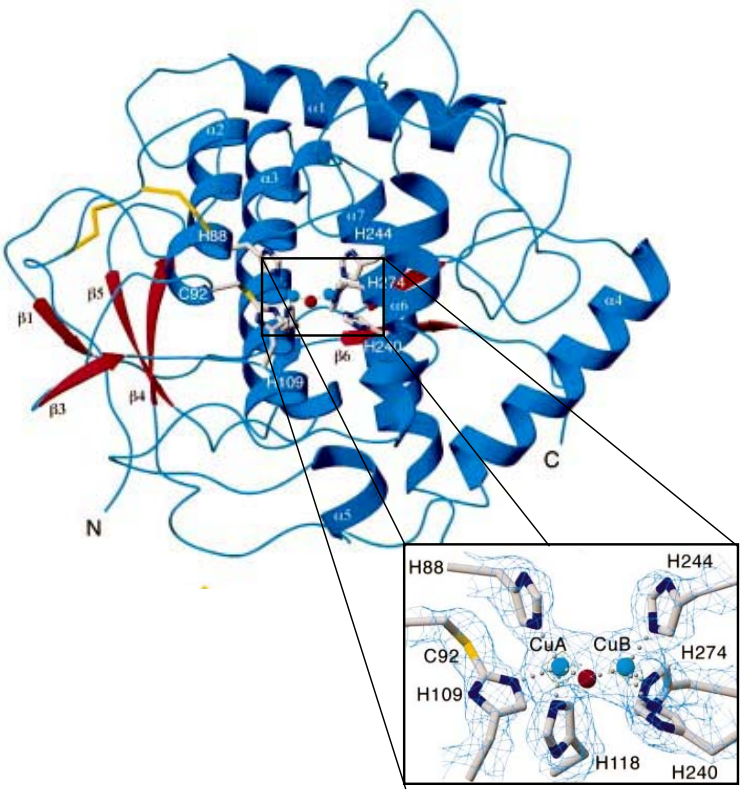

Figure 6. Ribbon representation of the three-dimensional structure of the catechol oxidase from *Ipomoea batatas* highlighting α-helices in blue, β-strands in red and disulfide bonds in yellow. In the inset, the red atom between the two copper binding sites is probably a hydroxide ion from the solvent (modified from Klabunde et al., 1998).

The main sequence feature of type-3 copper proteins such as tyrosinases and catechol oxidases (Decker & Tuczek, 2000) is the presence of two groups of three histidines in a conserved motif; these residues are involved in the binding of the two copper ion cofactors at the CuA and CuB sites (Decker, 2006) (Figure 6, inset). A summary of these features and the corresponding residues in the catechol oxidase from *Ipomoea batatas* is presented in Table 6.

Flurkey and co-authors (Flurkey & Inlow, 2008) identified in type-3 copper proteins the motifs marking the central globular domain left after N- and C-terminal processing. Their study suggested a key role for a conserved N-terminal arginine residue and for the C-terminal tyrosine motif (Table 6). These landmarks interact with each other, thus joining the N-terminal and the C-terminal

ends of the protein, and are located in a short β-strand both in *O. dofleini* haemocyanin and *Ipomoea batatas* catechol oxidase (Marusek et al., 2006, Flurkey & Inlow, 2008).

A closer look at the catalytic centre of the three-dimensional structures of type-3 copper proteins identified residues that could be involved in the determination of the different substrate specificities of tyrosinases and catechol oxidases. In tyrosinases, monophenolic compounds are docked to the CuA site and in the catechol oxidase from *Ipomoea batatas* the space surrounding the CuA is occupied by a phenylalanine residue (F_{261}, gate residue, Table 6). In haemocyanins the active site is completely occupied by a leucine or a phenylalanine in the protein from *O. dofleini* (L_{2830}) and *Limulu polyphemus* (F_{49}), respectively.

Table 6. Key conserved residues characteristic of type-3 copper proteins such as tyrosinases and catechol oxidases.

Sequence motif	**Position in *Ipomoea batatas* CO**	**Function**	**References**
N-terminal region			
R	R_{49}	interacts with the tyrosine motif	(Marusek et al., 2006, Flurkey & Inlow, 2008)
Central region			
H1A-X(n)-C-X(n)-H2A-X(8)-H3A	H_{88}-X(3)-C_{92}-X(16)-H_{109}-X8-H_{118}	CuA binding site	(Merkel et al., 2005, García-Borrón & Solano, 2002)
H1B-X(3)-H2B-X(n)-H3B	H_{240}-X(3)-H_{244}-X(29)-H_{274}	CuB binding site	(Merkel et al., 2005, García-Borrón & Solano, 2002)
HA3-X(n)-B-P-Φ-Φ-(D/N)-Φ and HB3-X(n)G-Y-X-Y	H_{118}-X(21)-L_{140}-P-F-W-N-W_{145} and H_{247}-X(55)-G_{330}-Y-K-Y_{333}	necessary for the globular structure	(García-Borrón & Solano, 2002)
F or L	F_{261}	gate residue	(Eicken et al., 1998)
R_{HA3+1}, E_{HA3+8}, D_{HB3-7}, D_{HB3+4}, B_{HB3+3}, B_{HB3+6}	R_{119}, E_{126}, D_{267}, D_{278}, V_{277}, M_{280}	possibly necessary for the folding	(García-Borrón & Solano, 2002)
$Φ_{HA1-7}$, F_{HA3-4}, $Φ_{HA3-1}$, $Φ_{H3+3}$, L_{HA3+4}, $Φ_{HA3+7}$, F_{HB3-4}, H_{HB3-1}, $Φ_{HB3+7}$,	F_{81}, F_{114}, F_{117}, Y_{121}, L_{122}, Y_{125}, F_{270}, H_{273}, W_{281}	aromatic shell around CuA and CuB	(García-Borrón & Solano, 2002)
C-terminal region			
Y/F-X-Y orY-X-Y/F	Y_{331}-K-Y_{333}	tyrosine motif	(Marusek et al., 2006)
Abbreviations: X, any residue, Φ, aromatic residue; B, hydrophobic residue.			

1.3 Sulfhydryl oxidase

Sulfhydryl oxidases (glutathione oxidase, EC 1.8.3.3) are enzymes catalysing the oxidation of thiol groups to disulfide bonds with the reduction of one molecule of oxygen to hydrogen peroxide (Figure 7). The classification of these enzymes is not well established and thiol oxidases (EC. 1.8.3.2) are also sometimes denominated sulfhydryl oxidases although their reaction produces water (Neufeld et al., 1958, Aurbach & Jakoby, 1962). Characteristic of the active site of thiol:disulfide oxidoreductases such as sulfhydryl oxidases is a reactive di-cysteine C-X-X-C motif, in which X is any amino acid.

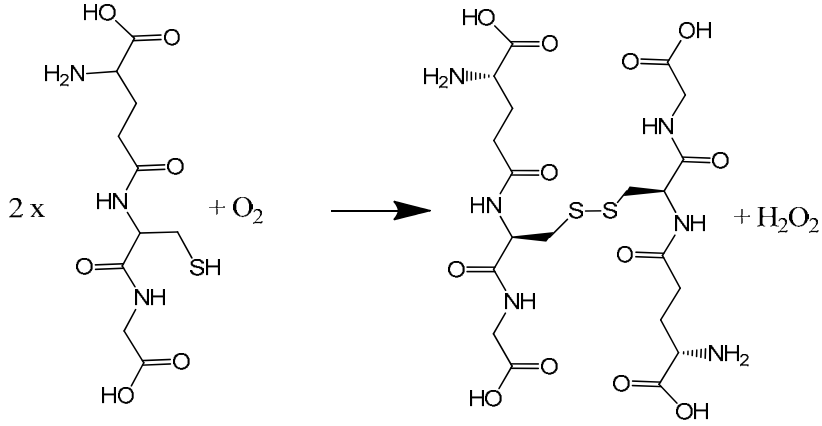

Figure 7. Oxidation of glutathione catalysed by sulfhydryl oxidase (EC 1.8.3.3).

Typical substrates for sulfhydryl oxidases are small thiol compounds, such as cysteine, dithiothreitiol and β-mercaptoethanol, and cysteine-containing peptides. Sulfhydryl oxidases are generally flavoenzymes, binding one molecule of FAD per subunit. Metal-dependent sulfhydryl oxidases have also been reported but the presence of metal ions has recently been attributed to adventitious binding (Brohawn et al., 2003). Whereas dithiothreitiol is a product of chemical synthesis (Evans et al., 1949, Cleland, 1964), glutathione is the most abundant small thiol compound produced in the cell (Forman et al., 2009). In the cell, glutathione is found in the cytoplasm in a 1–10 mM concentration (Meister, 1988). Glutathione at high concentration can become toxic and in organisms such as yeast and fungi a specific glutathione transporter is responsible for its secretion. Gluta-

thione is also secreted, mainly in the reduced form (Meister, 1988). The presence of surfactants and a low pH value of 3.5 induce the fungus *S. cerevisiae* to activate the secretion of glutathione (Perrone et al., 2005). Extracellular glutathione plays a protective role against reactive oxygen species and in humans low levels of glutathione are associated with tissue inflammation for example in cystic fibrosis patients (Winterbourn & Brennan, 1997, Kelly, 1999, Roum et al., 1993). Secreted glutathione has been reported to constitute a source of cysteines for mouse fibroblasts (Hanigan & Ricketts, 1993) and a defence mechanism in fungi for the chelation of metals such as cadmium and nickel (Joho et al., 1995, Courbot et al., 2004).

1.3.1 Distribution and physiological role

Enzymes with sulfhydryl oxidase activity have been reported both intracellularrly and in secreted form from bacterial, viral, fungal, plant and animal sources (Table 7).

Intracellular sulfhydryl oxidases of the Ero1 and Erv families are localised in the endoplasmic reticulum or mitochondrial intermembrane space and are involved in the oxidative folding of proteins. They are flavin-dependent and possess a di-cysteine motif either in the C- or N-terminal region, in addition to the central di-cysteine motif at the catalytic site (Fass, 2008). Multi-domain sulfhydryl oxidases have also been described and belong to the QSOX family. These enzymes comprise a thioredoxin and an Erv-type domain and can be involved in the intracellular oxidative folding of proteins or are secreted (Table 7).

Secreted sulfhydryl oxidases comprising one single domain have also been reported from fungi, but they do not share significant similarity with the other reported enzymes of the quiescin-sulfhydryl oxidase (QSOX) and Erv-families. They are more related to pyridine nucleotide–disulfide oxidoreductases (Thorpe et al., 2002, Hoober, 1999).

Table 7. Representative sulfhydryl oxidases from the literature, their localisation and possible physiological role.

Source	Enzyme	Organism	Location	Physiol. role	References
virus	E10R	*Vaccinia virus*	cytosol	oxidative folding of virion proteins	(Senkevich et al., 2000)
bacterium	FAD-dependent pyridine nucleotide-disulfide oxidoreductase	*Chromobacterium violaceum*	cytoplasm	biosynthesis of the anticancer agent FK228	(Wang et al., 2009, Cheng et al., 2007)
fungus	GSH oxidase	*Fusarium spp.*	extracellular	NR	(Kusakabe et al., 1983)
fungus	SOX	*Aspergillus niger*	extracellular	NR	(de la Motte &Wagner, 1987, Vignaud et al., 2002, Hammer et al., 1990)
fungus	thiol oxidases eroA and ervA	*Aspergillus niger*	ER	oxidative protein folding	(Harvey et al., 2010)
fungus	GSH oxidase	*Penicillium sp. K-6-5*	extracellular	NR	(Kusakabe et al., 1982)
fungus	Erv1	*Sacchar-myces cerevisiae*	IMS	oxidative protein folding	(Bien et al., 2010, Ang & Lu, 2009, Lange, 2001, Lee, 2000)
fungus	Erv2	*Saccharomyces cerevisiae*	ER	oxidative protein folding	(Wang et al., 2007, Vala et al., 2005, Gross et al., 2002, Gerber et al., 2001)

Source	Enzyme	Organism	Location	Physiol. role	References
fungus	Ero1	*Saccharomyces cerevisiae*	ER	oxidative protein folding	(Vitu et al., 2010, Gross et al., 2004, Frand & Kaiser, 1999, Hiniker & Bardwell, 2004)
plant	Erv1	*Arabidopsis thaliana*	IMS	promotes the import and oxidative folding of proteins	(Allen et al., 2005)
animal	QSOX	*Rattus norvegicus*	extracellular (seminal vesicle)	NR	(Ostrowski & Kistler, 1980, Ostrowski et al., 1979)
animal	QSOX	*Rattus norvegicus*	extracellular (skin)	sulfhydryl oxidation of proteins of epidermis	(Matsuba et al., 2002, Hashimoto et al., 2001, Hashimoto et al., 2000)
animal	QSOX	*Homo sapiens*	Golgi apparatus	oxidative protein folding	(Chakravarthi et al., 2007, Heckler et al., 2008)
animal	QSOX	*Bos taurus*	extracellular (milk)	oxidative protein folding	(Jaje et al., 2007, Zanata et al., 2005)
animal	QSOX	*Gallus gallus*	extracellular (egg)	protein disulfide bond formation	(Thorpe et al., 2002, Hoober et al., 1996, Hoober et al., 1999)

Abbreviations: ER, endoplasmic reticulum, IMS, mitochondiral intermembrane space.

