

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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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.

My warmest thanks go to Prof. Johanna Buchert for inviting me to be part of this ambitious project and to join such a stimulating scientific environment. My supervisor Doc. Markku Saloheimo is sincerely thanked for trusting me and for showing me passion for science. I thank Prof. Kristiina Kruus for contributing to my education and teaching me commitment. Dr. Raija Lantto, Dr. Harry Boer, Doc. Maija-Liisa Mattinen, Dr. Emilia Nordlund and Dr. Ritta Partanen are sincerely thanked for their contribution to the discussions during the ProEnz meetings. I also thank Doc. Taina Lundell and Doc. Tuomas Haltia for their examination of the thesis and their valuable comments. All the scientists of the Protein production team are also acknowledged for their critical comments during these years. I acknowledge Doc. Pekka Heino for his precious help during my PhD studies.

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Publications I–IV

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 <i>Aspergillus oryzae</i>
AoSOX1	Sulfhydryl oxidase Q2UA33 from <i>Aspergillus oryzae</i>
AoSOX2	Sulfhydryl oxidase Q2U4P3 from <i>Aspergillus oryzae</i>
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/jcfn)
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- <i>tert</i> -butylcatechol
TYR	Tyrosinase (monophenol, <i>o</i> -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).

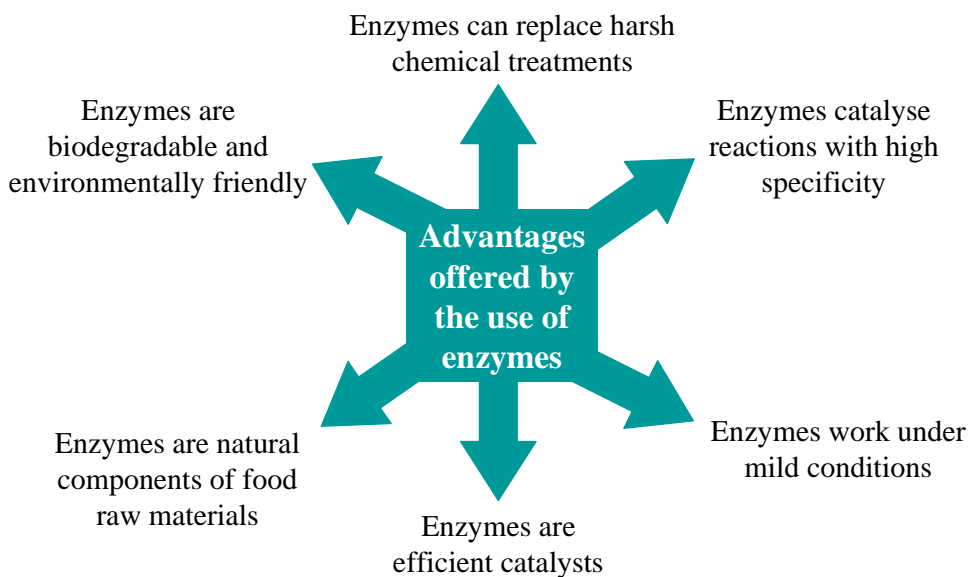


Figure 1. Schematic summary of the advantages offered by the use of enzymes in industrial processes.

1. Introduction

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	<i>Aspergillus niger</i> , <i>Penicillium spp.</i>	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	<i>Streptomyces spp.</i> , <i>Bacillus subtilis</i>	incorporation of amines in proteins, crosslinking, deamidation	meat, fish, dairy, wheat and soybean products	(Motoki & Seguro, 1998)
fructosyl-transferase	EC 2.4.1.9	<i>Aspergillus oryzae</i> , <i>Bacillus spp.</i>	fructosyl group transfer	production of sweeteners	(Nam et al., 2000, Sangeetha et al., 2005)
lipase	EC 3.1.1.3	<i>Candida rugosa</i> , <i>Aspergillus spp.</i> , <i>Rhizopus niveus</i>	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	<i>Trichoderma reesei</i> , <i>Aspergillus oryzae</i>	hydrolysis of cellulose	fruit juices, coffee	(Kapasakalidis et al., 2009, Delgado et al., 2008, Szakacs-Dobozi et al., 1988, Ghorai et al., 2009)

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Enzyme	Classification	Organism	Mode of action	Application	Reference
xylanase	EC 3.2.1.8	<i>Trichoderma spp.</i> , <i>Aspergillus spp.</i>	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	<i>Streptococcus thermophilus</i> , <i>Kluyveromyces spp.</i> , <i>Aspergillus spp.</i>	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	<i>Aspergillus oryzae</i> , <i>Aspergillus awamori</i> , <i>Aspergillus niger</i> , <i>Rhizopus spp.</i>	degradation of starch	baking, brewing	(Bamforth, 2009, Mondal & Datta, 2008)
protease	endopeptidases EC 3.4.21-99, exopeptidases EC 3.4.11-19	<i>Aspergillus oryzae</i> , <i>Rhizomucor spp.</i>	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	<i>Aspergillus spp.</i> , <i>Rhizopus spp.</i>	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	<i>Streptomyces spp.</i> , <i>Bacillus spp.</i> , <i>Aspergillus oryzae</i>	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 (Rhim 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, Gerard & Sutton, 2005, Tan et al., 2011).

1. Introduction

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

Enzyme	Classification	Organism	Mode of action (target aa)	Application	References
sulphydryl oxidase	EC 1.8.3.3	<i>Aspergillus niger</i> , <i>Penicillium sp. K-6</i>	formation of disulfide bonds (C)	baking	(Kusakabe et al., 1982, Haarasilta et al., 1991, Haarasilta & Vaisanen, 1989)
laccase	EC 1.10.3.2	<i>Trametes hirsuta</i> , <i>Polyporus pinisitus</i>	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	<i>Coprinus cinereus</i> , <i>Cochlearia armoracia</i> (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	<i>Agaricus bisporus</i> , <i>Neurospora crassa</i> and <i>Trichoderma reesei</i>	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	<i>Streptomyces spp.</i> and <i>Bacillus subtilis</i>	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.

1. Introduction

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	<i>Aspergillus oryzae</i> , <i>Bacillus spp.</i>	degradation of starch, production of sugars for yeast	improves bread volume, softness, firmness	(Goesaert et al., 2005, Kim et al., 2006, Cherl-Ho, 2009)
hemi-cellulase	<i>Trichoderma spp.</i> , <i>Aspergillus spp.</i> , <i>Bacillus spp.</i>	hydrolysis of arabinoxylans	improves bread volume and dough strength	(Dagdelen & Gocmen, 2007, Morita et al., 1998)
lipase	<i>Aspergillus oryzae</i> , <i>Aspergillus niger</i>	hydrolysis of triglycerides	increases dough stability, bread volume, texture and shelf-life	(Whitehurst & Van Oort, 2009, Gélinas et al., 1998)
tyrosinase	<i>Trichoderma reesei</i> , <i>Agaricus bisporus</i>	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	<i>Trametes hirsuta</i>	probable crosslinking 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	<i>Aspergillus niger</i> , <i>Aspergillus oryzae</i>	formation of protein crosslinks 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	<i>Glycine max</i> (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	<i>Aspergillus niger</i>	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, Soupe, 2000)
protease	<i>Aspergillus oryzae</i> , <i>Aspergillus niger</i>	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 (Halaoui 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.

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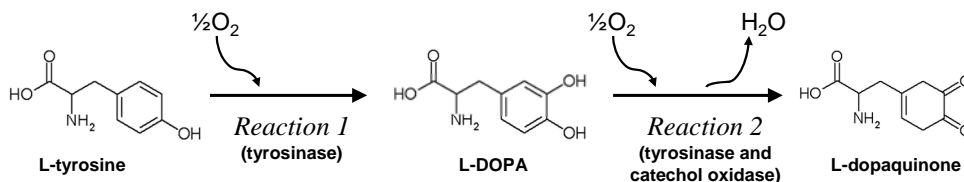


Figure 2. Reactions catalysed by tyrosinase (reactions 1 and 2) and catechol oxidase (reaction 2).

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, Halaoui 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	<i>Streptomyces castaneoglobisporus</i>	crystal structure in complex with caddie protein ORF378 (PDB: 1WX5)	(Matoba et al., 2006, Kohashi et al., 2004)
bacterium	TYR	NP659960, 609	<i>Rhizobium etli</i>	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	<i>Verrucomicrobium spinosum</i>	the first bacterial tyrosinase with a C-terminal domain	(Fairhead & Thony-Meyer, 2010)
fungus	TYR	CAL90884, 561	<i>Trichoderma reesei</i>	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	<i>Agaricus bisporus</i> (button mushroom)	isolated from fruiting bodies, C-terminally processed	(Wichers et al., 1996, Wichers et al., 2003)
plant	TYR	B21677, 593	<i>Malus x domestica</i> (apple)	solubilized and proteolyzed during ripening and storage	(Haruta et al., 1998, Murata et al., 1997)
plant	PPO	P93622, 607	<i>Vitis vinifera</i> (grapes)	catechol oxidase activity, crystal structure available (PDB: 2P3X1),	(Virador et al., 2010)
plant	CO	Q9ZP19, 496	<i>Ipomoea batatas</i> (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	<i>Homo sapiens</i>	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 (Halaoui 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).

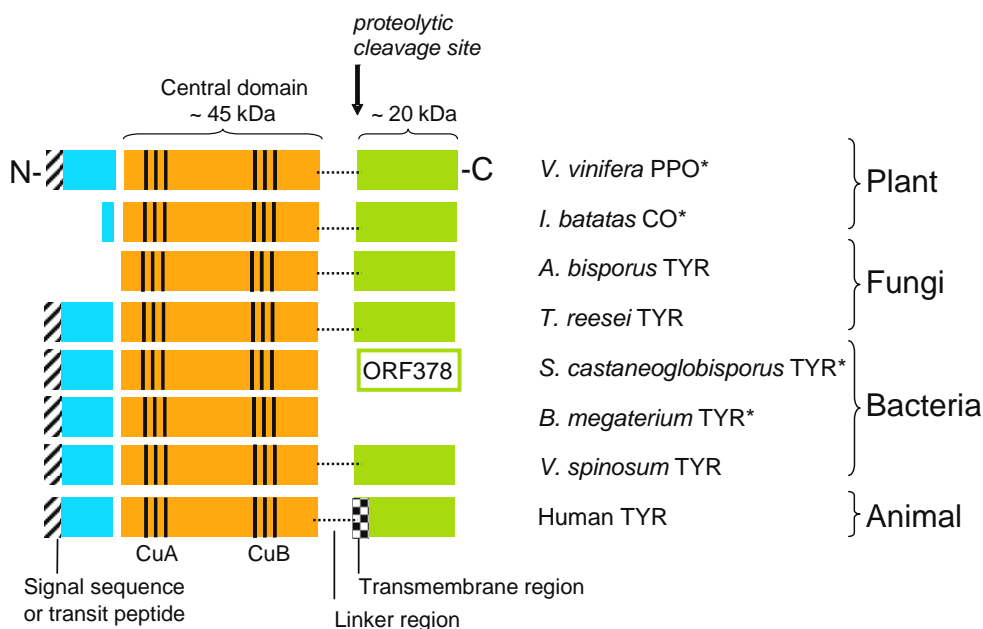


Figure 3. Domain organisation of representative tyrosinases and catechol oxidases from different organisms. The N-terminal domain is in blue and diagonal lines indicate the presence of a signal sequence or transit peptide. The central domain is in orange and vertical lines indicate the CuA and CuB sites. 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 form only