No clear role has yet been established for extracellular sulfhydryl oxidases. However, sulfhydryl oxidases have been suggested to be involved in the maturation of proteins along the secretory pathway (Tury et al., 2006) and in the formation of the extracellular matrix (Hoober, 1999, Tury et al., 2006). In addition, the production of hydrogen peroxide by sulfhydryl oxidases could have antimi-

crobial functions (Ostrowski & Kistler, 1980). Moreover, sulfhydryl oxidases might be involved in the synthesis of bioactive compounds such as non-ribosomal peptides (Wang et al., 2009).

1.3.2 Biochemical and molecular properties

Sulfhydryl oxidases have been reported from various sources, and different cellular compartments and secreted sulfhydryl oxidases such as that from *A. niger* (de la Motte & Wagner, 1987) have also been isolated. Few secreted sulfhydryl oxidases have been reported and thus the knowledge of their biochemical features, optimal activity conditions and physiological roles is rather limited. The most studied secreted sulfhydryl oxidases (Table 8) are the enzymes from *Penicillium* spp. and from *Aspergillus niger* (Kusakabe et al., 1982, de la Motte & Wagner, 1987).

Despite the evident similarities between the sulfhydryl oxidases that are secreted and the well-characterised enzymes of the QSOX family, e.g. both are secreted FAD-dependent and catalyse the oxidation of thiols, they have been reported to have a distinct evolutionary origin (Hoober, 1999). Moreover, the QSOX enzyme from chicken egg prefers reduced proteins as substrates whereas the fungal enzyme from *A. niger* (de la Motte & Wagner, 1987) is preferably active on small thiol compounds. The secreted fungal enzyme has a molecular weight of 53 kDa (de la Motte & Wagner, 1987) whereas enzymes belonging to the quiescin-sulfhydryl oxidase group (QSOX) have higher molecular weight around 80 kDa (Hoober, 1999) and are composed of two domains, e.g. a thioredoxin and an domain with structural similarities to ERV/ALR proteins.

The secreted fungal sulfhydryl oxidases reported in the literature are mainly active on small thiol compounds such as dithiothreitol, whereas the enzymes isolated from chicken egg and bovine milk also exhibit activity on peptides and protein-associated sulfhydryl groups (Table 8). The optimum pH conditions for the activity of these secreted sulfhydryl oxidases is in the neutral range, except for the enzyme from *A. niger* (Table 8).

Table 8. Secreted flavin-dependent sulfhydryl oxidases reported in the literature and their physico-chemical characteristics.

Organism	Enzyme	Biochemical features			Reference
		MW (kDa)	pH optimum	Substrates	
Aspergillus niger	SOX	53 (dimer)	5.0–5.5	DTT, GSH, cysteine, homocysteine, β-mercaptoethanol, reduced proteins (ribonuclease A), peptides (CG, EG)	(de la Motte & Wagner, 1987, Vignaud et al., 2002)
Bos taurus (bovine milk)	QSOX	62	7.5	DTT, D/L-cysteine, N-acetyl-L-cysteine, cysteamine	(Jaje et al., 2007, Sliwkowski et al., 1984)
Gallus gallus (egg white)	QSOX	80 (dimer)	7.0–8.0	GSH, DTT, cysteine, β-mercaptoethanol, proteins (ribonuclease A, lysozyme, riboflavin-binding protein, ovalbumin, aldolase, pyruvate kinase, insulin A and B chains), peptides (N-acetyl-EAQCGTS)	(Hoober et al., 1996, Hoober et al., 1999)
Penicillium sp. K-6-5	GSH oxidase	47	7.0–7.8	GSH, D/L-cysteine, N-acetyl-L-cysteine, L-cysteine methylester, thiophenol, DTT	(Kusakabe et al., 1982)

Abbreviations: MW, molecular weight of the subunit.

Concerning catalytic and structural stability, the sulfhydryl oxidase secreted by *A. niger* is reported to bind tightly the flavin cofactor since only irreversible denaturation allowed its release. This enzyme is also characterised by significant stability in acidic conditions (overnight at pH 3) (de la Motte & Wagner, 1987). The enzyme isolated from Penicillium cultures retained full activity after incubation for 30 minutes at temperatures up to 55°C (Kusakabe et al., 1982).

1.3.3 Sequence features

Flavin-dependent sulfhydryl oxidases have various conserved sequence features important for cofactor binding and catalytic activity. Sulfhydryl oxidases are thiol:disulfide oxidoreductases and their active site is characterised by two reac-

tive cysteine residues forming a C-X-X-C motif (Quan et al., 2007, Chivers et al., 1997).

The reported secreted sulfhydryl oxidases (Table 8) are flavin-dependent enzymes. Proteins binding nucleotides such as FAD or NAD are generally characterised by the α/ß fold named after Michael Rossmann (Rossmann et al., 1974, Rao & Rossmann, 1973). The Rossmann fold was first described in lactate dehydrogenase and indicates a nucleotide-binding domain found mainly in oxidoreductases (Kleiger & Eisenberg, 2002). This fold is formed by α and ß structures in a ß-α-ß-α-ß arrangement where the interaction between the first ß-strand and the following α-helix is stabilised by a G-X(3)-G/A (Table 9) sequence motif in the helix and a G-X(3)-G-X-X-G sequence motif in the connecting loop (Kleiger & Eisenberg, 2002). The sequence features characteristic of flavin-dependent sulfhydryl oxidases are summarised in Table 9.

Table 9. Conserved sequence motifs characterising flavin-dependent thiol:disulfide oxidoreductases and their position in the model enzyme from *A. niger*.

Residues	**Position in the SOX from *Aspergillus niger***	**Description**	**Reference**
V/I-X-G-X(1-2)- G-X(2)-G-X(3)-G/A	V_{31}-V-G-G-G-P-A-G-L-S-V-L_{42}	motif of the Rossman fold	(Kleiger & Eisenberg, 2002, Vallon, 2000)
R-X-G-G-R-X(2)-S/T	--	GG motif immediately after the dinucleotide binding site	(Kleiger & Eisenberg, 2002, Vallon, 2000)
O-O-B(3)-A-T-G	R_{132}-K-V-V-L-G-T-G_{139}	ATG motif found in flavoproteins with two dinucleotide binding domain	(Vallon, 2000)
T-X(4)-B-ϕ-B(2)-G-D	T_{309}-S-L-S-G-V-Y-A-V-G-D_{319}	typical of FAD-binding proteins of the GSH reductase family	(Eggink et al., 1990)
C-X-X-C	C_{161}-P-W-C_{164}	active site of thiol:disulfide oxidoreductases	(Chivers et al., 1997)

Abbreviations: X, any residue, B hydrophobic residue, ϕ,aromatic residue, O, charged residue.

1.4 Production of enzymes in filamentous fungi

The saprobic lifestyle of fungi has led to their ability to secrete enzymes at a high level. Fungi naturally secrete enzymes of industrial interest such as the cellulose-degrading enzymes produced by *T. reesei*, e.g. cellulases and hemicellulases, and the amylolytic enzymes produced by *Aspergilli* (Finkelstein & Christopher, 1992). The efficient secretory machinery of fungi has been exploited for the production of both homologous and heterologous enzymes. With the development of gene technology, the promoter and terminator regions of highly expressed genes have been exploited to drive the production of heterologous protein candidates in high amounts, e.g. the cellobiohydrolase I *cbhI* promoter in *T. reesei* (Keranen & Penttila, 1995) and the glucoamylase *glaA* promoter in *A. niger* are examples of heavily used promoters (Finkelstein & Christopher, 1992, Fowler et al., 1990).

The cultivation of fungi can be performed in inexpensive media in large culture volumes and give high production levels in optimal conditions. The production of hydrolases by *T. reesei* can reach amounts exceeding 100 grams per litre (Cherry & Fidantsef, 2003) and a production level of more than 20 grams of glucoamylase per litre has been obtained in *A. niger* (Berka et al., 1991). The secretion of the target protein by fungi has the benefit of facilitating downstream processing since neither breakage of the cells nor separation of the target protein from the intracellular proteins are required.

However, production levels may be much lower for non-fungal proteins, even only milligrams per litre, making the system in some cases not suitable for large-scale industrial production (Gouka et al., 1997). Different approaches have been developed in order to improve the production of heterologous proteins by fungal hosts, such as (a) use of a stronger host-specific promoter (Marui et al., 2010), (b) engineering of the promoter to enhance the binding of positive transcriptional regulators (Liu et al., 2003), (c) increasing the gene copy number (Shiba et al., 2001), (d) use of a fusion construct with a highly expressed secreted native protein (Nyyssonen et al., 1993) such as the cellulose *cbhI* from *T. reesei* and the *A. niger* glucoamylase *gluA*, (e) codon optimisation of the gene of interest (Shumiao et al., 2010, Tokuoka et al., 2008), (f) selection of a protease-free host (Yoon et al., 2011), (g) control of fungal morphology and fermentation conditions to reduce culture viscosity (Dai et al., 2004, Talabardon & Yang, 2005).

The production of enzymes, especially those aimed at the food industry, in filamentous fungi is a well established process. Fungi from different *genera* are

exploited and many have been shown to be non-pathogenic for healthy individuals and have been given the GRAS status (Generally Regarded As Safe), e.g. *A. oryzae* and *T. reesei*. Some of them do however secrete low levels of toxic secondary metabolites under certain cultivation conditions, e.g. *A. niger*, *A. oryzae* and *Fusarium venenatum* (Olempska-Beer et al., 2006).

Knowledge of the physiology of filamentous fungi is continuously improving, as is understanding of the molecular basis of enzyme production. The use of enzymes and their production using filamentous fungi are already widely used in industrial scale. However, well-known enzymes can be employed in new applications and new enzymatic activities can be discovered, opening the doors to completely new production processes.

2. Aims of the study

The aim of the study was to discover novel oxidative enzymes potentially able to crosslink proteins. The target enzymes were tyrosinases and sulfhydryl oxidases, enzymes with a potential application in the industrial food production. By analysing the fungal genomes available, proteins with sequence features characteristic of tyrosinases and sulfhydryl oxidases were identified and several representatives were selected for heterologous production in the filamentous fungus *Trichoderma reesei*. The novel enzymes produced were then biochemically characterised with regard to activity and stability. The potential industrial application of the enzymes was evaluated.

The detailed aims of the study are:

1. Identification of novel tyrosinases and sulfhydryl oxidases through analysis of the available fungal genomes (Publications I and II)
2. Heterologous production of a potential tyrosinase in the filamentous fungus *T. reesei* and biochemical characterisation of the enzyme (Publication I)
3. Heterologous production of two potential sulfhydryl oxidases in the filamentous fungus *T. reesei* and their biochemical characterisation (Publications II and III)
4. Evaluation of the application potential of one sulfhydryl oxidase as an improver in wheat dough alone and in combination with ascorbic acid (Publication IV).

3. Materials and methods

Table 10. Methods employed in this study, purpose of their utilization and the publication in which they are presented in more detail.