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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 & Tucek, 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	<i>Streptomyces castaneoglobisporus</i>	Monophenols: L-tyrosine. Diphenols: L-DOPA. Others: NR.	(Matoba et al., 2006, Kohashi et al., 2004, Ikeda et al., 1996)
bacterium	TYR	<i>Rhizobium elii</i>	Monophenols: L-tyrosine, n-acetyl-L-tyrosine. Diphenols: L-DOPA, catechol, caffeic acid. Others: NR.	(Cabrera-Valladares et al., 2006)
bacterium	TYR	<i>Verrucosporium spinosum</i>	Monophenols: L-tyrosine. Diphenols: L-DOPA. Others: NR.	(Fairhead, Thony-Meyer, 2010)
fungus	TYR	<i>Trichoderma reesei</i>	Monophenols : tyramine, phenol, L-tyrosine, <i>p</i> -cresol, <i>p</i> -tyrosol, <i>p</i> -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	<i>Agaricus bisporus</i> (button mushroom)	Monophenols L-tyrosine, phenol, <i>p</i> -cresol, tyramine, <i>p</i> -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	<i>Malus x domestica</i> (apple)	Monophenols: L-tyrosine, <i>p</i> -cresol, tyramine, <i>p</i> -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	<i>Vitis vinifera</i> (grapes)	Monophenols: NR. Diphenols: catechol, 4- <i>tert</i> -butylcatechol, 4-methylcatechol. Others: NR.	(Virador et al., 2010, Sanchez-Ferrer et al., 1988)
plant	CO	<i>Ipomoea batatas</i> (sweet potato)	Monophenols: NR. Diphenols: catechol, caffeic acid, 4-methylcatechol, L-DOPA. Others: NR.	(Eicken et al., 1998)
animal	TYR	<i>Homo sapiens</i>	Monophenols :L-tyrosine. Diphenols: L-DOPA.Others: NR.	(Takara et al., 2008, Okombi et al., 2006)

Abbreviations: NR, not reported

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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 optima of 9 and 8, respectively (Selinheimo et al., 2006, Eicken et al., 1998).

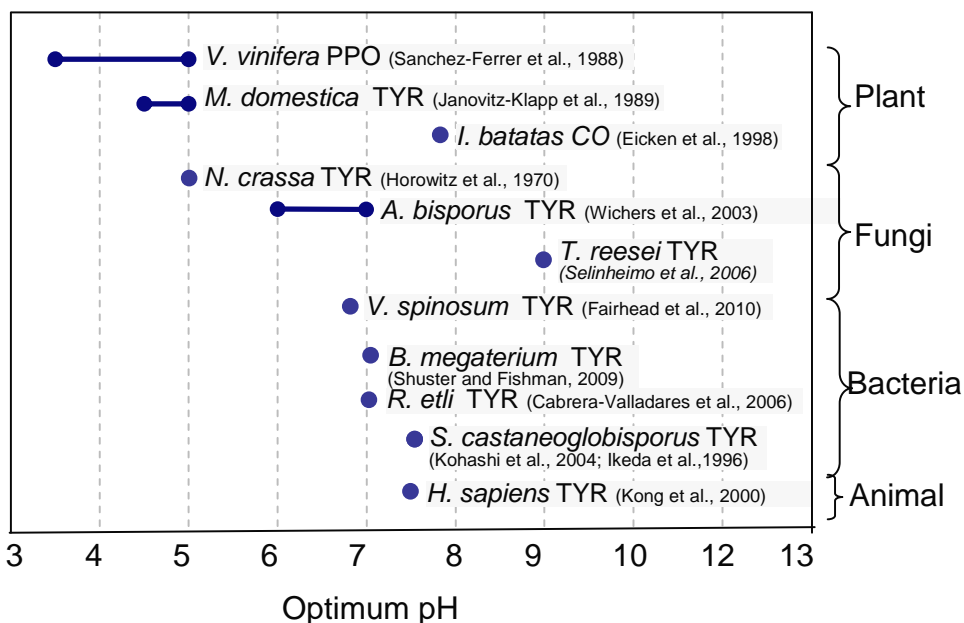


Figure 4. Optimum pH values of catechol oxidases and tyrosinases from different sources.

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.

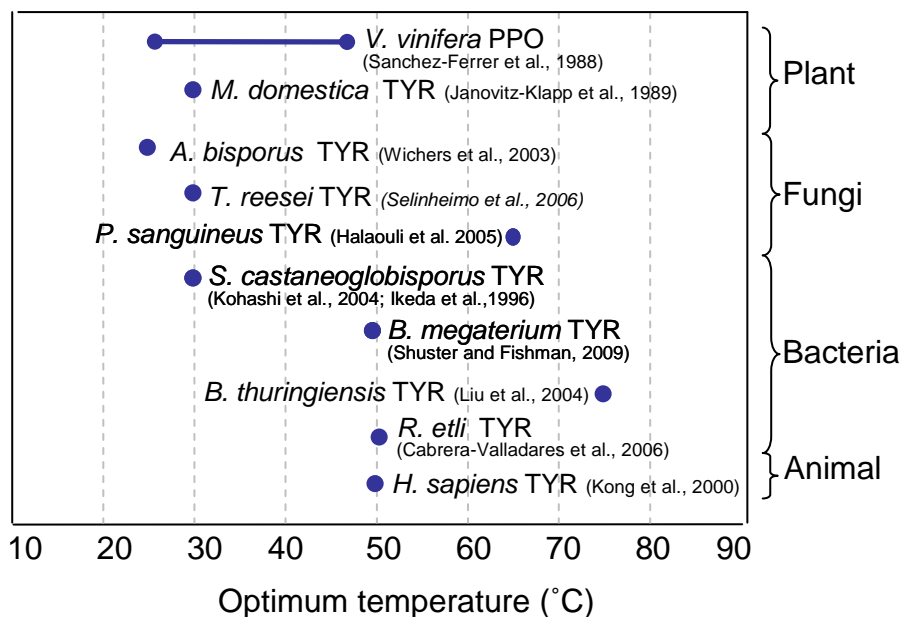


Figure 5. Optimum temperature of representative tyrosinases and catechol oxidases from different sources.

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 dofloeni*, 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).

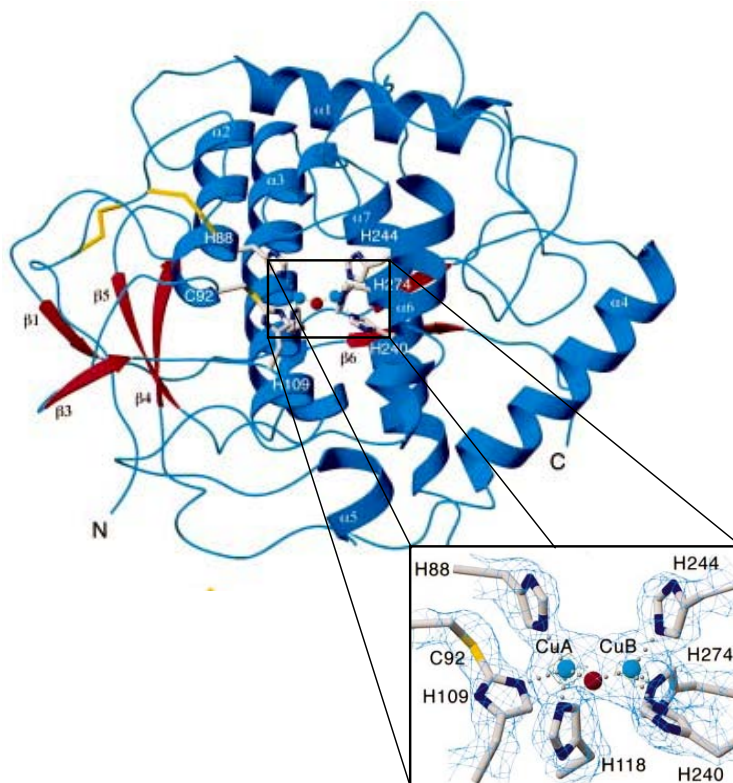


Figure 6. Ribbon representation of the three-dimensional structure of the catechol oxidase from *Ipomoea batatas* highlighting α -helices in blue, β -strands in red and disulfide bonds in yellow. In the inset, the red atom between the two copper binding sites is probably a hydroxide ion from the solvent (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 (F₂₆₁, gate residue, Table 6). In haemocyanins the active site is completely occupied by a leucine or a phenylalanine in the protein from *O. dofleini* (L₂₈₃₀) and *Limulu polyphemus* (F₄₉), respectively.

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

Sequence motif	Position in <i>Ipomoea batatas</i> CO	Function	References
<i>N-terminal region</i>			
R	R ₄₉	interacts with the tyrosine motif	(Marusek et al., 2006, Flurkey & Inlow, 2008)
<i>Central region</i>			
<u>H</u> 1A-X(n)- <u>C</u> -X(n)- <u>H</u> 2A-X(8)- <u>H</u> 3A	H ₈₈ -X(3)-C ₉₂ -X(16)-H ₁₀₉ -X8-H ₁₁₈	CuA binding site	(Merkel et al., 2005, García-Borrón & Solano, 2002)
<u>H</u> 1B-X(3)- <u>H</u> 2B-X(n)- <u>H</u> 3B	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 - 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)
<i>C-terminal region</i>			
Y/F-X-Y or Y-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.

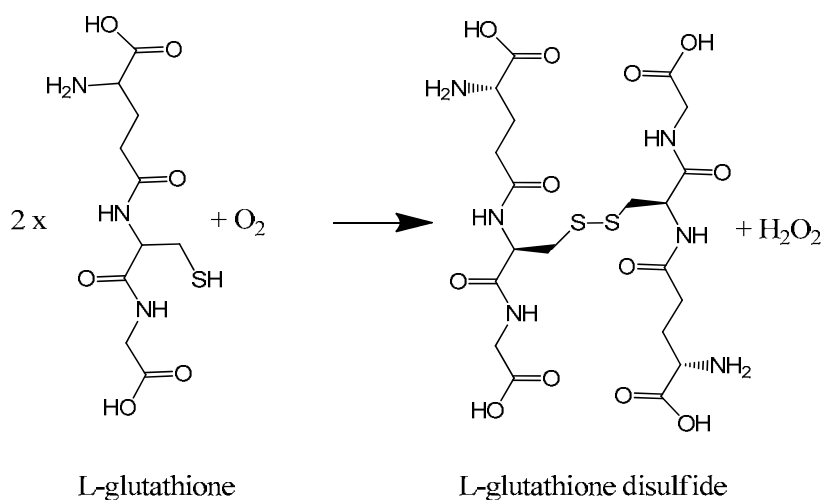


Figure 7. Oxidation of glutathione catalysed by sulfhydryl oxidase (EC 1.8.3.3).