Method	Aim	Described in
Analysis of the fungal genomes	- identification of candidate proteins - evaluate the distribution of a target protein	I, II
Alignment of protein sequences	- determination of the level of identity; prediction of the signal peptide and identification of conserved residues	I,II,III
Extraction of genomic DNA and polymerase-chain reaction	- target gene amplification and cloning - selection of positive *Trichoderma reesei* transformants	I, II, III I, II, III
Transformation of *Trichoderma reesei* and selection of the antibiotic-resistant transformants	- heterologous expression of the target protein and isolation of positive transformants	I, II, III
Cultivation of *Trichoderma reesei* transformants on plates containing tyrosine	- selection of the transformant with highest tyrosinase/catechol oxidase production	I
UV-vis spectroscopy	- measurement of tyrosinase/catechol oxidase activity - detection of sulfhydryl oxidase activity by colorimetric assay with Elman's reagent (DNTB) - determination of the flavoenzymatic nature of the sulfhydryl oxidases and identification of the cofactor - measurement of ascorbic acid oxidation - measurement of sulfhydryl content of wheat flour	I II, III II, III IV IV

Fluorescence spectroscopy	-	measurement of sulfhydryl oxidase activity by HVA-peroxidase coupled assay	II, III
	-	determination of protein intrinsic fluorescence	I, II, III
	-	monitoring of protein unfolding in the presence of denaturant	II, III
(with HPLC chromatography)	-	conversion of the results of HVA-peroxidase coupled assay to nkatals per millilitre	II
Oxygen-consumption measurement	-	measurement of sulfhydryl oxidase activity	II, III
	-	inhibition study of sulfhydryl oxidases	II, III
SDS PAGE analysis	-	evaluation of the level of expression of the heterologous protein and of the level of purity of protein solutions	I, II, III
Western Blot	-	detection of the protein tag	II, III
Isoelectrofocusing	-	determination of the isoelectric point	I
Ion-exchange and size-exclusion chromatography	-	purification of AoSOX1 and AoSOX2	II, III
Circular dichroism	-	evaluation of the secondary structure of the enzyme and determination of the melting temperature	I, II, III
Moisture-air oven method	-	determination flour moisture	IV
Farinograph	-	determination of optimal water absorption of the flour	IV
Kjeldahl-method	-	protein content determination of the flour	IV
Data analysis and graphics (Origin 7.5 software)	-	linear and non-linear fitting of data for determination of kinetic constants of the enzymes	I, II, III

4. Results and discussion

4.1 Discovery of tyrosinases (Publication I)

4.1.1 Fungal genome analysis for the identification of potential tyrosinases

The distribution of genes coding for potentially secreted tyrosinases was analysed in the genome sequences of fungi in order to identify novel candidate enzymes with biochemical features of interest for industrial use.

An in-house database containing the publicly available genome sequences of 30 fungi (Arvas et al., 2007) was searched for families of proteins containing at least a tyrosinase Interpro entry (IPR002227) and 134 such proteins with an N-terminal signal sequence were retrieved. Two conserved three-histidine motifs, characteristic of type-3 copper proteins such as tyrosinases and catechol oxidases (HA1-X(n)-HA2-X(8)-HA3 and HB1-X(3)-HB2-X(n)-HB3 pattern for the CuA and CuB site), were present in 114 of the sequences retrieved. The alignment of the sequences allowed the identification of conserved residues previously reported in the literature as typical of tyrosinases (Table 6) (Gerdemann et al., 2002, Flurkey & Inlow, 2008, Klabunde et al., 1998, Cuff et al., 1998, García-Borrón & Solano, 2002).

All the sequences analysed carried the landmarks of the central globular domain of tyrosinases such as the C-terminal tyrosine motif Y/FXY and a conserved N-terminal arginine residue aligning to the residue R40 of the tyrosinase from *Trichoderma reesei* (Flurkey et al., 2008). The length of the sequences retrieved as described above and in Publication I, was first analysed in order to find extremely long or short sequences due to a incorrect ORF prediction during genome analysis. The length distribution of the sequences retrieved clearly suggested the existence of two major groups of proteins with average lengths of

approximately 400 and 560 residues and carrying a suitable histidine pattern for copper binding (Figure 8).

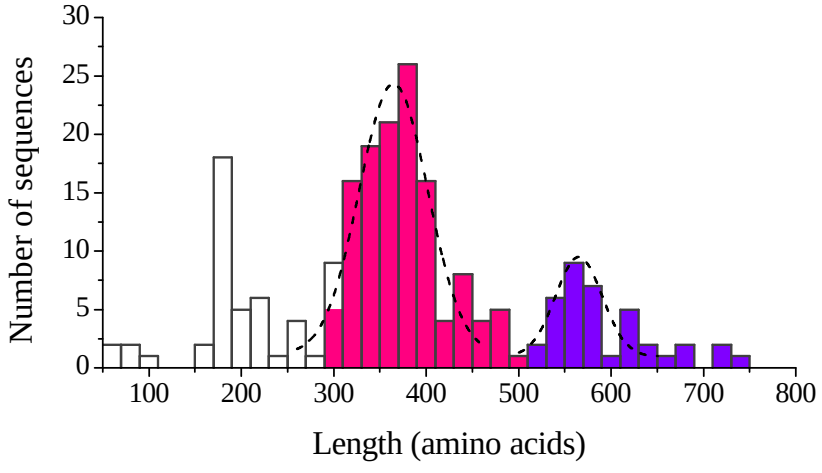

Figure 8. Length distribution of the sequences retrieved carrying a predicted tyrosinase domain (IPR0002227) and an N-terminal signal sequence. Sequences bearing a conserved histidine pattern (coloured bars) are divided into two groups with length of 300–500 amino acids (pink bars) and 500–800 amino acids (blue bars).

4.1.2 Analysis of sequences of long tyrosinases

Proteins with a length between 500 and 800 residues were named ‘long tyrosinases’ (Publication I Table 1) and they showed sequence similarities with the tyrosinases previously reported in the literature (Table 5), e.g. with the intracellular enzymes from *Agaricus bisporus* (556 and 568 amino acids of CAA59432 and CAA11562, respectively), *Neurospora crassa* (542 aa, EAA35696) and the tyrosinase from *T. reesei* (561 aa). The level of sequence identity between long tyrosinases and the tyrosinase from *T. reesei* ranges from 22.7% (Q1DQ30 from *Coccidioides immitis*) to 47% (NECHA0066755 from *Nectria haematococca*) (Publication I Table 1).

These proteins carried the highly conserved histidine pattern in the two copper binding regions, i.e. HA1-X(20-23)-HA2-X(8)-HA3 and HB1-X(3)-HB2-X(n)-HB3, in which n varies from 20 to 33 residues (Table 5 and Publication I table 1). Moreover, a conserved C-terminal tyrosinase motif was found and the YG motif, suggested to be a signature for the C-terminal cleavage site, was conserved in all the sequences except for TRIRE0050793 from *T. reesei* (Flurkey &

Inlow, 2008). Long tyrosinases were characterised by a low cysteine content, since only one cysteine was conserved in position HA2-2 and was possibly involved in the formation of a thioether bond with the nearby residue HA2, similarly to the tyrosinase from *N. crassa*, the catechol oxidase from *Ipomoea batatas* and the haemocyanin from *Octopus dofloeini* (Klabunde et al., 1998, Cuff et al., 1998, Lerch, 1983, Lerch, 1982). This thioether bond is thought to be formed post-translationally and to be involved in enzyme activation, more efficient copper binding, enzyme maturation and tuning of the redox potential (Lerch, 1983, Lerch, 1982, Lerch, 1978, Nakamura et al., 2000). This thioether bond has however not been detected in the tyrosinase from *Streptomyces* spp., mouse and human (Marusek et al., 2006).

The length of the region following the C-terminal tyrosine motif of long tyrosinases varied between the 346 residues of the sequence Q7S218 from *N. crassa* and the 85 residues of the sequence TRIRE0050793 from *T. reesei*. The alignment of long tyrosinases and the analysis of this C-terminal stretch evidenced few sequence features conserved in all the long tyrosinases. The function of these residues is not known. In 13 out of 27 long tyrosinases retrieved the tyrosine motif was followed by a proline-glutamic acid dipeptide, whereas in the other sequences these residues could be replaced by an amino acid of small size such as glycine or alanine. As an exception, the tyrosinase from *T. reesei* was the only one with a glutamine-glycine dipeptide. Generally, the analysis of the C-terminal region of long tyrosinases showed an overall low level of conservation.

4.1.3 Analysis of sequences of short tyrosinases

A novel finding of this study was the identification of a second major group of sequences with the features of tyrosinases and with a length of 300–500 amino acids (Publication I Table 1).

These ‘short tyrosinases’ had a lower level of sequence identity to the tyrosinase from *T. reesei*, i.e. between 10 and 20%, and they had several unique sequence features: (a) The presence of a stop codon a few residues after the C-terminal tyrosine motif Y/F-X-Y and thus the lack of the whole linker and C-terminal domain, similarly to the tyrosinase from *S. castaneoglobisporus;* (b) a novel histidine pattern HA1-X(7)-HA2-X(8)-HA3 of the CuA site with a seven-residue distance between the residues HA1 and HA2 (Publication I Table 1). This distance was significantly shorter than in the long tyrosinases, for example 23 residues in the *Trichoderma reesei* tyrosinase and 15 residues in the *S. casta-*

neoglobisporus tyrosinase that lacked the C-terminal domain but was associated with a caddie protein. (c) A novel conserved pattern of six cysteines, absent in the previously characterised enzymes (Lerch, 1983, Wichers et al., 2003, Lerch, 1982). Two of these cysteines were located in the N-terminal region (aligning to C_{72} and C_{100} in the sequence Q2UNF9 from *Aspergillus oryzae* named AoCO4), three between the copper binding regions (C_{159}, C_{223} and C_{261} in AoCO4) and one adjacent to the C-terminal tyrosine motif (C_{404} in AoCO4). The conserved N-terminal arginine corresponding to R_{40} in *T. reesei* tyrosinase was located between the first two conserved cysteine residues, e.g. R80 in AoCO4. In addition, short tyrosinases lacked the cysteine residue candidate for the formation of a thioether bridge with the histidine residue HA2 (Figure 9, Publication I Table 1).

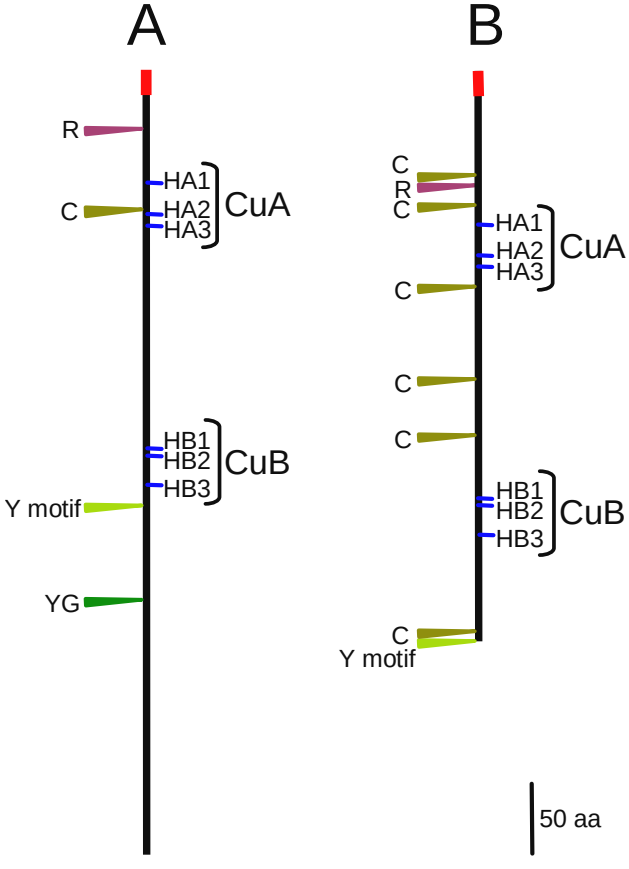

Figure 9. Schematic representation of the distribution of some conserved residues between long (A) and short (B) tyrosinases. The histidines of the CuA and CuB sites as are in blue. The N-terminal conserved arginine (R) is in purple and the tyrosine (Y) motif is in light green (Flurkey & Inlow, 2008). Conserved cysteine (C) residues are in olive green. The signal sequence is in red. The relative distances are in proportion and tyrosinase from *T. reesei* and Q2UNF9 (later called catechol oxidase AoCO4) from *A. oryzae* were used as models.