Typical substrates for sulfhydryl oxidases are small thiol compounds, such as cysteine, dithiothreitol 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 dithiothreitol 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.

Glutathione 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 intracellularly 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, Hooper, 1999).

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

Source	Enzyme	Organism	Location	Physiol. role	References
virus	E10R	<i>Vaccinia virus</i>	cytosol	oxidative folding of virion proteins	(Senkevich et al., 2000)
bacterium	FAD-dependent pyridine nucleotide-disulfide oxidoreductase	<i>Chromobacterium violaceum</i>	cytoplasm	biosynthesis of the anticancer agent FK228	(Wang et al., 2009, Cheng et al., 2007)
fungus	GSH oxidase	<i>Fusarium spp.</i>	extracellular	NR	(Kusakabe et al., 1983)
fungus	SOX	<i>Aspergillus niger</i>	extracellular	NR	(de la Motte & Wagner, 1987, Vignaud et al., 2002, Hammer et al., 1990)
fungus	thiol oxidases eroA and ervA	<i>Aspergillus niger</i>	ER	oxidative protein folding	(Harvey et al., 2010)
fungus	GSH oxidase	<i>Penicillium sp. K-6-5</i>	extracellular	NR	(Kusakabe et al., 1982)
fungus	Erv1	<i>Saccharomyces cerevisiae</i>	IMS	oxidative protein folding	(Bien et al., 2010, Ang & Lu, 2009, Lange, 2001, Lee, 2000)
fungus	Erv2	<i>Saccharomyces cerevisiae</i>	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	<i>Saccharomyces cerevisiae</i>	ER	oxidative protein folding	(Vitu et al., 2010, Gross et al., 2004, Frand & Kaiser, 1999, Hiniker & Bardwell, 2004)
plant	Erv1	<i>Arabidopsis thaliana</i>	IMS	promotes the import and oxidative folding of proteins	(Allen et al., 2005)
animal	QSOX	<i>Rattus norvegicus</i>	extracellular (seminal vesicle)	NR	(Ostrowski & Kistler, 1980, Ostrowski et al., 1979)
animal	QSOX	<i>Rattus norvegicus</i>	extracellular (skin)	sulfhydryl oxidation of proteins of epidermis	(Matsuba et al., 2002, Hashimoto et al., 2001, Hashimoto et al., 2000)
animal	QSOX	<i>Homo sapiens</i>	Golgi apparatus	oxidative protein folding	(Chakravarthi et al., 2007, Heckler et al., 2008)
animal	QSOX	<i>Bos taurus</i>	extracellular (milk)	oxidative protein folding	(Jaje et al., 2007, Zanata et al., 2005)
animal	QSOX	<i>Gallus gallus</i>	extracellular (egg)	protein disulfide bond formation	(Thorpe et al., 2002, Hooper et al., 1996, Hooper et al., 1999)

Abbreviations: ER, endoplasmic reticulum, IMS, mitochondrial 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 (Hooper, 1999, Tury et al., 2006). In addition, the production of hydrogen peroxide by sulfhydryl oxidases could have antimicro-

bial 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 (Hooper, 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 (Hooper, 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	
<i>Aspergillus niger</i>	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)
<i>Bos taurus</i> (bovine milk)	QSOX	62	7.5	DTT, D/L-cysteine, N-acetyl-L-cysteine, cysteamine	(Jaje et al., 2007, Sliwkowski et al., 1984)
<i>Gallus gallus</i> (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)	(Hooper et al., 1996, Hooper et al., 1999)
<i>Penicillium sp. K-6-5</i>	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-

1. Introduction

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/β 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 β structures in a β - α - β - α - β arrangement where the interaction between the first β -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 <i>Aspergillus niger</i>	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 Rossmann 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 *gluA* 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

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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	<ul style="list-style-type: none"> - identification of candidate proteins - evaluate the distribution of a target protein 	I, II
Alignment of protein sequences	<ul style="list-style-type: none"> - 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	<ul style="list-style-type: none"> - target gene amplification and cloning - selection of positive <i>Trichoderma reesei</i> transformants 	I, II, III I, II, III
Transformation of <i>Trichoderma reesei</i> and selection of the antibiotic-resistant transformants	<ul style="list-style-type: none"> - heterologous expression of the target protein and isolation of positive transformants 	I, II, III
Cultivation of <i>Trichoderma reesei</i> transformants on plates containing tyrosine	<ul style="list-style-type: none"> - selection of the transformant with highest tyrosinase/catechol oxidase production 	I
UV-vis spectroscopy	<ul style="list-style-type: none"> - 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).

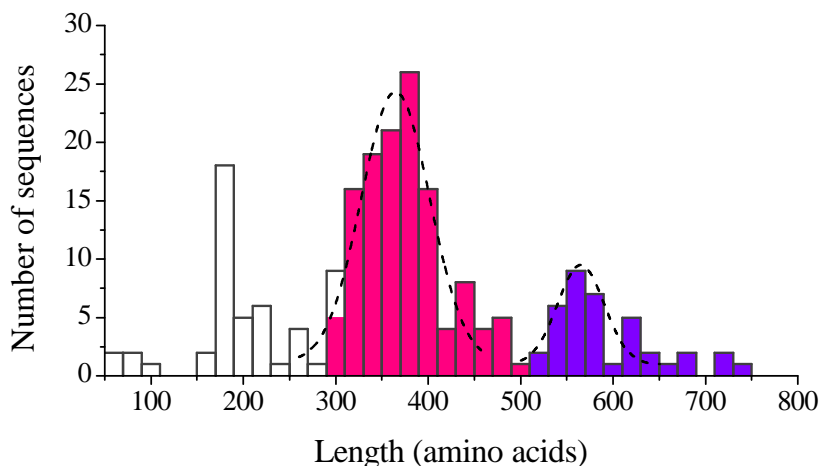


Figure 8. Length distribution of the sequences retrieved carrying a predicted tyrosinase domain (IPR0002227) and an N-terminal signal sequence. Sequences bearing a conserved histidine pattern (coloured bars) are divided into two groups with length of 300–500 amino acids (pink bars) and 500–800 amino 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 dofloei* (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 C₇₂ and C₁₀₀ in the sequence Q2UNF9 from *Aspergillus oryzae* named AoCO4), three between the copper binding regions (C₁₅₉, C₂₂₃ and C₂₆₁ in AoCO4) and one adjacent to the C-terminal tyrosine motif (C₄₀₄ in AoCO4). The conserved N-terminal arginine corresponding to R₄₀ 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).

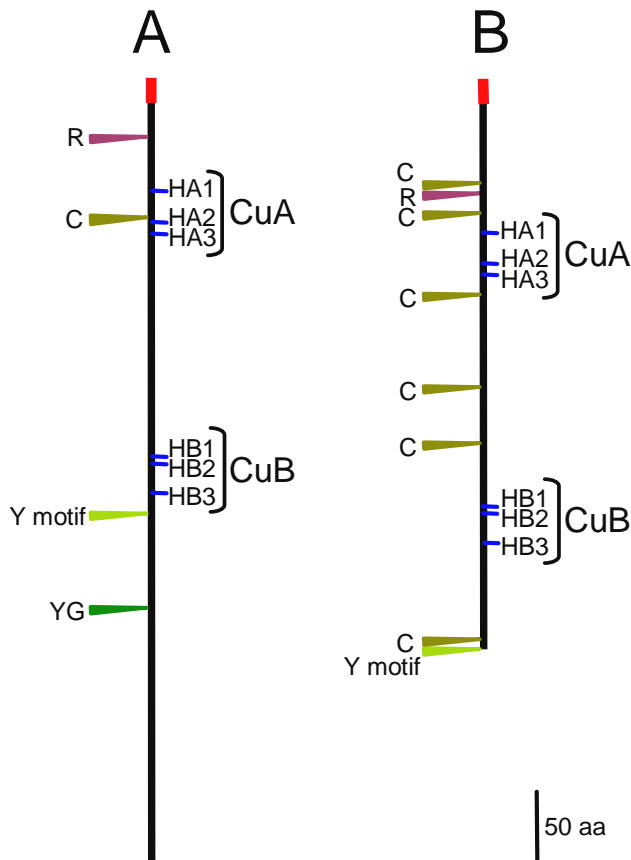


Figure 9. Schematic representation of the distribution of some conserved residues between long (A) and short (B) tyrosinases. The histidines of the CuA and CuB sites as are in blue. The N-terminal conserved arginine (R) is in purple and the tyrosine (Y) motif is in light green (Flurkey & Inlow, 2008). 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).

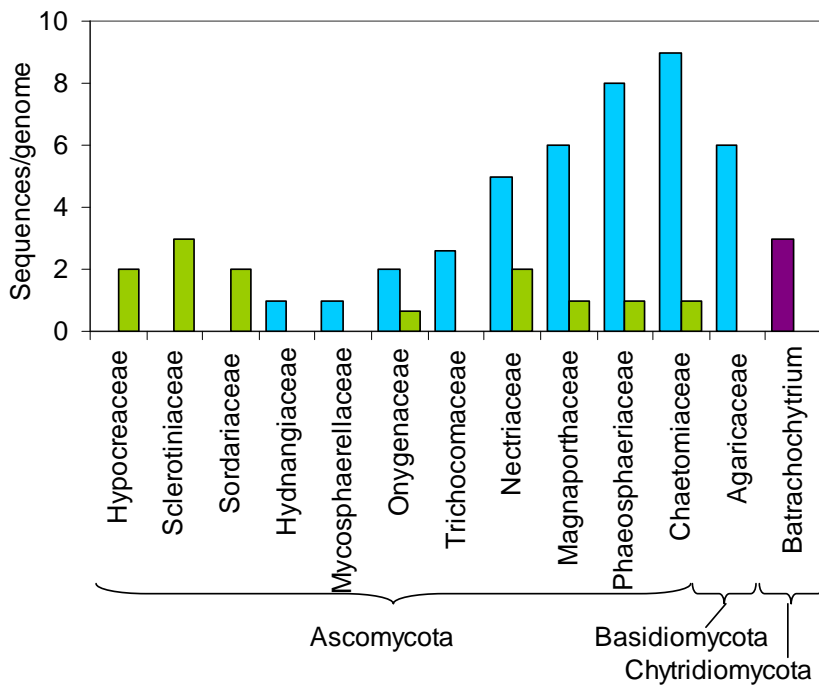


Figure 10. Averaged species distribution of the sequences of long (green) and short (blue) tyrosinase among the fungal families analysed. Sequences from *Batrachochytrium dendrobatidis* are marked by a purple bar.

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).