4.1.4 Phylogenetic analysis of sequences of long and short tyrosinases

The majority of sequences retrieved were in the subphylum Pezizomycotina of Ascomycetes, but short putative tyrosinases were also found in Basidiomycota, Agaricomycotina species, and in Chytridiomycota, genus *Batrachochytrium* (Figures 10 and 11).

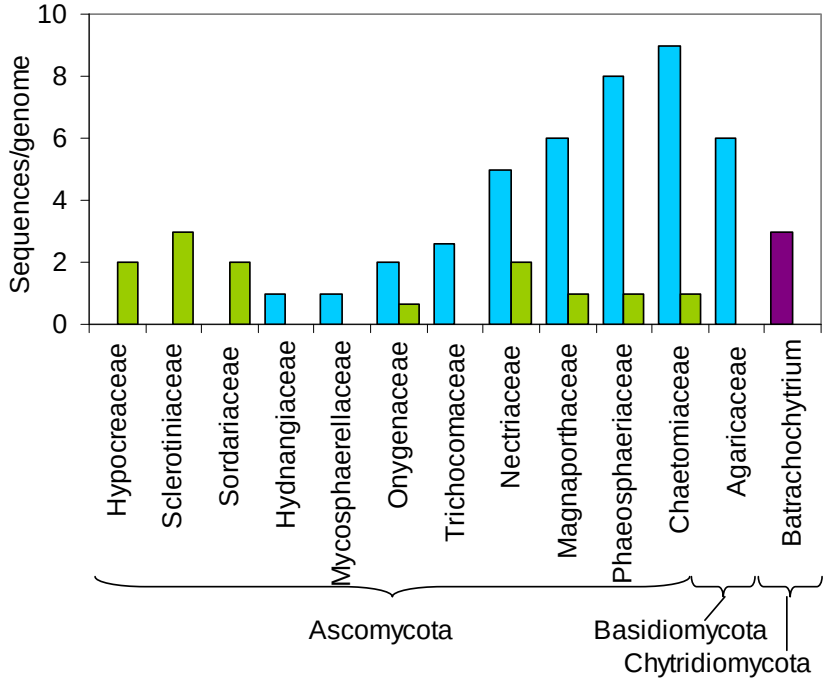

Figure 10. Averaged species distribution of the sequences of long (green) and short (blue) tyrosinase among the fungal families analysed. Sequences from *Batrachochytrium dendrobatidis* are marked by a purple bar.

The two groups of short and long tyrosinases showed different phylogenetic origins and separated clearly into two branches of the phylogenetic tree (Figure 11 and Publication I Online Resource 1).

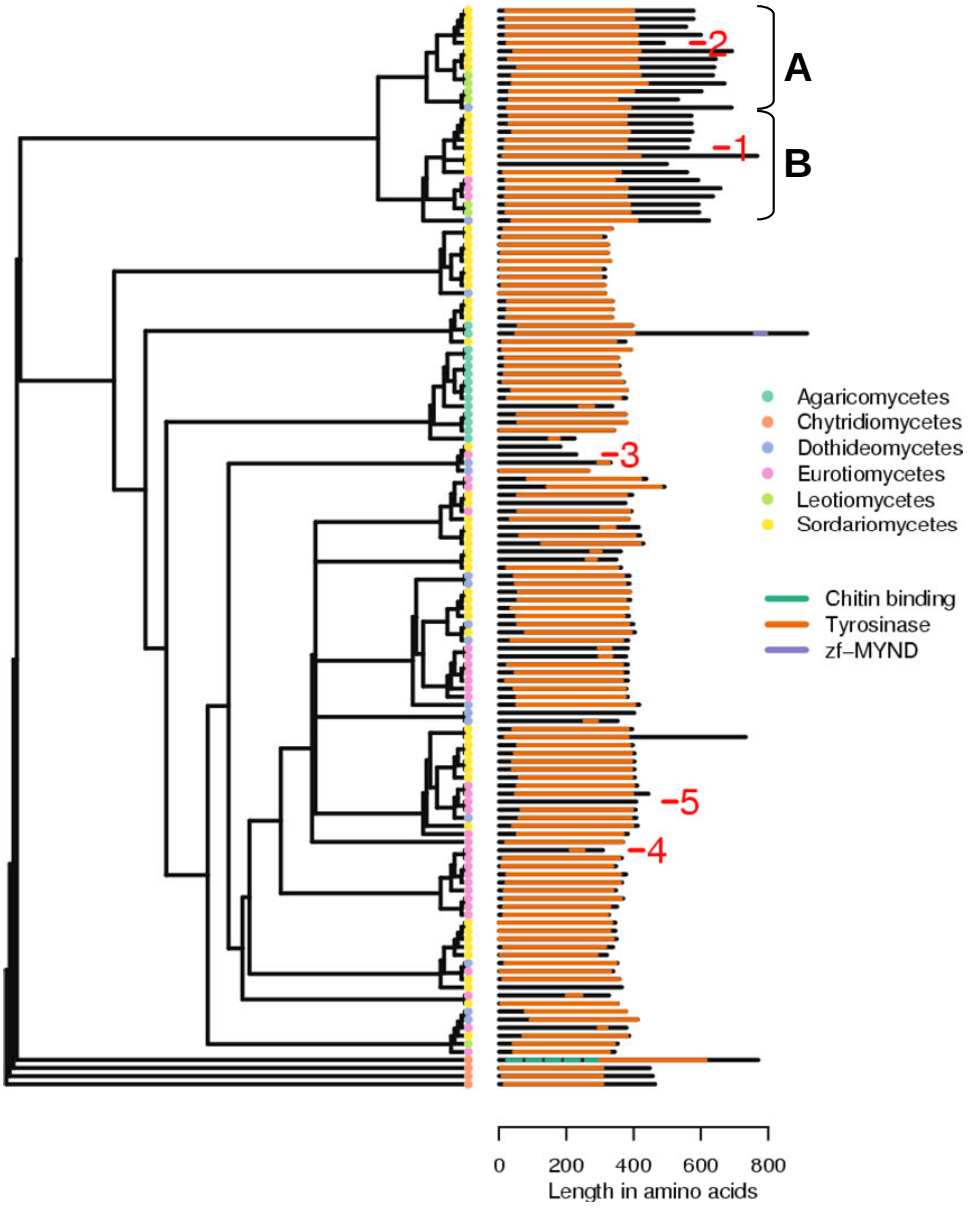

Figure 11. Phylogenetic analysis of potential secreted tyrosinase sequences. Fungal classes are indicated by a coloured dot at the end of each branch. On the right, a line represents the length of the protein and the computationally predicted domains (coloured region). Numbers indicate proteins of specific interest: (1) TRIRE0045445 from *T. reesei* (Selinheimo et al., 2006), (2): tre50793 from *T. reesei*; (3) Q2UCH2 from *A. oryzae*, (4) Q2UFM6 from *A. oryzae* and (5) Q2UNF9 (AoCO4) from *A. oryzae*. A more detailed tree is provided as a supplementary file in Publication I.

A large majority of sequences retrieved belonged to the short tyrosinase group (Figure 11, Publication I Table I). Numerous members of the group of long tyrosinases were found in the Ascomycota families of Sclerotiniaceae, Nectriaceae and Hypocreaceae and no representatives were found in the genomes of Basidiomycota species belonging to the subphylum Agaricacomycotina, and in the only representative of Chytridiomycota, which was the species *Batrachochytrium dendrobatidis* with four putative tyrosinase sequences (Figure 10, 11 and 13). By contrast, short tyrosinase sequences were predominantly found in Trichocomaceae and Nectriaceae and a few representatives also in Basidiomycota (Figure 10, 11 and 13).

Since some fungal families possessed both short and long tyrosinases (Figure 11) it is reasonable to deduce that both forms were probably present in the common ancestor of fungi.

The phylogenetic analysis revealed the existence of two groups of long tyrosinases, clades A and B (Figure 11) that were both present in the common ancestor of Ascomycota and probably produced by gene duplication. This also suggested possibly different physiological roles for the two groups, since species possessing genes for long tyrosinases usually have a member of each group. For example *T. reesei* (Hypocreaceae family in Figure 10) has the protein model TRIRE0050793 in addition to the characterised protein TRIRE0045445 (Selinheimo et al., 2006), belonging to the different group of long tyrosinases (sequences 1 and 2 in Figure 11 and in Publication I Online Resource 1).

Detailed sequence analysis of the predicted proteins belonging to the clade A of long tyrosinases in the phylogenetic tree in Figure 11 and Publication I Online Resource 1, e.g., FGSG.05628 from *Fusarium graminearum*, TRIRE00050793 from *T. reesei* and Q7SFK3 from *N. crassa*, indicated the presence of an additional tyrosine motif Y-X-Y in the N-terminus (residues 55–58 in the alignment in Figure 12). However, this motif is located between the predicted cleavage site of the signal peptide and putative Kex-2 cleavage site (K-R and R-R in position 69–70 in Figure 12) and might be removed during the protein maturation process. Additionally, only in this sub-group of proteins an additional arginine residue is conserved a few residues after that corresponding to R_{40} of *T. reesei* tyrosinase whereas it is replaced by an asparagine in the long tyrosinases of the clade B (residue 108 in the alignment in Figure 12). No role for these conserved motifs has hitherto been established.

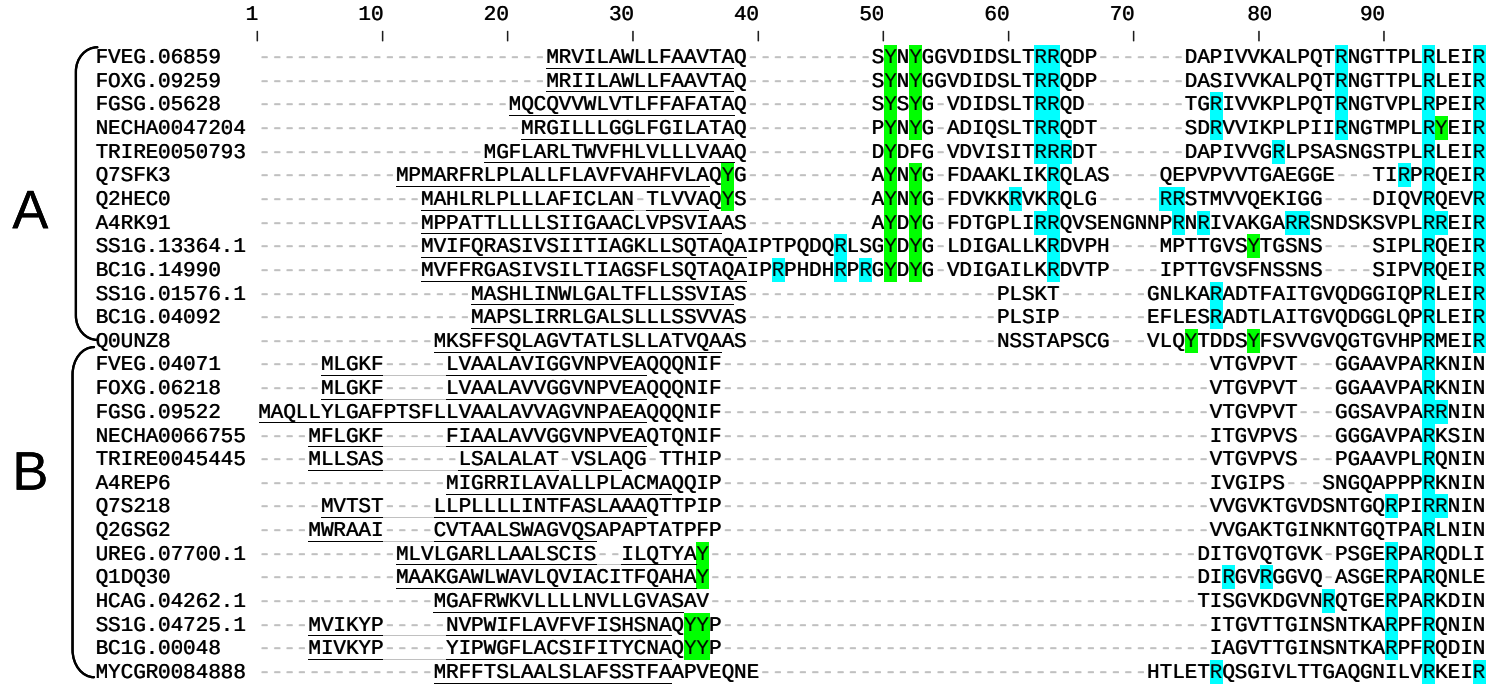

Figure 12. Sequence Multiple alignment of the N-terminal sequence of the long tyrosinases in the order in which they appear in the phylogenetic tree in Figure 11 and in Publication I Online Resource 1 (sequence identifiers as in Publication I Table 1). The only characterised protein is TRiRE0045445 (TrTyr2, Selinheimo et al., 2006). Tyrosine and arginine residues are highlighted in green and blue, respectively. Predicted signal sequences are underlined.

4.1.5 Analysis of the putative tyrosinase sequences from *Batrachochytrium dendrobatidis*

The sequence features of the four sequences retrieved from *B. dendrobatidis* were separately analysed (Figure 13) and were ascribed to the group of short tyrosinases (Publication I Table 1). Interestingly, these proteins showed sequence similarities to both short and long tyrosinases since they presented (a) the conserved six cysteine pattern and (b) the histidine pattern typical of short tyrosinases, but their sequence presents a C-terminal stretch following the tyrosine motif. However, the copper binding sites A and B are separated by a shorter distance in the sequences from *B. dendrobatidis*, e.g. 46 amino acids shorter in BDEG06104 than in the short tyrosinase AoCO4 (Q2UNF9) (Figure 13).

Figure 13. Partial alignment of sequences from *B. dendrobatidis* with a representative short tyrosinase Q2UNF9 (AoCO4) from *A. oryzae* and the long tyrosinase TRIRE0045445 (TrTyr2) from *T. reesei* (Selinheimo et al., 2006). The putative N-terminal conserved arginine residue is in green, histidine residues of the copper binding sites are in red and the whole regions are in bold, the conserved cysteine residues are in blue, and the putative C-terminal tyrosine motif is underlined.

4.2 Catechol oxidase AoCO4 from *Aspergillus oryzae* (Publication I)

4.2.1 Production and purification of the catechol oxidase AoCO4 from *Aspergillus oryzae*

A new group of short putative tyrosinases lacking the whole linker and C-terminal region was characterised. No information was available on the three short tyrosinase genes of *A. oryzae* retrieved in our study, although the industrially exploited filamentous fungus *A. oryzae* has been reported to produce melanin (Te Biesebeke & Record, 2008), and three genes encoding long tyrosinases have previously been characterised, i.e. MelB (Obata et al., 2004), MelD (Masayuki et al., 2004) and MelO (Fujita et al., 1995).

Three novel putative short tyrosinases from *A. oryzae* (Machida et al., 2005) were retrieved, i.e. sequences Q2UCH2, Q2UFM6 and Q2UNF9 (numbers 3, 4 and 5 in Figure 11 and in Publication I Online Resource 1). Only the last one of these had a complete histidine pattern for a type-3 copper centre and was thus selected for heterologous expression and biochemical characterisation. This protein, named AoCO4, has the common central domain of tyrosinases and a level of sequence identity to known tyrosinases between 12 and 20% (Table 11). Interestingly, two histidine residues, H_{111} and H_{127} were candidates as the copper ligand residue HA1 and thus the histidine pattern for short and long tyrosinases could be identified (Publication I, Figure 1).

The gene *Q2UNF9* coding for AoCO4 was amplified by PCR from the genomic DNA of *A. oryzae* and cloned to an expression vector for heterologous expression in *T. reesei*. The resulting expression construct was transformed into a *T. reesei* production strain, and transformants were selected for resistance to hygromycin. The transformants were subsequently screened for tyrosinase activity on plates containing L-tyrosine (55.2 mM) and copper (0.1 mM). The appearance of a black colouration around the colonies indicated oxidation of tyrosine. Positive transformants were purified to uninuclear clones through single spore cultures and were grown in shake flask (medium volume 50 ml) in medium supplemented with copper (1 mM). Enzyme activity was measured with catechol as substrate and the best transformants were selected for further studies.

Table 11. Amino acid sequence identity of the protein AoCO4 to selected characterised and non-characterised proteins.

Source	Organism	Protein	Reference	Identity (%)
Biochemically characterised				
bacterium	*Streptomyces castaneoglobisporus*	TYR	(Ikeda et al., 1996, Machida et al., 2005)	20.4
fungus	*Trichoderma reesei*	TYR	(Selinheimo et al., 2006)	14.7
bacterium	*Verrucomicrobium spinosum*	TYR	(Fairhead & Thony-Meyer, 2010)	14.4
fungus	*Neurospora crassa*	TYR	(Lerch, 1978)	13.3
plant	*Ipomoea batata*	CO	(Eicken et al., 1998)	13.0
fungus	*Agaricus bisporus*	TYR (AbPPO1)	(Flurkey et al., 2008, Wichers et al., 1996, Espin & Wichers, 1999)	12.5
fungus	*Agaricus bisporus*	TYR (AbPPO2)	(Flurkey et al., 2008, Wichers et al., 2003, Espin & Wichers, 1999)	12.4
Non-characterised proteins (best hits)				
fungus	*Aspergillus niger*	An01g09220	(Pel et al., 2007)	72.4
fungus	*Penicillium chrysogenum*	Pc22g18500	(van den Berg et al., 2008)	68.8
fungus	*Talaromyces stipitatus*	putative TYR EED15869.1	direct annotation (J. Craig Venter Institute, USA)	68.3
fungus	*Coccidioides immitis*	hypothetical CIMG07314	direct annotation (Broad Institute of MIT and Harvard, USA)	64.0

The production of AoCO4 was optimised by growing the transformant giving the highest activity in shake flasks (volume 50 ml) at different copper concentrations (0–6 mM); the maximum activity was produced at 1 mM copper concentration (Figure 14). The level of activity detected when AoCO4 was produced in shake-flask cultivation was however low, since a loss of activity was detected along with decrease of the pH in the culture (Figure 12). Either the AoCO4 enzyme was inactivated by low pH or it was degraded by acidic proteases emerging in the culture. The highest activity produced in shake flasks corresponded to approximately 230 mg/l of AoCO4 protein. The production of AoCO4 in a 10 L

bioreactor in inducing medium at pH 5.5 supplemented with 0.5 mM copper led to a seven-fold higher yield than obtained in shake flask cultures (approx. 1.5 g/l). AoCO4 was purified from the culture medium after removal of the cells by filtration and buffer exchange to 20 mM sodium acetate buffer pH 4.8. Purification was performed by chromatography (Publication I Table 2).

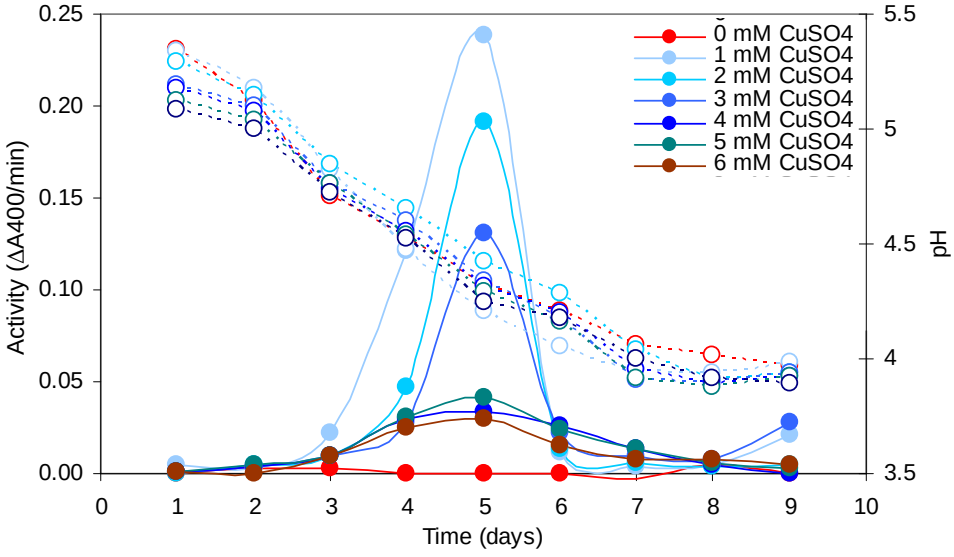

Figure 14. Production of AoCO4 in shake flask culture in the presence of different concentrations of copper. The maximum activity (filled circles) was reached on the 5th day and a sharp decrease was observed as the pH of the culture medium (empty circles) decreased below 4.5.

Two forms of AoCO4 with molecular masses of 39349 and 40482 could be partially separated during purification since they eluted at different salt concentrations during the first separation step, i.e. 60 and 90 mM NaCl. N-terminal sequencing of both forms of AoCO4 indicated different proteolytic processing during maturation (Table 11, Publication I). The form of AoCO4 eluting at a salt concentration of 60 mM started at residue Q_{25} and the major activity was eluted at 90 mM and corresponded to a more extensively processed form that started at R_{69}, after a potential recognition site for KEx2-like proteases (K_{68}-R_{69}). This form was thus lacking 51 residues after the signal peptide cleavage site (A_{18}-F_{19}). Both forms were active but only the latter major form of AoCO4 was biochemically characterised.

4.2.2 Biochemical characterisation of the catechol oxidase AoCO4 from *Aspergillus oryzae*

The activity of the purified AoCO4 was tested on different phenolic compounds. The enzyme was active on both mono and diphenolic substrates with highest activity on the diphenolic compound 4-*tert*-butylcatechol (TBC), which was further selected as the substrate for the biochemical characterisation (Publication I). In all conditions tested, the purified enzyme had no activity on the typical substrates for tyrosinases such as L-tyrosine and L-DOPA. According to its substrate specificity (Table 12) AoCO4 could not be classified as a tyrosinase (EC 1.14.18.1) but instead was ascribed to the catechol oxidase family (EC 10.3.1).

Table 12. Summary of the biochemical properties of the purified catechol oxidase AoCO4 from *A. oryzae*.

Feature	**Technique**	**Result**
Molecular weight	Calculation, SDS PAGE, MALDI TOF-MS and N-terminal sequencing	42716 Da, 53 kDa, two forms of 39.3 kDa (from R_{69}) and 40.5 kDa (from Q_{25})
Substrate specificity	Activity measurement	Monophenols: aminophenol, guaiacol, phenol, tyrosol, p-creasol. Diphenols: 4-*tert*-butylcatechol, catechol, caffeic acid and hydrocaffeic acid. Others: aniline, catechins;
pI	Isoelectric focusing	5.2
pH optimum	Activity measurement	pH 5–7
pH stability	Activity measurement, circular dichroism	>75% activity retained in a 5–9 pH range
Temperature stability	Activity measurement	T½ at 50°C at pH 7: 20 hrs; T½ at 60°C at pH 7: 2 hrs, Tm: 70°C
Cofactor binding	UV-vis spectroscopy	absorption maxima at 280 nm and 330 nm
Presence of aromatic residues	Fluorescence	maximum at 330 nm when excited at 280 nm
Secondary structure	Circular dichroism	negative peaks at 200 nm and 220 nm in the far-UV spectrum

As suggested by circular dichroism, absorbance and fluorescence analyses (Table 12, Publication I Figures 3 and 4), the purified AoCO4 was correctly folded

and incorporated the copper ions required for the catalytic activity. AoCO4 was characterised by a significant temperature stability of the secondary structure, with a melting temperature of 70°C (Table 12, Publication I Figure 4b). This might be related to the presence of the six cysteine residues and the possible formation of disulfide bonds that have been shown to improve the temperature stability of many proteins, e.g. xylanases (Yang et al., 2007) and ribonuclease A (Pecher & Arnold, 2009).

The phylogenetic analysis and the fact that AoCO4 could be produced in an active form suggested that the evolutionary process allowed the loss of the C-terminal extension found in long tyrosinases (Masayuki et al., 2004), and partially in sequences from *Batrachochytrium dendrobatidis.* The results of this study do not support the hypothesis that the C-terminal domain is required for correct protein folding in terms of copper incorporation and secondary structure formation. However, it cannot be excluded that the C-terminal domain in long tyrosinases would keep the enzyme inactive during the secretion process and prevent its action on intracellular elements. The recent characterisation of a tyrosinase from the bacterium *Verrucomicrobium spinosum* supports the suggested role of the C-terminal domain in copper incorporation and that its removal activates the enzyme (Fairhead & Thony-Meyer, 2010). In the case of AoCO4, it might not be necessary to keep the enzyme inactive inside the cell since it has no activity on tyrosine and thus might constitute no harm to other proteins and the cell metabolism. On the other hand, since no activity was detected on tyrosine the physiological role of AoCO4 is probably not related to melanin synthesis, as for canonical tyrosinases, but it could possibly be involved in detoxification of the extracellular environment, since it is active on aminophenol and it is a secreted protein.