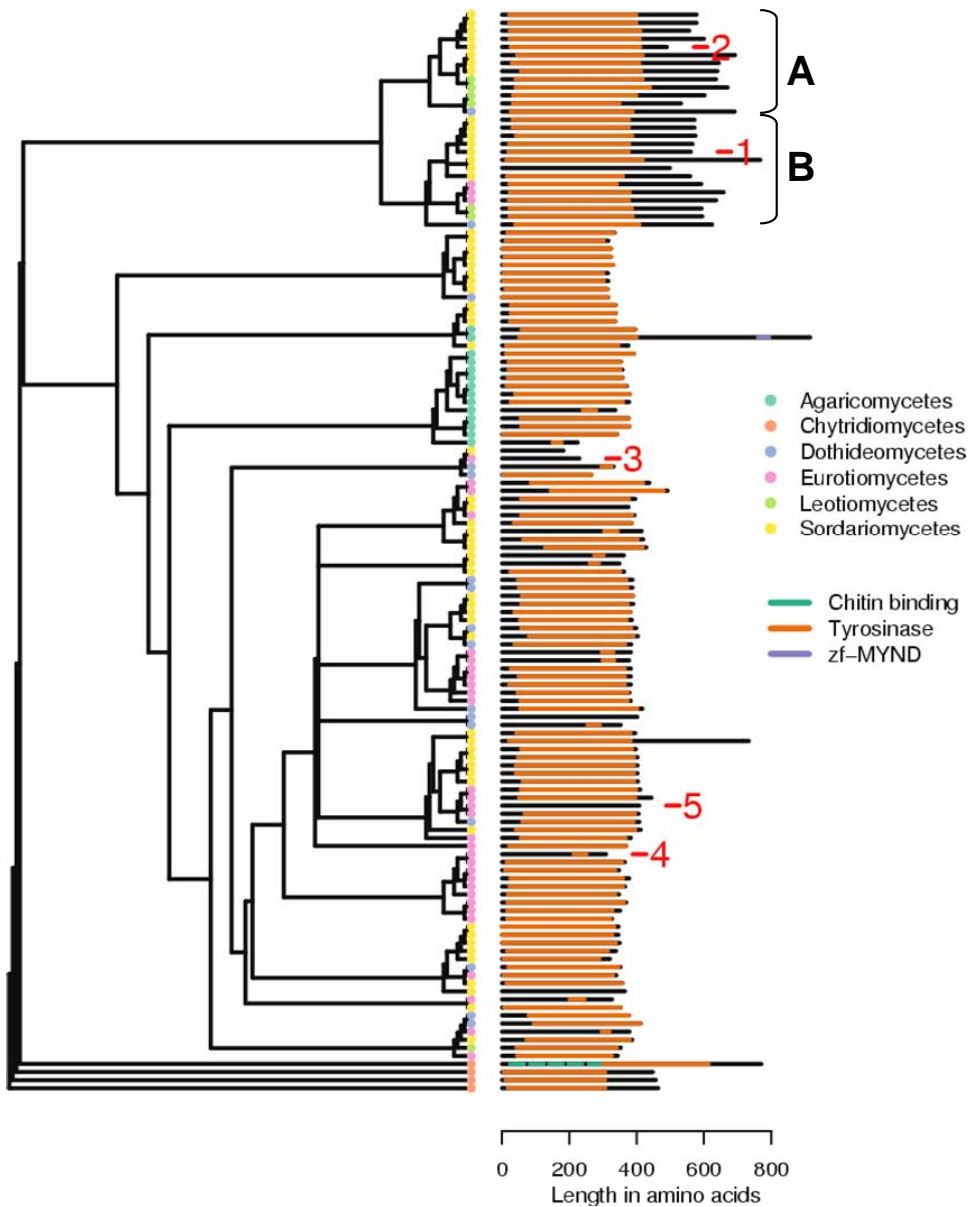


Figure 11. Phylogenetic analysis of potential secreted tyrosinase sequences. Fungal classes are indicated by a coloured dot at the end of each branch. On the right, a line represents the length of the protein and the computationally predicted domains (coloured region). Numbers indicate proteins of specific 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.

4. Results and discussion

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 Agaricomycotina, 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 R₄₀ 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.

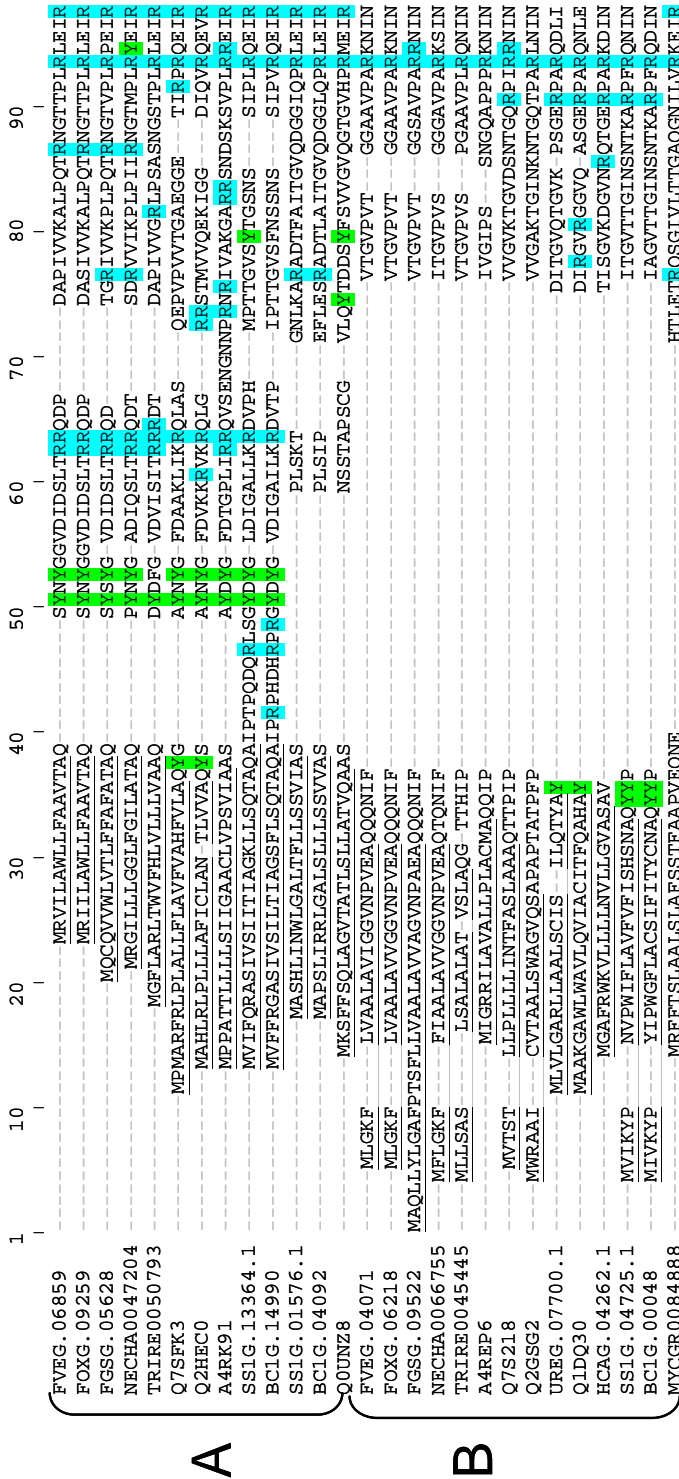


Figure 12. Sequence Multiple alignment of the N-terminal sequence of the long tyrosinases in the order in which they appear in the phylogenetic tree in Figure 11 and in Publication I Online Resource 1 (sequence identifiers as in Publication I Table 1). The only characterised protein is TRIRE0045445 (TrTyr2, 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).

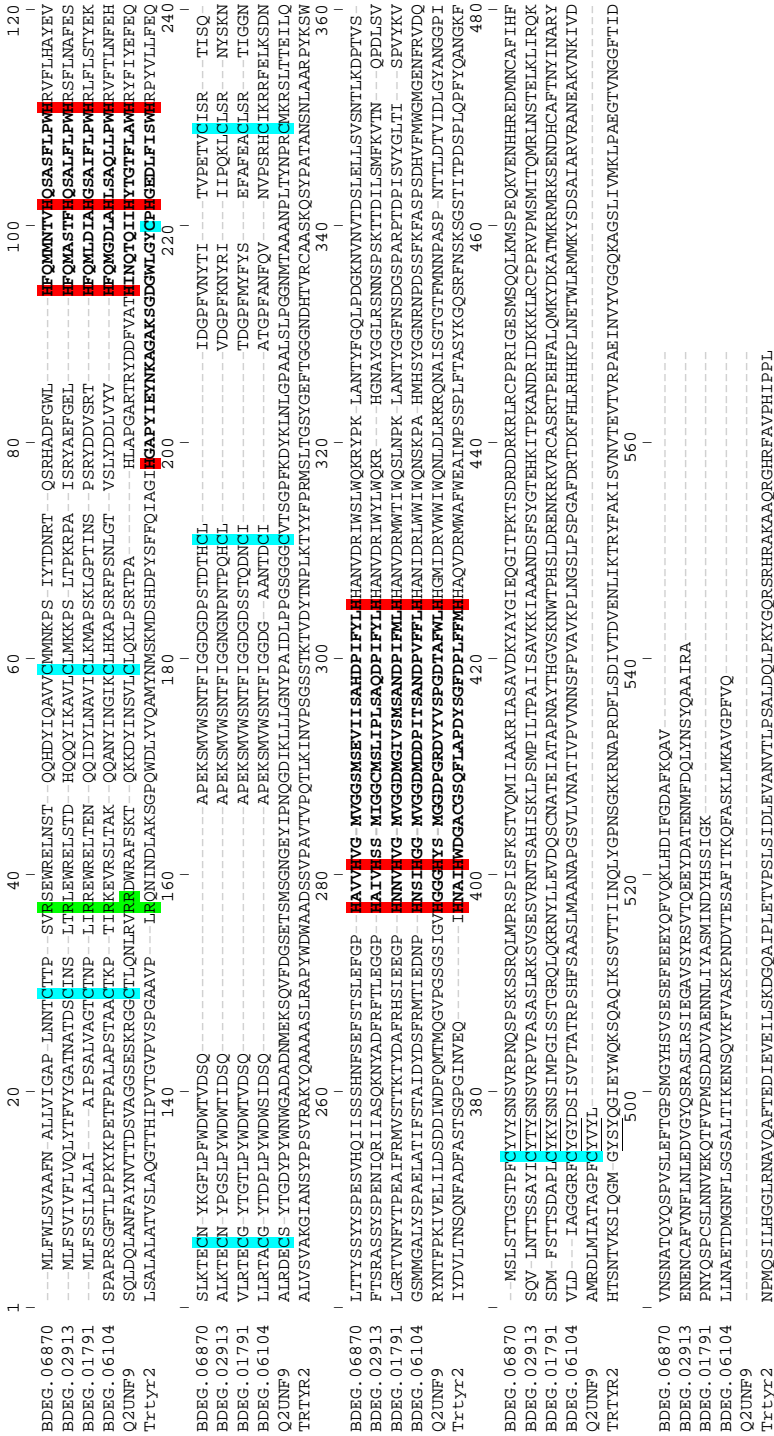


Figure 13. Partial alignment of sequences from *B. dendrobatidis* with a representative short tyrosinase Q2UNF9 (AoCo4) from *A. oryzae* and the long tyrosinase TRIRE0045445 (TTr2) from *T. reesei* (Selinheimo et al., 2006). The putative N-terminal conserved arginine residue is in green, histidine residues 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, H₁₁₁ and H₁₂₇ 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 (%)
<i>Biochemically characterised</i>				
bacterium	<i>Streptomyces castaneoglobisporus</i>	TYR	(Ikeda et al., 1996, Machida et al., 2005)	20.4
fungus	<i>Trichoderma reesei</i>	TYR	(Selinheimo et al., 2006)	14.7
bacterium	<i>Verrucomicrobium spinosum</i>	TYR	(Fairhead & Thony-Meyer, 2010)	14.4
fungus	<i>Neurospora crassa</i>	TYR	(Lerch, 1978)	13.3
plant	<i>Ipomoea batata</i>	CO	(Eicken et al., 1998)	13.0
fungus	<i>Agaricus bisporus</i>	TYR (AbPPO1)	(Flurkey et al., 2008, Wichers et al., 1996, Espin & Wichers, 1999)	12.5
fungus	<i>Agaricus bisporus</i>	TYR (AbPPO2)	(Flurkey et al., 2008, Wichers et al., 2003, Espin & Wichers, 1999)	12.4
<i>Non-characterised proteins (best hits)</i>				
fungus	<i>Aspergillus niger</i>	An01g09220	(Pel et al., 2007)	72.4
fungus	<i>Penicillium chrysogenum</i>	Pc22g18500	(van den Berg et al., 2008)	68.8
fungus	<i>Talaromyces stipitatus</i>	putative TYR EED15869.1	direct annotation (J. Craig Venter Institute, USA)	68.3
fungus	<i>Coccidioides immitis</i>	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

4. Results and discussion

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).

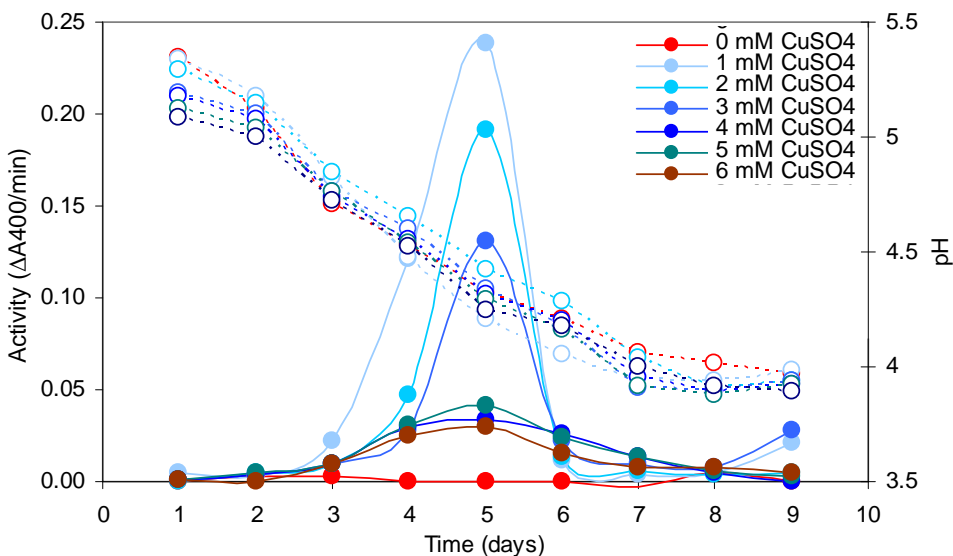


Figure 14. Production of AoCO4 in shake flask culture in the presence of different concentrations of copper. The maximum activity (filled circles) was reached on the 5th day and a sharp decrease was observed as the pH of the culture medium (empty circles) decreased below 4.5.

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 Q₂₅ and the major activity was eluted at 90 mM and corresponded to a more extensively processed form that started at R₆₉, after a potential recognition site for KEx2-like proteases (K₆₈-R₆₉). This form was thus lacking 51 residues after the signal peptide cleavage site (A₁₈-F₁₉). 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- <i>tert</i> -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	T _{1/2} at 50°C at pH 7: 20 hrs; T _{1/2} at 60°C at pH 7: 2 hrs, T _m : 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.

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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
<i>N-terminal region</i>			
(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)
<i>Central region</i>			
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)- <u>C-X-X-C</u> -(D/H)-G-Φ-E	177–180	typical of thiol:disulfide oxidoreductases with a thioredoxin fold	(Fass, 2008)
<i>C-terminal region</i>			
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 sulphhydryl 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 sulphhydryl 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		
<i>AoSOX1 homologs</i>				
<i>Aspergillus flavus</i>	thioredoxin reductase	EED47993	direct submission (J. Craig Venter Institute, USA)	99.5
<i>Neosartorya fischeri</i>	thioredoxin reductase	XP_001266180	direct submission (The Institute for Genomic Research, USA)	69.9
<i>Aspergillus fumigatus</i>	thioredoxin reductase	XP_747990	(Nierman et al., 2005)	68.9
<i>AoSOX2 homologs</i>				
<i>Aspergillus flavus</i>	thioredoxin reductase	ABY86217	direct submission (J. Craig Venter Institute, USA)	97.7
<i>Penicillium chrysogenum</i>	-	Pc12g03690	(van den Berg et al., 2008)	67.4
<i>Neosartorya fischeri</i>	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.

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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 (K₃₃₇K₃₃₈, K₃₅₈R₃₅₉, K₃₆₈R₃₆₉ in AoSOX1 and K₃₅₇R₃₅₈, K₃₈₄R₃₈₅ and R₃₈₈R₃₈₉ 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)

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AoSOX1	1	MAPKSLFYT-----LFSSLTVALAASVPQTDYEVIVVGGGPAGLTA	41
AoSOX2	1	-----MMFTCNWTALLCALLSILSVCV-ADIPQTNVDVIIIGGGPSGLSA	44
AoSOX1	42	LSGLSRVRRKTALFDSHEYRNAATRNMHDVIGNDGTVPSEFRGLAREQIS	91
AoSOX2	45	ASALSRVLRKSVLFDSGEYRNNPTRHMHDVIGNDHVVPAEFRKKTAREQIS	94
AoSOX1	92	RYDTATFIDKRVNTIETVSDPEATNTSYFRAQDADGKAYTARKVVLGTGLV	141
AoSOX2	95	FY NT TFIDQKVTKLEKTGDNA-----FQATVGD-QQYTARKVILGSGVK	138
AoSOX1	142	DILPDVPLGLEAWGKGVYWCPCWCDGYEHRDQPFILGALPDVVGVSLEVY	191
AoSOX2	139	DDLDPVPLGLEGFKGIFWCPCWCDGFEHRNQSMGVLGDISEAYGAVRELH	188
AoSOX1	192	-TLNTDIIAFVNGTQTPDQEAELAKKYPNWEAOLEAYNVRLNETIASFE	240
AoSOX2	189	PTLNKDIRIYANGTNTTEQIAILDKNHPNWRKVFQAYNVTVNNKPILNIT	238
AoSOX1	241	RIQDGSVWKDRNGTRQIDIFRVHFTNGSSVDRNAFITNYPSEQRSDLPKQ	290
AoSOX2	239	RIQDGA>VNDPAIRLEFDKQIYFADNSSEVRGAFITNYGTSQRSDLPAQ	288
AoSOX1	291	LGLAMLGKIDATTNPGMRTSLPGVWAIGDCNSD NSTNV PHAMFSGKRAA	340
AoSOX2	289	LGVEMLGSKIN-TLRKGLQTSVPGVWVGVDANS NSTNV PHGMSSGKSA	337
AoSOX1	341	VFAHVEMAKEE--SNTAIDKR-----DDFVKEVEKRMGNDMEKIY NR-SRGL	384
AoSOX2	338	VYCHVELAQEELDRDAGLEKRETEFDAESFHETTEROMGSEIQDLYKRLSR-	389

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 sulphhydryl 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).

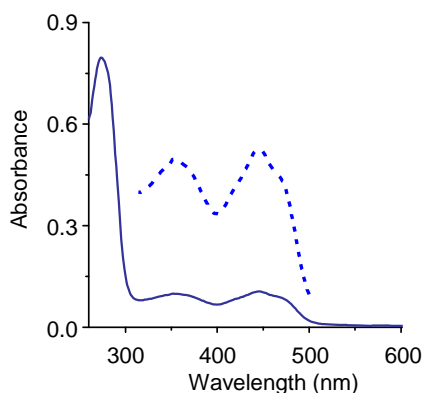


Figure 16. The flavin cofactor bound to AoSOX2 is indicated in the absorbance spectrum of the purified enzyme by the peaks at approx. 370 and 440 nm. The dotted line is a 5X magnification of the absorbance spectrum.

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.

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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), β -ME (219); Dithiols: DTT (5451).	Monothiols: GSH (370), L-Cys (106), D-Cys (116), β -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	T _m : 57°C C _m : 2.5 M GndHCl	T _m : 75°C C _m : 4 M GndHCl

Abbreviations: T_m, melting temperature; C_m, 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, 24×10^{-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).

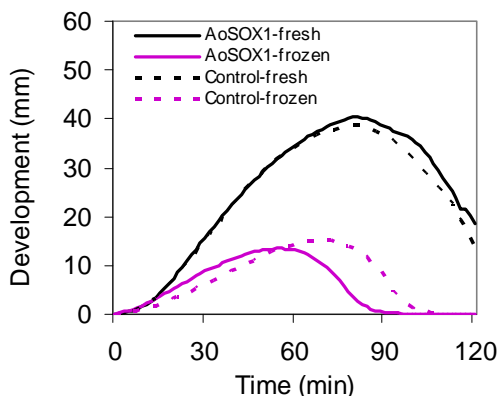


Figure 17. Development curves of fresh (black lines) and frozen (red lines) doughs made from commercial flour containing ascorbic acid with (continuous line) and without (dotted line) addition of 100 U/g sulphhydryl oxidase in flour. Fermentation was monitored by Rheofermentometer F3.

Doughs prepared with flour not containing ascorbic acid and treated with different amounts of sulphhydryl 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 sulphhydryl oxidase on non-yeasted fresh dough with and without ascorbic acid

In order to evaluate the effects of the sulphhydryl 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 sulphhydryl

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).