4.3 Discovery of sulfhydryl oxidases (Publication II)

4.3.1 Fungal genome analysis for the identification of novel potential sulfhydryl oxidases

The publicly available fungal genomes were searched for secreted sulfhydryl oxidases, e.g. proteins carrying a predicted disulfide oxidoreductase domain of class II and, in particular, FAD-dependent protein signatures (InterPro entry IPR000103 and IPR013027) and a signal peptide. The 48 protein sequences retrieved were aligned and 18 of them had the di-cysteine motif C-X-X-C char-

acteristic of thiol:disulfide oxidoreductases such as sulfhydryl oxidases (Publication II, Table 1).

Predicted sulfhydryl oxidases were abundant in *Aspergillus* spp. and the closely related species *Neosartorya fischeri*; the only characterised protein detected was the sulfhydryl oxidase from *A. niger* (AnSOX, CAK40401) (de la Motte & Wagner, 1987). The proteins retrieved presented, as expected, conserved sequence motifs typical of a nucleotide-binding domain such as the Rossmann fold (Table 13). The C-terminal stretches of the sequences analysed were characterised by a predicted intrinsic disorder (Table 13).

A previously uncharacterised proline-tryptophan pair was found in the di-cysteine C-X-X-C motif of 12 of the predicted sulfhydryl oxidases identified. The influence of the dipeptide located between the two conserved cysteine residues has been studied in DsbA, a protein of *E. coli* required for disulfide formation in proteins (Quan et al., 2007). The presence of an aromatic amino acid in the C-terminal position has been related to catalytic efficiency (Quan et al., 2007, Lundstrom et al., 1992). An aromatic residue in the C-terminal position of the dipeptide within the C-X-X-C motif is found also in the motif CLFC characterising four sequences that grouped on top of the alignment in Publication II Figure 1, i.e. A4QYP9 from *Magnaporthe grisea*, A1DN23 from *Neosartorya fischeri*, Q0CME9 from *A. terreus* and Q5MBU7 from *A. fumigatus*. Protein Q4WQJ0 from *A. fumigatus*, with 21.8% identity to AnSOX, had a previously uncharacterised dipeptide alanine-valine in the di-cysteine motif (bottom sequence in the alignment in Figure 1 Publication II). Conserved sequence motifs and their suggested roles are reported in Table 13.

Table 13. Conserved sequence features found in the putative sulfhydryl oxidase carrying a signal peptide and di-cysteine C-X-X-C motif.

Sequence feature	Position*	Description	Reference
N-terminal region			
(V/I)-(V/I)-G-X-G-X(2)-G-X(3)-A/L	38–49	typical of the Rossmann fold	(Rossmann et al., 1974, Wierenga et al., 1986)
Central region			
R-K-B-B-L-A-T-G	153–155	common to FAD and NADPH-binding proteins	(Vallon, 2000)
R-K-B(2) L-(A/G)-T-G-X(2) D-X(&) G-X-X-(E/D)-X-Φ-(G/A)-X-G-X-Φ(2)-C-X-X-C-(D/H)-G-Φ-E	177–180	typical of thiol:disulfide oxidoreductases with a thioredoxin fold	(Fass, 2008)
C-terminal region			
T-X-B-X-G-B-Φ-(A/G)-B-G-D	340–350	GD motif involved in the binding of the ribityl moiety of FAD	(Vallon, 2000)
A-B-X-X-G	362–366	G-helix located within the flavin-binding domain	(Vallon, 2000, Eggink et al., 1990)
C-terminus, after E_{350} in AnSOX and K_{349} in AoSOX1	380–430	rich in glutamic acid and characterised by intrinsic disorder	-

Abbreviations: X, any residue; B. hydrophobic residue; Φ, aromatic residue.
*position relative to the alignment in Publication II Figure 1

4.4 Sulfhydryl oxidases AoSOX1 and AoSOX2 from *Aspergillus oryzae* (Publications II–III)

4.4.1 Production and purification of the sulfhydryl oxidases AoSOX1 and AoSOX2 from *Aspergillus oryzae*

Two of the secreted putative sulfhydryl oxidases identified were selected to be heterologously expressed in a fungal host. Proteins AoSOX1 (Q2UA33) and AoSOX2 (Q2U4P33) from *A. oryzae* shared 50.6% sequence identity and 68% sequence similarity and had the sequence features of flavin-dependent sulfhydryl

oxidases (Table 11 and Figure 1 Publication II). Proteins AoSOX1 and AoSOX2 had 64.7 and 47.3% levels of sequence identity to the characterised sulfhydryl oxidase from *A. niger*. The proteins with the highest level of amino acid identity to AoSOX1 and AoSOX2 were all from fungal origin and, since only a few studies have been conducted on secreted sulfhydryl oxidases, they were all not characterised in the literature but annotated from the genome sequence (Table 14).

Table 14. Level of amino acid identity of AoSOX1 and AoSOX2 to fungal characterised and non-characterised protein models.

Organism	Protein		Reference	Identity (%)
	Annotation	Identifier		
AoSOX1 homologs				
Aspergillus flavus	thioredoxin reductase	EED47993	direct submission (J. Craig Venter Institute, USA)	99.5
Neosartorya fischeri	thioredoxin reductase	XP_001266180	direct submission (The Institute for Genomic Research, USA	69.9
Aspergillus fumigatus	thioredoxin reductase	XP_747990	(Nierman et al., 2005)	68.9
AoSOX2 homologs				
Aspergillus flavus	thioredoxin reductase	ABY86217	direct submission (J. Craig Venter Institute, USA)	97.7
Penicillium chrysogenum	-	Pc12g03690	(van den Berg et al., 2008)	67.4
Neosartorya fischeri	cytoplasmic thioredoxin reductase	XP_001258605	direct submission (The Institute for Genomic Research, USA)	65.5

Following the isolation of the genomic DNA of *A. oryzae*, the genes coding for the predicted proteins AoSOX1 and AoSOX2 (*Q2UA33* and *Q2U4P3*, respectively) were amplified by polymerase chain reaction and cloned to an expression vector for heterologous expression in *T. reesei*. The resulting constructs were transformed into a *T. reesei* production strain. Transformants were selected first for resistance to hygromycin, then purified to uninuclear clones through single spore cultures, and eventually analysed by polymerase chain reaction to detect the presence of the gene of interest. The cloning was planned in order to introduce a C-terminal six-histidine tag to facilitate the subsequent purification step.

Positive transformants were grown in shake flasks (culture volume 50 ml) and assayed the activity on the substrate glutathione in the medium. The maximum production level was reached after 5 days of cultivation and AoSOX1 and AoSOX2 was produced in sufficient amounts for the next steps of purification and biochemical characterisation (Table 15).

Table 15. Production levels of AoSOX1 and AoSOX2 in *T. reesei* after 5 days of cultivation in shake-flask and percentage of the total secreted proteins in the culture medium.

Protein	**Production level (mg/l)**	**Relative amount (%)**
AoSOX1	70	5
AoSOX2	180	8

First attempts to purify AoSOX1 and AoSOX2 were based on His-tag affinity chromatography, but no significant binding to the copper-chelated resin was detected. To detect the presence of the histidine-tag, Western blot analysis with specific anti-histidine tag antibody was carried out in order to reveal whether the histidine tag was buried within the protein molecule. According to the analysis the tag was not detected. It was thus concluded that both AoSOX1 and AoSOX2 were subjected to proteolysis in the C-terminal region during secretion. Accordingly, the C-terminal peptide containing the tag was not detected by MALDI TOF-MS analysis of the tryptic peptides. A more careful analysis of the amino acid sequences of AoSOX1 and AoSOX2 detected potential cleavage sites for Kex2-like proteases just before the tag ($K_{337}K_{338}$, $K_{358}R_{359}$, $K_{368}R_{369}$ in AoSOX1 and $K_{357}R_{358}$, $K_{384}R_{385}$ and $R_{388}R_{389}$ in AoSOX2, Figure 15).

Both sulfhydryl oxidases AoSOX1 and AoSOX2 were purified in a two-step chromatographic procedure comprising a first separation by anion exchange and second by size-exclusion chromatography. A good yield and level of purification were achieved for AoSOX1, whereas poorer results were obtained for AoSOX2 (Table 2 Publication II, Table 1 Publication III).

Proteins AoSOX1 and AoSOX2, both in the culture medium and in the purified form, were in-gel digested with trypsin after being subjected to SDS PAGE analysis and were identified with a sequence coverage of 24.5% for AoSOX1 and 23.1% for AoSOX2 by peptide-mass fingerprinting performed with a MALDI TOF-MS instrument (Figure 15).

The second step of purification based on size-exclusion chromatography was also used to determine the molecular weights of AoSOX1 and AoSOX2 in na-

tive conditions. Both AoSOX1 and AoSOX2 appeared to be dimeric proteins with a molecular weights in solution of approximately 89 and 78 kDa (Table 16) migrating in SDS PAGE as a double band of approx. 45 kDa (Table 16 and Publication II and III). The presence of a double band was possibly due to heterogeneous glycosylation. Various N-glycosylation sites were predicted in both proteins (Figure 15). Since the peptides containing them were not detected during protein identification, it was suggested that the sites may be occupied by glycans. This hypothesis is supported by the higher molecular weight detected by SDS PAGE and MALDI TOF-MS than the calculated value (Table 16). Moreover, in the case of AoSOX1 the removal of the glycans by treatment with PNGase F resulted in a single protein band of lower molecular weight (Publication I Figure 2a).