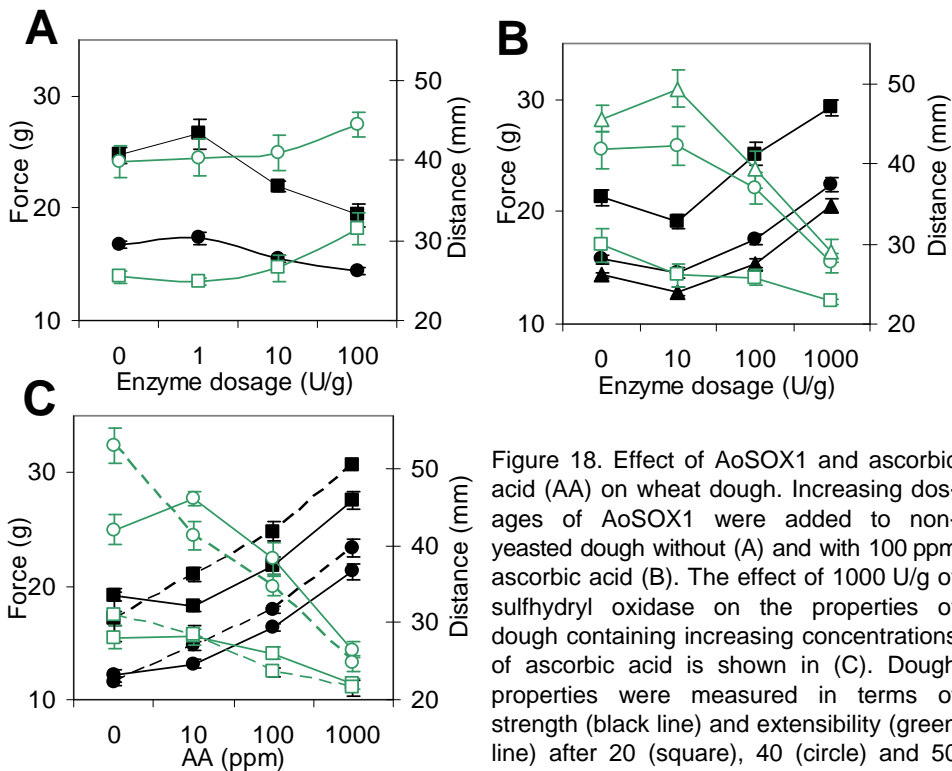


Figure 18. Effect of AoSOX1 and ascorbic acid (AA) on wheat dough. Increasing dosages of AoSOX1 were added to non-yeasted dough without (A) and with 100 ppm ascorbic acid (B). The effect of 1000 U/g of sulfhydryl oxidase on the properties of dough containing increasing concentrations of ascorbic acid 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).

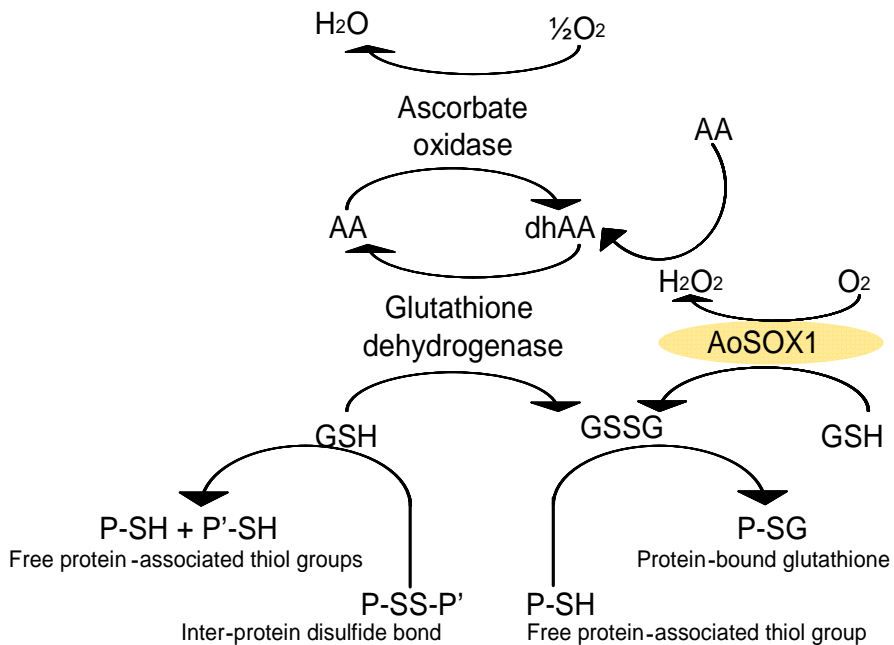


Figure 19. Proposed interaction between the mechanism of action of ascorbic acid in wheat dough and the reaction catalysed by the sulfhydryl oxidase AoSOX1. Abbreviations: GSH and GSSG, reduced and oxidised glutathione respectively, AA, ascorbic acid, dhAA, dehydroascorbic acid.

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

5. Conclusions and future prospects

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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PUBLICATION I

**Discovery of a new tyrosinase-like enzyme
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Discovery of a new tyrosinase-like enzyme family lacking a C-terminally processed domain: production and characterization of an *Aspergillus oryzae* catechol oxidase

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Abstract A homology search against public fungal genome sequences was performed to discover novel secreted tyrosinases. The analyzed proteins could be divided in two groups with different lengths (350–400 and 400–600 residues), suggesting the presence of a new class of secreted enzymes lacking the C-terminal domain. Among them, a sequence from *Aspergillus oryzae* (408 aa, *AoCO4*) was selected for production and characterization. *AoCO4* was expressed in *Trichoderma reesei* under the strong *cbh1* promoter. Expression of *AoCO4* in *T. reesei* resulted in high yields of extracellular enzyme, corresponding to 1.5 g L⁻¹ production of the enzyme. *AoCO4* was purified with a two-step purification procedure, consisting of cation and anion exchange chromatography. The N-terminal analysis of the protein revealed N-terminal processing taking place in the Kex2/furin-type protease cleavage site and removing the first 51 amino acids from the putative N-terminus. *AoCO4* activity was tested on various substrates, and the highest activity was found on 4-*tert*-butylcatechol. Because no activity was detected on L-tyrosine and on L-dopa, *AoCO4* was classified as a catechol oxidase. *AoCO4* showed the highest activity within an acidic and neutral pH range, having an optimum at pH 5.6. *AoCO4* showed good pH stability within a neutral and alkaline pH range and good thermostability up to 60°C. The UV-visible and circular dichroism spectroscopic analysis suggested that the folding of the protein was correct.

Keywords Fungal · Catechol oxidase · Tyrosinase · Secreted · *Aspergillus* · *Trichoderma*

Introduction

Tyrosinases (monophenol, *o*-diphenol/oxygen oxidoreductase, EC 1.14.18.1) belong to type 3 copper proteins, which also include catechol oxidases (EC 1.10.3.1) and oxygen carrier proteins, hemocyanins (Halaouli et al. 2006). Tyrosinases are able to catalyze the *o*-hydroxylation of mono- (cresolase activity) and diphenolic compounds (catechol oxidase activity) to the corresponding *o*-quinones reducing molecular oxygen to water. Enzymes possessing mainly a diphenolase activity are classified as catechol oxidases. Primary structure or physico-chemical characteristics, other than the catalytic activity, do not allow the distinction between catechol oxidase and tyrosinase; both enzymes are also called polyphenol oxidases (Gerdemann et al. 2002; Marusek et al. 2006; Flurkey and Inlow 2008). Most of the recent research has focused on tyrosinases, while knowledge on catechol oxidases has been limited; in fact, only a few catechol oxidases from plants have been studied in detail (Eicken et al. 1998; Rompel et al. 1999). Cresolase and catechol oxidase activities are found ubiquitously in nature both in prokaryotic and eukaryotic microbes, mammals, and plants (Kwon et al. 1987; Claus and Decker 2006; Mayer 2006). Tyrosinases are mainly associated with browning and pigmentation reactions, and they play a key role in melanogenesis (Hearing and Tsukamoto 1991; Martinez and Whitaker 1995).

Fungal and plant tyrosinases consist of three regions, an N-terminal catalytic domain, a linker region, and a C-terminal domain. The catalytic domain contains six conserved histidine residues involved in the coordination of

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two copper ions. In addition, several conserved residues, mainly aromatic, have been identified in polyphenol oxidase primary structures. A tyrosine motif (Y/F-X-Y or Y-X-Y/F), located C-terminally to the copper-binding sites, is highly conserved from bacterial to human polyphenol oxidases and is supposed to mark both the end of the globular active domain and the start of the linker region (García-Borrón and Solano 2002). Previously characterized tyrosinases are produced in an inactive form retaining both domains. The activation occurs by a proteolytic attack in the linker region and a release of a C-terminal domain of 15–20 kDa. The mature protein corresponds to a typical size of 35–50 kDa (Marusek et al. 2006).

Fungal tyrosinases have potential utilization in food and non-food applications as they can catalyze the formation of covalent bonds between peptides, proteins and carbohydrates, thus showing interesting cross-linking abilities (Aberg et al. 2004; Halaouli et al. 2005; Freddi et al. 2006; Mattinen et al. 2008). Tyrosinases have proved to be applicable enzyme in structure engineering of meat-derived food products, as well in baking applications (Lantto et al. 2007; Selinheimo et al. 2007a). Tailoring polymers, e.g., grafting of silk proteins onto chitosan via tyrosinase reactions, has also been reported (Freddi et al. 2006; Anghileri et al. 2007).

The fungal tyrosinases characterized so far are typically intracellular enzymes. The first extracellular fungal tyrosinase was characterized recently from the filamentous fungus *Trichoderma reesei* (Selinheimo et al. 2006). This tyrosinase showed good cross-linking abilities of α -casein proteins as compared to tyrosinases from fungal and plant origins. Tyrosinases from *T. reesei* and *Pycnoporus sanguineus* were found to cross-link α -caseins directly, while tyrosinases from *Agaricus bisporus*, apple, and potato were capable to cross-link α -caseins only in the presence of L-dopa (Selinheimo et al. 2007b). In order to discover novel secreted tyrosinases, we performed an extensive homology search against the public fungal genome sequences. Sequence comparison of the candidate fungal tyrosinase is presented in this paper. Furthermore we report here cloning, expression, and characterization of a novel catechol oxidase from *Aspergillus oryzae*.

Materials and methods

Tyrosinase sequence analysis

In-house database (Arvas et al. 2007) was used to retrieve potential tyrosinase protein sequences. Briefly, 49 fungal genome sequences were gathered, and their putative protein families were predicted by MCL clustering (Enright et al. 2002). Predicted protein sequences of 30 representative

genomes were then analyzed with InterProScan (Quevillon et al. 2005) and TargetP (Emanuelsson et al. 2007) to detect MCL protein families with Interpro tyrosinase hits (IPR002227) and secretory pathway signal peptides. The ClustalW (Larkin et al. 2007) alignment of these proteins was visually inspected, false positives were discarded, and sequences were realigned. Parsimonious phylogenetic tree for the aligned protein sequences with jackknifing support values was predicted with TNT (Goloboff 1999) using sectorial searches and multiple replications. Trees using each of the four *Batrachochytrium dendrobatidis* proteins as a root were calculated, and the tree with the highest average of jackknifing support scores (67.5) was selected. Trees were visualized with R library ape (Robb 1984; Paradis et al. 2004).

Isolation of the *AoCO4* gene from *A. oryzae* and its expression in *T. reesei*

The putative tyrosinase *AoCO4* gene, *Q2UNF9*, was amplified from the genomic DNA of *A. oryzae* strain VTT-D-88348 by PCR with the Dynazyme EXT thermostable polymerase (Finnzymes, Helsinki, Finland). Cloning was performed with the Gateway recombination system (Invitrogen). Forward primer: 5'-GAC AAG TTT GTA CAA AAA AGC AGG CTA TCA TGG TCA CAT CTA CCC TCC TGC CA-3' and reverse primer: 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAG TGT CCC CCT CCG CCG TAC CC-3'. The PCR program comprised an initial denaturation step of 3 min at 94°C, followed by 25 cycles of 30 s at 94°C, 45 s at 52°C decreasing by 1°C per cycle, and 1.5 min at 72°C. This was followed by a final elongation step of 10 min at 72°C. The *AoCO4* gene was transferred into the *T. reesei* expression vector pMS186 to create plasmid pGF007, and the sequence of the product was verified. Vector pGF007 was transformed into a variant of the *T. reesei* VTT-D-00775 where the cellobiohydrolase I gene had been disrupted (Penttilä et al. 1987). Transformants were selected as described by Selinheimo et al. (2006). Transformants showing deeper color formation were isolated to single-spore cultures and grown in shake flasks in 50 and 300 mL volume of *Trichoderma* minimal medium supplemented with 4% lactose, 2% spent grain, 100 mM piperazine-*N,N*-bis(3-propanesulfonic acid), and 0.5 mM CuSO₄. The enzyme was also subsequently produced in a 10 L bioreactor in the same medium at pH 5.5.

Protein and enzyme activity assay

The protein concentration was determined with the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA) using BSA as standard. The activity of *A. oryzae* catechol oxidase

AoCO₄ was measured using 15 mM 4-*tert*-butylcatechol (TBC) or 15 mM catechol as substrates. Activity assays were carried out in 0.1 M sodium phosphate buffer (pH 7) at 25°C, monitoring *o*-*tert*-butylquinone formation at 400 nm ($\epsilon_{400\text{ nm}}=1,200\text{ M}^{-1}\text{cm}^{-1}$; Garcia-Molina et al. 2007). When catechol was used as a substrate, the activity was measured as arbitrary units (U mL⁻¹, where 1 U= $\Delta\text{Abs}_{400\text{ nm}}\text{min}^{-1}$), since the extinction coefficient of formed quinones was not available.

Enzyme purification

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

Determination of the isoelectric point

The isoelectric point of the enzyme from culture supernatant and purified enzyme was determined by IEF as described by Selinheimo et al. (2006). Bands containing catecholase activity were visualized by staining the gel with 15 mM TBC in 0.1 M sodium phosphate buffer (pH 7.0), and proteins were visualized by Coomassie Blue staining.

Tryptic digestion and MS analysis

MALDI-TOF MS was performed using an Ultraflex TOF/TOF instrument (Bruker Daltonik, Bremen, Germany). Proteins were analyzed in the linear positive mode using sinapic acid (Fluka Chemie AG, Buchs, Switzerland) as a

matrix. Peptides were analyzed in the reflector positive mode using α -cyano-4-hydroxycinnamic acid (Aldrich, Steinheim, Germany). SDS-PAGE-separated proteins were stained with Coomassie Brilliant Blue, and protein bands of interest were cut off from the gel. The proteins were “in-gel” digested essentially as described by Rosenfeld et al. (1992). Proteins were reduced with dithiothreitol and alkylated with iodoacetamide before digestion with trypsin (Sequencing Grade Modified Trypsin, V5111; Promega). Peptides generated by enzymatic cleavage were analyzed by MALDI-TOF MS for mass fingerprinting.

Protein sequencing

The N-terminus of the protein was sequenced according to Edman degradation chemistry using a PE Biosystems Procise Sequencer (PE Biosystems, Foster City, CA, USA) as described by Kiiskinen et al. (2002).

Fluorescence, circular dichroism and UV-visible spectroscopy

Intrinsic fluorescence intensity measurements were carried out using Varian Cary Eclipse Fluorescence Spectrophotometer. The intrinsic emission of protein (20 μM) was seen at the excitation wavelength of 280 nm (Gheibi et al. 2005). Far-UV (250–190 nm) circular dichroism (CD) spectra were recorded on a JASCO model J-720 CD spectrometer according to Boer and Koivula (2003). Measurements were performed with 2 μM of purified enzyme in 0.1 M sodium phosphate buffer pH 7 at 25°C, spectra were accumulated four times, and the values were corrected for buffer contribution. CD spectrum was reported in ellipticity as millidegrees (mdeg). Thermal induced unfolding was monitored by following the change in signal at 222 nm with the CD spectropolarimeter over a temperature range of 30–85°C with a heating rate of 2°C min⁻¹. UV-visible absorption spectra of 0.1 mM purified catechol oxidase were monitored in 0.1 M sodium phosphate buffer pH 7 at 25°C using a Varian Cary 100 Bio spectrophotometer using a quartz cuvette of 1 cm path length.

pH and temperature behavior

Determination of pH optimum for AoCO₄ was carried out on 15 mM TBC, dissolved in 50 mM sodium acetate buffer at a pH range 4.0–5.6, in 50 mM sodium phosphate buffer at a pH range 5.8–8.0, and in 50 mM TRIS/HCl buffer at a pH range 7.5–8.6; the catechol oxidase activity was determined on TBC. The stability of the enzyme at different pHs was determined by incubating the enzyme at room temperature for 1, 2, and 3 days in 50 mM McIlvaine universal buffer (50 mM NaHPO₄ containing 25 mM citric

acid) at a pH range 4.0–7.6 and 50 mM TRIS/HCl buffer at a pH range 7.5–8.6. The residual catechol oxidase activity was determined with TBC as a substrate. Temperature stability was determined at 30°C, 40°C, 50°C, and 60°C. The enzyme was placed in 20 mM TRIS/HCl buffer pH 7.2 in 1 mg mL⁻¹ concentration, and it was incubated at different temperatures. The residual activity was determined after certain time intervals with TBC as a substrate.

Substrate specificity

The substrate specificity of AoCO4 was evaluated based on visual observations after 30 min of incubation at 25°C with different potential substrates: L-tyrosine, *p*-coumaric acid, tyramine, phloretic acid, phenol, aminophenol, tyrosol, 4-mercaptoethanol, guaiacol, creasol, aniline, caffeic acid, hydrocaffeic acid, dopamine, L-dopa, D-dopa, catechol, TBC, ABTS, (–)-epicatechin, and (+)-catechin. All the substrates tested were dissolved in 0.1 M sodium phosphate buffer pH 7.0 to a final concentration of 15 mM, but L-tyrosine final concentration was 2 mM.

Results

Sequence analysis of tyrosinase genes in fungal genomes

From the alignment of the putative secreted tyrosinase sequences, two groups were identified, differing mainly in length and thus named “long” and “short” tyrosinases. The sequences considered, identified by their accession number, are listed in Table 1. The uncharacterized proteins ascribed to the long tyrosinases group are 500 to 767 residues long, similar to the unprocessed gene product of the secreted tyrosinase from *T. reesei* (561 amino acids) and the characterized intracellular enzymes from *A. bisporus* (CAA59432, 556 aa and CAA11562, 568 aa) and *Neurospora crassa* (EAA35696, 542 aa). A multiple sequence alignment of the long tyrosinase sequences allowed the identification of the histidine residues, the C-terminal tyrosine motif, and characteristic conserved residues. A high level of conservation of the histidine residues was found in the first copper-binding region CuA with the H-X(20-23)-H-X(8)-H motif, while in the second, CuB, the motif H-X(3)-H-X(20-33)-H showed more variability (Table 1). All the candidate proteins belonging to the first group possess only one conserved cysteine residue located two residues before the second histidine of the CuA site (HA2-2) that could be involved in a thioester bond with the following histidine residue, as seen in tyrosinase from *N. crassa*, in catechol oxidase from *Ipomoea batatas*, and in hemocyanin from *Octopus dofleini* (Lerch 1982; Klabunde et al. 1998; Cuff 1998).

The C-terminal domains of the long tyrosinases showed no relevant level of homology; however, the Y-G motif noticed by Flurkey and Inlow (2008) is conserved.

As a novel finding, we discovered a second group of uncharacterized secreted tyrosinases, comprising clearly shorter proteins, 300–500 amino acids in length. These so-called short tyrosinases have the conserved histidine residues of tyrosinases and the same histidine pattern of the CuB-binding site. Interestingly, three main differences are evident between long and short tyrosinase sequences. Firstly, a novel histidine pattern H-X(7)-H-X(8)-H at the CuA-binding site is found, where only seven residues separate the first two histidines (HA1 and HA2; Table 1). Secondly, most of the short tyrosinase sequences have a stop codon just one to two residues after the tyrosine motif; thus, the lack of the C-terminal domain accounts for the difference in length. Thirdly, short tyrosinases do not present a cysteine residue in close proximity of HA2 (Table 1); therefore, a thioester bond between these two residues is not possible. However, six cysteine residues are clearly conserved in all short tyrosinases: two of them located N-terminally (C72 and C100 in AoCO4), three between CuA and CuB (C159, C223, and C261 in AoCO4), and one right before the tyrosine motif (C404 in AoCO4). Short and long tyrosinase sequences analyzed have the tyrosine motif and an arginine residue aligning to R40 of *T. reesei* tyrosinase, supposed to delimit the enzyme structural core (Flurkey and Inlow 2008). Short tyrosinases present the conserved N-terminal arginine residue located between the first two conserved cysteine residues, being R80 in AoCO4.

In order to study the evolution of the secreted tyrosinases, we performed a phylogenetic analysis on their sequences. As a result, the short and long tyrosinase sequences cluster in two clearly separate branches of the phylogenetic tree (Online Resource 1). The phylogenetic tree suggests that the common ancestor of all fungi except Chytridiomycota had both the long and the short form. Chytridiomycota are an early-diverging clade within the kingdom Fungi and represented in the data set by *B. dendrobatidis* (James et al. 2006). Analysis of *B. dendrobatidis* sequences retrieved features characteristic of short tyrosinases, like the six conserved cysteine residues and the second histidine pattern, though a shorter distance between the two copper-binding sites was found, e.g., 46 amino acids shorter in BDEG06104 than in AoCO4. However, sequences from *B. dendrobatidis* have a C-terminal region following the tyrosinase domain, whose length falls between the long and the short form (Online Resource 1). Analyzed sequences belong mainly to Pezizomycotina (Ascomycetes) subphyla, but examples of short tyrosinase sequences are found also in the Basidiomycetes, like the Agaricomycotina subphyla, e.g., in *Coprinopsis cinerea*.