Dimeric enzymes with sulfhydryl oxidase activity and non-covalently binding a flavin cofactor have been reported, e.g. from the filamentous fungus *A. niger* (de la Motte & Wagner, 1987), from the plant *Arabidopsis thaliana* (Levitan et al., 2004) and the yeast *Saccharomyces cerevisiae* (Lee, 2000)

```
AoSOX1     1 MAPKSLFYT---------LFSSLTVALAASVPQTDYEVIVVGGGPAGLTA   41
                   :.:|          |.|.|:|.: |.:|||:|:|||:|||||:||:|
AoSOX2     1 -----MMFTCNWTALLCALLSILSVCV-ADIPQTNYDVIIIGGGPSGLSA   44

AoSOX1    42 LSGLSRVRRKTALFDSHEYRNAATRNMHDVIGNDGTVPSEFRGLAREQIS   91
             .|.||||.||:.||||.||||..||:|||||||||..||:|||..||||||
AoSOX2    45 ASALSRVLRKSVLFDSGEYRNNPTRHMHDVIGNDHVVPAEFRKTAREQIS   94

AoSOX1    92 RYDTATFIDKRVNTIETVSDEATNTSYFRAQDADGKAYTARKVVLGTGLV  141
             .|:..||||::|..:|...|.|     |:|...| :.||||||:||:|:.
AoSOX2    95 FYNMTTFIDQKVTKLEKTGDNA-----FQATVGD-QQYTARKVILGSGVK  138

AoSOX1   142 DILPDVPGLQEAWGKGVYWCPWCDGYEHRDQPFGILGALPDVVGSVLEVY  191
             |.||||||||||.:|||::|||||||:|||:|..|:||.:.:..|:|.|::
AoSOX2   139 DDLPDVPGLQEGFGKGIFWCPWCDGFEHRNQSMGVLGDISEAYGAVRELH  188

AoSOX1   192 -TLNTDIIAFVNGTQTPDQEAELAKKYPNWEAQLEAYNVRLENETIASFE  240
             |||.||..:.|||.|.:|.|.|.|.:|||....:||||.:.|:.|.:..
AoSOX2   189 PTLNKDIRIYANGTNTTEQIAILDKNHPNWRKVFQAYNVTVNNKPILNIT  238

AoSOX1   241 RIQDGSWVKDRNGTRQIDIFRVHFTNGSSVDRNAFITNYPSEQRSDLPKQ  290
             |||||:.|.|.....:.|.|:::|.:.||..|.||||||.:.|||||.|
AoSOX2   239 RIQDGAVVNDPAIRLEFDKFQIYFADNSSEVRGAFITNYGTSQRSDLPAQ  288

AoSOX1   291 LGLAMLGNKIDATTNPGMRTSLPGVWAIGDCNSDNSTNVPHAMFSGKKAA  340
             ||:.|||:||: |...|::||:||||.:||.||||||||||.|.|||.||
AoSOX2   289 LGVEMLGSKIN-TLRKGLQTSVPGVWGVGDANSDNSTNVPHGMSSGKSAA  337

AoSOX1   341 VFAHVEMAKEE--SNTAIDKR------DDFVKEVEKRMGNDMEKIYNR-SRGL 384
             |:.|||:|:||  .:..::||      :.|.:..|::||:::::.:|.| ||.
AoSOX2   338 VYCHVELAQEELDRDAGLEKRETEFDAESFHETTERQMGSEIQDLYKRLSRR- 389
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Figure 15. Alignment of AoSOX1 and AoSOX2 amino acid sequences. The signal peptide (italic), the conserved di-cysteine motif (blue), the C-terminal potential cleavage sites for Kex2-like proteases (green) and the predicted N-glycosylation sites (bold) are shown. Identical amino acids are connected by a vertical line and similar ones by dots. Tryptic peptides detected by MALDI TOF-MS and used for protein identification are underlined.

4.4.2 Biochemical characterisation of the sulfhydryl oxidases AoSOX1 and AoSOX2 from *Aspergillus oryzae*

Solutions containing the purified enzymes AoSOX1 and AoSOX2 had a characteristic bright yellow colour and the flavoenzymatic nature of both AoSOX1 and AoSOX2 was confirmed by the UV-Vis absorbance spectrum (Figure 16, Table 16), similarly to the glutathione oxidase from *Penicillium* spp. (Kusakabe et al., 1982). The flavin cofactor was removed from AoSOX1 after denaturation with SDS (0.2%) and heat treatment (10–30 minutes at 95°C in the dark) and an extinction coefficient at 450 nm of 12160 M^{-1} cm^{-1} could be calculated, Figure 4 Publication II).

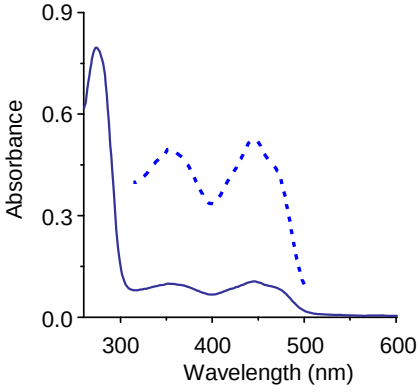

Figure 16. The flavin cofactor bound to AoSOX2 is indicated in the absorbance spectrum of the purified enzyme by the peaks at approx. 370 and 440 nm. The dotted line is a 5X magnification of the absorbance spectrum.

The best reducing substrates for AoSOX1 and AoSOX2, among the tested, were glutathione and DTT, respectively (Table 16). These compounds are however improbable natural substrates for these enzymes, the physiological role of which have not yet been established.

Inhibition studies of AoSOX1 and AoSOX2 by different salts showed a drastic inhibition by zinc sulphate, similarly to the glutathione oxidase isolated from *Penicillium* (Kusakabe et al., 1982). Cysteine residues are able to chelate divalent metal ions such as zinc and considering the paucity of cysteine residues in AoSOX1 and AoSOX2, the inhibition by zinc might confirm the presence of reactive cysteine residues at the catalytic centre, i.e. the di-cysteine motif CPWC. Only minor inhibition was caused by the other compounds tested (Publication II Table 4, Publication III, Table 3).

Both AoSOX1 and AoSOX2 showed good stability in different pH and temperature conditions (Table 16). However, the melting temperature of AoSOX2, as assayed by circular dichroism, was about 20˚C higher than for AoSOX1 and AoSOX2 also retained activity after one hour of incubation at 60˚C (Table 16, Publication II and III). The key biochemical features of AoSOX1 and AoSOX2 are reported in Table 16.

Table 16. Summary of the biochemical features of the purified enzymes AoSOX1 and AoSOX2 from *A. oryzae.*

Feature	**Technique**	**AoSOX1**	**AoSOX2**
Molecular weight	Calculation, SDS PAGE, MALDI TOF-MS and N-terminal sequencing	42.5 kDa,45 kDa,: 43.96 kDa, 89 kDa	41.5 kDa, 45 kDa, 42.41 kDa, 78 kDa
Substrate specificity	Activity measurement (V_{max}/K_m,s^{-1})	Monothiols: GSH (55010), L-Cys (364), D-Cys (163), ß-ME (219); Dithiols: DTT (5451).	Monothiols: GSH (370), L-Cys (106), D-Cys (116), ß-ME (136); Dithiols: DTT (1342).
pH optimum	Activity measurement	7.5–8.0	7.5–8.0
Stability to pH	Activity measurement	>80% activity retained after 24 h incubation at pH 5–8.5, activity loss pH<4	>80% activity retained after 24 h incubation at pH 4–8, activity loss pH<3
Stability to temperature	Activity measurement	>70% activity retained after 24 h incubation at 40°C	>65% activity retained after 1 h incubation at 60°C
Cofactor binding	UV-Vis spectroscopy	absorption maxima at 275, 370 and 440 nm, a shoulder at 365 nm	absorption maxima at 275, 354 and 445 nm, a shoulder at 470 nm
Presence of aromatic residues/ flavin cofactor	Fluorescence spectroscopy	fluorescence maximum at 334 nm when excited at 290 nm and at 525 nm when excited at 450 nm	fluorescence maximum at 337 nm when excited at 290 nm and at 525 nm when excited at 450 nm
Secondary structure	Circular dichroism	negative peaks at 210 and 220 nm in the far-UV spectrum	negative peaks at 210 and 225 nm in the far-UV spectrum
Stability to denaturation	Circular dichroism, fluorescence spectroscopy	Tm: 57ºC Cm: 2.5 M GndHCl	Tm: 75ºC Cm: 4 M GndHCl

Abbreviations: Tm, melting temperature; Cm, melting concentration.

Fluorescence studies confirmed the presence of aromatic residues in the primary structure of AoSOX1 and AoSOX2 and circular dichroism revealed α-helical elements in their secondary structure (Table 16 and Publication II and III). In

order to briefly characterise the flavin binding site of AoSOX2, the purified enzyme was incubated in the presence of sulfite and the absorbance spectrum of AoSOX2 showed a decrease in the absorbance peaks of the cofactor with a maximum variation at 445 nm (Publication III Figure 4). The formation of the covalent colourless complex between the flavin and sulfite ion (Massey et al., 1969) has long been considered specific of oxidases and recently found characteristic of proteins with a positively charged residue near the flavin (Leferink et al., 2009).

4.5 Application of the sulfhydryl oxidase AoSOX1 in wheat dough (Publication IV)

The sulfhydryl oxidase AoSOX1 was evaluated as a possible improver in fresh and frozen wheat doughs. The experiments were carried out with flours containing ascorbic acid (42 ppm, 24 x 10^{-8} moles/g flour) and without ascorbic acid. One unit (U) of sulfhydryl oxidase activity was defined as the amount of enzyme able to catalyse the reduction of 1 nanomole of oxygen per second. The effects caused by sulfhydryl oxidase were evaluated with respect to the development of fresh and frozen yeasted dough and to the strength and extensibility of non-yeasted fresh dough.

4.5.1 Effect of sulfhydryl oxidase on yeasted frozen dough with and without ascorbic acid

The addition of AoSOX1 had no detectable effect on the development of fresh doughs prepared either with flour containing ascorbic acid or without any improver (Publication IV Figure 2 and 3). On the other hand, the activity of the sulfhydryl oxidase was evident in doughs subjected to a long frozen storage.

Frozen doughs containing ascorbic acid in combination with the sulfhydryl oxidase had a clear reduction in maximum dough height, Hm, and time of maximum development T1 and T'2 after six weeks of frozen storage (Figure 17 and Publication IV Supplementary data 3 and Publication IV Figure 1A, B and E). The presence of AoSOX1 did not affect the gas retention properties of the dough and the tolerance (time the dough has the maximum height, Publication IV Figure 1C, D, F).

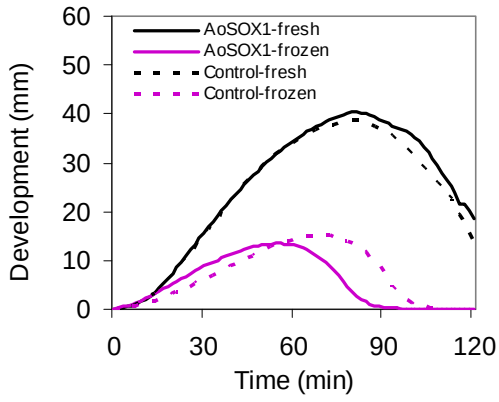

Figure 17. Development curves of fresh (black lines) and frozen (red lines) doughs made from commercial flour containing ascorbic acid with (continuous line) and without (dotted line) addition of 100 U/g sulfhydryl oxidase in flour. Fermentation was monitored by Rheofermentometer F3.

Doughs prepared with flour not containing ascorbic acid and treated with different amounts of sulfhydryl oxidase were significantly softer than the control dough after frozen storage. AoSOX1 at high concentration accelerated the deleterious effects caused by freezing, and the characteristics found after 4 weeks of frozen storage in the control doughs were detected already after 1 week in doughs containing 100 U/g of AoSOX1, e.g. gas retention properties (H'm, Publication IV Figure 2C) and total volume (Publication IV Figure 2D). Dough tolerance was however not affected.

4.5.2 Effect of sulfhydryl oxidase on non-yeasted fresh dough with and without ascorbic acid

In order to evaluate the effects of the sulfhydryl oxidase AoSOX1 on the extensibility and strength of wheat dough, further studies were carried out in a simplified system, i.e. yeast-free water-flour dough. Measurements were performed with a Kieffer dough and gluten extensibility rig fitted onto a TA.XT2 texture analyzer.

When the ascorbic-acid free flour was used, loss of strength and increase in dough extensibility were observed with increase of enzyme dosage (Figure 18A and Publication IV Figure 3A). A concentration of 100 U/g of AoSOX1 resulted in a reduction in strength of 22% and an increase in extensibility of 23% after 20 minutes of relaxation time. Opposite effects were observed when the sulfhydryl

oxidase was added to dough prepared with flour containing ascorbic acid (Figure 18B and Publication IV Figure 3B), and stronger and less extensible doughs were obtained. In a dough containing 100 ppm ascorbic acid, a sulfhydryl oxidase concentration of 100 and 1000 U/g increased the strength by 7 and 43% and reduced the extensibility by 15 and 57% after 50 minutes, respectively.

The results suggested a clear interaction between the sulfhydryl oxidase and the ascorbic acid system in the flour. In order to clarify whether this was dependent on the amount of ascorbic acid, a constant amount of sulfhydryl oxidase (1000 U/g) was added to doughs containing increasing amounts of ascorbic acid (0–1000 ppm). The synergistic effect was not dependent on the concentration of ascorbic acid and a constant ca. 10–15% increase in strength after 20 and 40 minutes of relaxation time was measured. (Figure 18C and Publication IV Figure 3C). About 10% reduction of extensibility was observed after 40 minutes in doughs containing ascorbic acid (Publication IV Figure 3C).

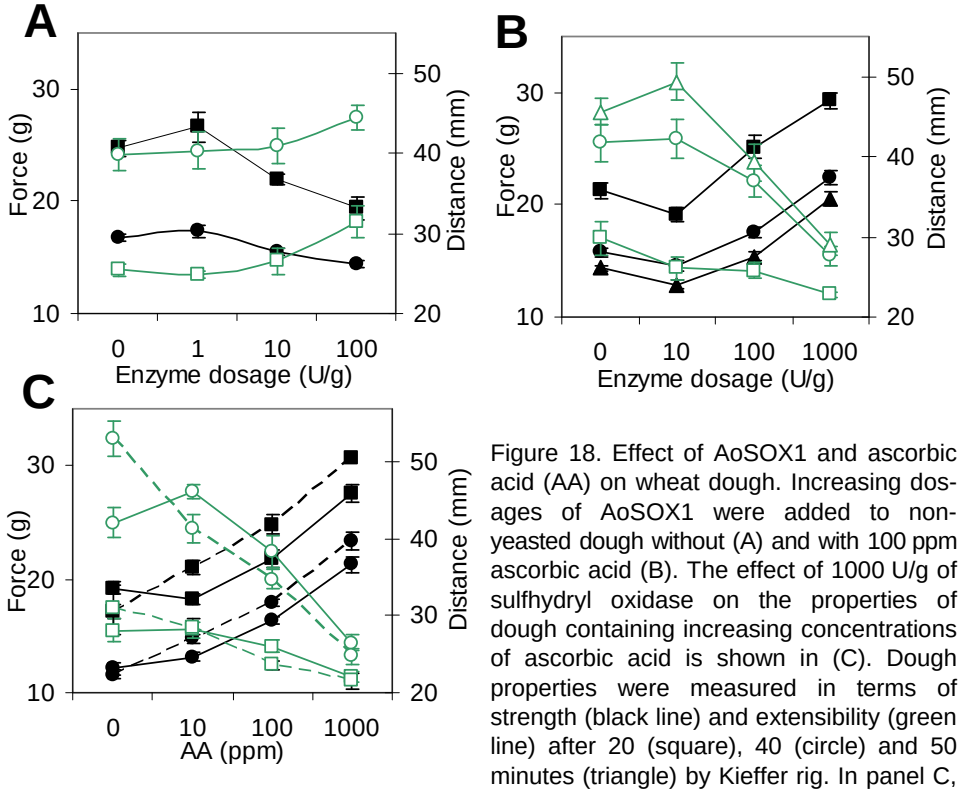

Figure 18. Effect of AoSOX1 and ascorbic acid (AA) on wheat dough. Increasing dosages of AoSOX1 were added to non-yeasted dough without (A) and with 100 ppm ascorbic acid (B). The effect of 1000 U/g of sulfhydryl oxidase on the properties of dough containing increasing concentrations of ascorbic acid is shown in (C). Dough properties were measured in terms of strength (black line) and extensibility (green line) after 20 (square), 40 (circle) and 50 minutes (triangle) by Kieffer rig. In panel C, control doughs are indicated by a continuous line and doughs containing sulfhydryl oxidase by a dashed line.

4.5.3 Mechanism of action of the combined use of sulfhydryl oxidase and ascorbic acid

On the basis of the results obtained, suggestions for the mechanism of action of AoSOX1 in wheat dough were made and evaluated. Sulfhydryl oxidase could potentially affect the ascorbic acid system in many ways. Sulfhydryl oxidase could act directly (1) by converting the ascorbic acid to dehydroascorbic acid, the actual improver, or (2) by acting similarly to the enzyme glutathione dehydrogenase of flour, e.g. coupling the oxidation of glutathione with the reduction of dehydroascorbic acid to ascorbic acid (Walther & Grosch, 1987). The effect due to the sulfhydryl oxidase could also be indirect, (3) by producing reactive species such as hydrogen peroxide able to affect the action of the ascorbic acid.

Ascorbic acid and dehydroascorbic acid were not substrates for this enzyme (Publication IV) and therefore AoSOX1 could not be directly affecting the mechanism of the ascorbic acid.

In order to test the influence of the reaction catalysed by AoSOX1 on ascorbic acid and dehydroascorbic acid, the oxidation of glutathione by sulfhydryl oxidase was carried out in the presence of these compounds. It was possible to monitor the concentration of ascorbic acid in the reaction mixture spectrophotometrically as absorbance at 265 nm.

Increasing concentrations of sulfhydryl oxidase incubated in the presence of glutathione and dehydroascorbic acid negatively affected the spontaneous reduction of the latter compound to ascorbic acid (dotted line in Publication IV Figure 4A). This suggested that a reactive species produced by the enzymatic reaction, i.e. hydrogen peroxide, was able to remove the ascorbic acid formed by converting it into a UV-transparent compound such as dehydroascorbic acid. High concentrations of sulfhydryl oxidase in the reaction mixtures were also reflected in lower final absorbance values at 265 nm after 2 minutes (Publication IV Figure 54, inset).

In order to directly evaluate the effect of the hydrogen peroxide produced by the sulfhydryl oxidase on the oxidation of ascorbic acid, ascorbic acid was incubated in the presence of glutathione and different amounts of enzyme for two hours at pH 6. A progressive decrease in the concentration of ascorbic acid was detected indicating its oxidation by the hydrogen peroxide produced by AoSOX1-catalysed reaction (Publication IV, Figure 4).

After combining the results obtained, a mechanism of action of the sulfhydryl oxidase in wheat dough containing ascorbic acid was suggested (Figure 19).

The sulfhydryl oxidase promoted the action of the ascorbic acid in the dough by two indirect ways: firstly, by contributing to removal of reduced glutathione able to loose the protein network by reducing the disulfide bonds between gluten proteins, and secondly, by producing hydrogen peroxide that is able to oxidise the ascorbic acid to the actual improver, dehydroascorbic acid (Figure 19).

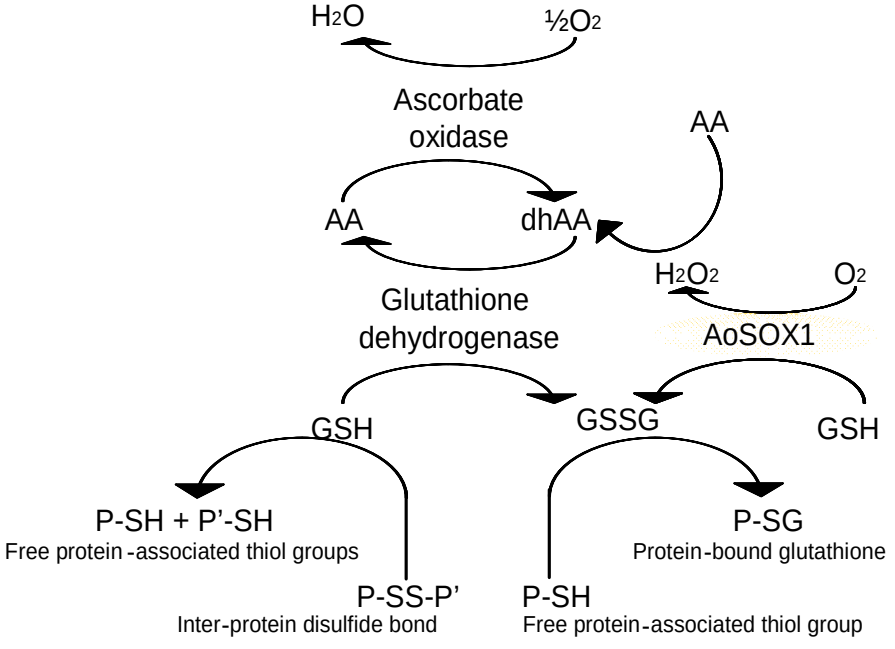

Figure 19. Proposed interaction between the mechanism of action of ascorbic acid in wheat dough and the reaction catalysed by the sulfhydryl oxidase AoSOX1. Abbreviations: GSH and GSSG, reduced and oxidised glutathione respectively, AA, ascorbic acid, dhAA, dehydroascorbic acid.

The positive effect of the reaction catalysed by AoSOX1 on the hardening of wheat dough caused by ascorbic acid indicates the potential of this enzyme and AoSOX1 for industrial applications. The combined use of AoSOX1 and ascorbic acid could constitute a valid tool for improvement of the properties of baked wheat products. Similar positive effects in wheat dough have been obtained by combining ascorbic acid and potassium bromate, a substance currently considered to be health-hazardous (Kurokawa et al., 1990) and withdrawn from the market in many countries. The use of AoSOX1 from *A. oryzae* could thus provide a healthier alternative to the use of potassium bromate in baked products.

5. Conclusions and future prospects

A wide variety of different enzymes is available in nature, each catalysing a specific reaction in a highly efficient manner. The use of enzymes in industrial processes offers many advantages such as low energy requirements, high reaction specificity and possibilities for more environmentally friendly processes. This study aimed at the discovery of novel tyrosinases and sulfhydryl oxidases with a potential crosslinking activity and possible application potential, especially in the food industry.

In the first part of the study, a search of the available fungal genomes identified a novel family of proteins with the sequence features of tyrosinases but shorter in length and lacking the linker and C-terminal domain. A member of this family, the enzyme biochemically characterised as catechol oxidase AoCO4, was produced in *Trichoderma reesei* (production level 1.5 g/l) and biochemically characterised. AoCO4 was active on mono and diphenolic compounds such as catechol, caffeic acid and tyrosol, but showed a relatively low level of activity on the tested substrates. AoCO4 had no activity on the typical substrates of tyrosinases and was thus classified as a catechol oxidase. Despite the lack of the C-terminal domain, AoCO4 was active and produced in correctly folded form binding the copper cofactor and thus contributed to the ongoing discussion concerning the role of the C-terminal domain of tyrosinases with regard to enzyme activation, correct folding and cofactor incorporation. Future structural studies will address the resolution of the three-dimensional structure of AoCO4 and characterisation of other members of the novel family of short protein sequences with the sequence features of tyrosinases.

A second genome search led to the identification of numerous potential secreted sulfhydryl oxidases in fungi. This study reports the heterologous production and biochemical characterisation of two of them, AoSOX1 and AoSOX2 from *Aspergillus oryzae* (production levels 70 and 180 mg/l, respectively).

AoSOX1 and AoSOX2 were FAD-dependent enzymes active on small thiol compounds such as glutathione, dithiothreitol and cysteine, and analysis by circular dichroism revealed the presence of α-helical elements in their secondary structure. The activity of AoSOX1 and AoSOX2 was drastically inhibited by zinc, suggesting the presence of reactive cysteine residues at the active site. AoSOX1 and AoSOX2 showed good pH and temperature stability and thus good potential for industrial applications.

The application of the enzyme AoSOX1 was tested for improving the properties of fresh and frozen wheat dough. AoSOX1 showed no effect on the fermentation of fresh yeasted dough either in the presence or absence of ascorbic acid. However, the presence of AoSOX1 in frozen doughs without ascorbic acid resulted in doughs softer than the control, whereas the combined use of AoSOX1 and ascorbic acid led to a dough harder than the control. Tests in yeast-free water-flour doughs confirmed that sulfhydryl oxidase had a weakening effect on the dough when used alone, i.e. it increases the extensibility and lowers the strength. The presence of the sulfhydryl oxidase enhanced the hardening effect of the ascorbic acid in a dose-dependant manner by increasing the dough strength and reducing the extensibility. This effect was ascribed to an increased formation of dehydroascorbic acid, the actual improver, by the action of the hydrogen peroxide produced in the reaction catalyzed by AoSOX1. In addition, the activity of the sulfhydryl oxidase possibly contributed to a more efficient removal of the reduced glutathione that is able to weaken the dough protein network.

AoSOX1 had a possible application in the baking industry as an alternative to potassium bromate, a bread improver currently withdrawn from the market due to the hazardous effects on human health. Further studies will be performed in order to evaluate the effects of the combined use of AoSOX1 and ascorbic acid on the final baked product.

This study reported the discovery and production of three novel enzymes and showed that genome analysis can be a powerful tool for this task. In the near future the crosslinking activity of all the three novel enzymes identified in this study, i.e. AoCO4, AoSOX1 and AoSOX2, should be assessed on model proteins as substrates. The potential applications of sulfhydryl oxidases are numerous and not limited to their potential crosslinking activity. Sulfhydryl oxidases can be utilised by the food industry either to act on protein complex structures as crosslinking enzymes or to oxidise small thiol compounds. The action of sulfhydryl oxidases on small thiol compounds indicates their potential use to tailor the flavour of food and beverages, for example of fermented products such as wine

and beer. Moreover, these enzymes have a potential utilization in the removal of off-flavours either from industrial products or residual by-products such as wastewaters. In the beverage and milk industry, the hydrogen peroxide produced by sulfhydryl oxidases can be used to control undesired microbial contaminations and thus produce safer products. The high specificity of the reaction catalysed by sulfhydryl oxidases makes them useful for the production of thiol-containing bioactive and pharmaceutical compounds, and wherever the oxidation of sulfhydryl groups is required but harsh oxidising conditions are not suitable for the process, e.g. folding of therapeutic proteins and antibodies.

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