Table 1 Sequence analysis of the candidate long and short tyrosinase-like proteins

Group	Phylum	Family	Species	Sequence ^a	Length ^b	TP length ^b	Identity to TrTyr ^{2c}	HA1-HA2 ^d	HA2-HA3 ^d	HB1-HB2 ^d	HB2-HB3 ^d	XHA2-2 ^e
Long	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2HEC0	644	22	23.7	23	8	3	35	C
Long	Ascomycota	Hypocreaceae	<i>Trichoderma reesei</i>	tre45445	561	18	100.0	23	8	3	24	C
Long	Ascomycota	Hypocreaceae	<i>Trichoderma reesei</i>	tre50793	490	20	22.9	23	8	3	23	C
Long	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4RKK91	640	25	26.3	23	8	3	23	C
Long	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.09522	575	31	45.0	23	8	3	24	C
Long	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.05628	556	18	23.8	23	8	3	23	C
Long	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.06218	572	21	45.0	23	8	3	24	C
Long	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.09259	578	15	23.6	23	8	3	23	C
Long	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.04071	572	21	45.9	23	8	3	24	C
Long	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.06859	578	15	24.1	23	8	3	23	C
Long	Ascomycota	Nectriaceae	<i>Nectria haematococca</i>	NECHA.0066755	566	22	47.1	23	8	3	24	C
Long	Ascomycota	Nectriaceae	<i>Nectria haematococca</i>	NECHA.0047204	600	19	24.5	23	8	3	23	C
Long	Ascomycota	Onygenaceae	<i>Coccidioides immitis</i>	Q1DQ30	658	24	22.7	20	8	3	25	C
Long	Ascomycota	Onygenaceae	<i>Histoplasma capsulatum</i>	HCAG.04262.1	636	21	27.2	20	8	3	25	C
Long	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UNZ8	691	23	22.9	23	8	3	24	C
Long	Ascomycota	Sclerotiniaceae	<i>Botrytis cinerea</i>	BCIG.00048	595	24	30.3	23	8	3	24	C
Long	Ascomycota	Sclerotiniaceae	<i>Botrytis cinerea</i>	BCIG.14990	670	26	28.6	23	8	3	23	C
Long	Ascomycota	Sclerotiniaceae	<i>Botrytis cinerea</i>	BCIG.04092	532	21	26.9	22	8	3	27	C
Long	Ascomycota	Sclerotiniaceae	<i>Sclerotinia sclerotiorum</i>	SSIG.04725.1	593	24	30.1	23	8	3	24	C
Long	Ascomycota	Sclerotiniaceae	<i>Sclerotinia sclerotiorum</i>	SSIG.13364.1	636	26	28.2	23	8	3	23	C
Long	Ascomycota	Sclerotiniaceae	<i>Sclerotinia sclerotiorum</i>	SSIG.01576.1	602	21	27.6	22	8	3	27	C
Long	Ascomycota	Sordariaceae	<i>Neurospora crassa</i>	Q7S218	767	22	26.4	23	8	3	25	C
Long	Ascomycota	Sordariaceae	<i>Neurospora crassa</i>	Q7SFK3	692	25	24.0	23	8	3	35	C
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2H5C8	363	23	16.0	7	8	3	23	T
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2GQ28	376	18	15.1	7	8	3	22	F
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2H818	314	15	15.0	7	8	3	22	S
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2GZJ4	386	19	14.7	7	8	3	22	Y
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2HF52	358	22	14.5	7	8	3	25	D
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2H5H1	404	23	14.3	7	8	3	22	F
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2GQI5	396	18	14.0	7	8	3	22	N
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2GNG2	353	19	13.8	7	8	3	22	A
Short	Ascomycota	Chaetomiaceae	<i>Chaetomium globosum</i>	Q2H717	420	18	12.3	7	8	3	23	M
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4RNE4	335	18	16.2	7	8	3	22	D
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4RLH2	321	22	14.6	7	8	3	25	A
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4RAA0	415	19	14.5	7	8	3	23	P
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4QVW4	404	18	14.4	7	8	3	23	T
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4RNQ7	387	18	13.9	7	8	3	22	N

Table 1 (continued)

Group	Phylum	Family	Species	Sequence ^a	Length ^b	TP length ^b	Identity to TrTyr2 ^c	HA1-HA2 ^d	HA2-HA3 ^d	HB1-HB2 ^d	HB2-HB3 ^d	XHA2-2 ^e
Short	Ascomycota	Magnaporthaceae	<i>Magnaporthe grisea</i>	A4QUP4	429	18	12.4	7	8	3	23	S
Short	Ascomycota	Mycosphaerellaceae	<i>Mycosphaerella graminicola</i>	MYCGR0040497	353	19	14.9	7	8	3	22	D
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.11106	316	15	15.4	7	8	3	22	Q
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.03531	337	23	15.2	7	8	3	22	E
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.03801	350	21	15.1	7	8	3	25	D
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.08007	316	16	14.7	7	8	3	25	D
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.03348	390	20	14.3	7	8	3	22	S
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.04510	330	18	14.1	7	8	3	22	W
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.01988	398	18	14.0	7	8	3	23	T
Short	Ascomycota	Nectriaceae	<i>Fusarium graminearum</i>	FGSG.11528	390	21	13.3	7	8	3	22	S
Short	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.16980	338	24	16.1	7	8	3	22	D
Short	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.16885	346	21	14.8	7	8	3	25	D
Short	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.04315	403	18	14.5	7	8	3	23	M
Short	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.07401	412	20	13.7	7	8	3	23	T
Short	Ascomycota	Nectriaceae	<i>Fusarium oxysporum</i>	FOXG.13704	324	18	13.7	7	8	3	22	W
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.13776	338	24	16.6	7	8	3	22	D
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.11137	324	18	15.3	7	8	3	22	W
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.12936	346	21	15.0	7	8	3	25	D
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.11735	315	20	14.6	7	8	3	22	Q
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.01614	382	20	14.5	7	8	3	22	S
Short	Ascomycota	Nectriaceae	<i>Fusarium verticillioides</i>	FVEG.07441	403	18	14.4	7	8	3	23	T
Short	Ascomycota	Nectriaceae	<i>Nectria haematococca</i>	NECHA0085924	403	18	13.6	7	8	3	23	I
Short	Ascomycota	Omygenaceae	<i>Coccidioides immitis</i>	Q1DQU9	444	18	15.9	7	8	3	23	T
Short	Ascomycota	Omygenaceae	<i>Histoplasma capsulatum</i>	HCAG.00691.1	327	20	13.3	7	8	3	22	M
Short	Ascomycota	Omygenaceae	<i>Histoplasma capsulatum</i>	HCAG.04336.1	379	15	13.3	7	8	3	22	E
Short	Ascomycota	Omygenaceae	<i>Histoplasma capsulatum</i>	HCAG.06499.1	438	22	11.6	7	8	3	24	F
Short	Ascomycota	Omygenaceae	<i>Uncinocarpus reesi</i>	UREG.07862.1	411	17	15.2	7	8	3	23	G
Short	Ascomycota	Omygenaceae	<i>Uncinocarpus reesi</i>	UREG.01037.1	383	19	14.9	7	8	3	22	Y
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UXF5	388	17	15.8	7	8	3	23	S
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UUJ6	408	19	15.3	7	8	3	23	T
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UVC7	399	20	14.9	7	8	3	22	Y
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UEG5	388	15	14.4	7	8	3	23	F
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0U440	384	19	14.2	7	8	3	22	T
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0UID1	417	15	13.7	7	8	3	23	F
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0U9F8	402	21	13.4	7	8	3	23	M
Short	Ascomycota	Phaeosphaeriaceae	<i>Phaeosphaeria nodorum</i>	Q0U1I9	412	18	11.8	7	8	3	22	N
Short	Ascomycota	Trichocomaceae	<i>Aspergillus clavatus</i>	A1CCY9	383	20	15.2	7	8	3	23	V

Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus clavatus</i>	A1CDX9	327	15	13.8	7	8	3	22	Q
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus clavatus</i>	A1CIU9	378	16	13.7	7	8	3	22	N
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus clavatus</i>	A1C4G0	351	18	15.9	7	8	3	22	N
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus fumigatus</i>	Q4WFT7	383	20	14.6	7	8	3	23	V
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus nidulans</i>	AN5311	383	20	14.5	7	8	3	23	F
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus nidulans</i>	AN9388	340	18	14.5	7	8	3	25	S
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus nidulans</i>	ANI0821	369	20	14.2	7	8	3	22	N
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus nidulans</i>	AN0231	366	19	14.8	7	8	3	22	V
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus niger</i>	A2QFP6	383	20	14.2	7	8	3	23	Y
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus niger</i>	A2QTR2	365	20	14.1	7	8	3	22	N
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus niger</i>	A2QUC2	394	19	13.1	7	8	3	22	H
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus niger</i>	A2Q9V3	406	18	15.2	7	8	3	23	E
Short	<i>Ascomycota</i>	<i>Trichocomaceae</i>	<i>Aspergillus oryzae</i>	Q2UNF9	408	18	14.7	7	8	3	23	I
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Aspergillus terreus</i>	Q0CYP3	379	19	15.8	7	8	3	23	F
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Neosartorya fischeri</i>	A1DJF5	383	20	14.6	7	8	3	23	F
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Neosartorya fischeri</i>	A1CVG0	348	21	14.4	7	8	3	22	Y
Short	Ascomycota	<i>Trichocomaceae</i>	<i>Neosartorya fischeri</i>	A1CVX2	348	13	14.1	7	8	3	22	H
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.08048.1	371	22	16.0	7	8	3	22	R
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.02471.1	380	22	15.0	7	8	3	22	H
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.06016.1	358	23	15.0	7	8	3	22	W
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.10470.1	378	20	14.6	7	9	3	22	H
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.00628.1	379	20	14.4	7	8	3	22	Q
Short	Basidiomycota	<i>Agaricaceae</i>	<i>Coprinopsis cinerea</i>	CC1G.00642.1	377	30	13.0	7	8	3	22	Y
Short	Basidiomycota	<i>Batrachosporium</i>	<i>Batrachosporium dendrobatidis</i>	BDEG.01791	448	23	19.7	7	8	3	23	I
Short	Basidiomycota	<i>Batrachosporium</i>	<i>Batrachosporium dendrobatidis</i>	BDEG.02913	464	18	18.7	7	8	3	23	T
Short	Basidiomycota	<i>Batrachosporium</i>	<i>Batrachosporium dendrobatidis</i>	BDEG.06870	457	17	16.8	7	8	3	23	T
Short	Basidiomycota	<i>Hydnangiaceae</i>	<i>Laccaria bicolor</i>	LACB10252993	343	24	13.7	7	8	3	22	E

^a Only retrieved sequences possessing the six-histidine pattern are listed. Protein Q2UNF9, selected for production, is in italics

^b The number of residues of the protein and of the target peptide (TP Length) are reported

^c The level of identity to the tyrosinase from *T. reesei* (TrTyr2, tre45445) is reported in percentage

^d The amino acid located two residues before HA2 (XHA2-2) is in the last column

^e Copper-binding domains are described by the distance in amino acids between the conserved histidine residues (in order HA1, HA2, and HA3 in the CuA site and HB1, HB2, and HB3 in the CuB site). Residue H127 of AoCO4 was considered as HA1

No examples of potential secreted tyrosinases were found in the Saccharomycetes (yeasts) genomes analyzed. Concerning Pezizomycotina in genus *Aspergillus*, only short tyrosinases were found; on the contrary, *T. reesei* has only long forms, while *Fusarium* spp. have typically both forms (Online Resource 1). The phylogenetic tree reveals the presence of two different conserved long forms, which were already present in the common ancestor of Ascomycota. Species possessing the long tyrosinase proteins seems to present a member of each of these two groups, suggesting that they might have somehow different conserved roles. For instance, *Trichoderma* has two tyrosinase genes (number 1, tre50793, and number 2, tre45445, in Online Resource 1), and they ascribe each one to a different group. Ten long sequences belonging to the upper branch of tree in Online Resource 1, e.g., *Fusarium graminearum* FGSG.05628, *T. reesei* tre50793, and *N. crassa* Q7SFK3 have an additional N-terminal tyrosine motif, located few residues after the predicted target peptide cleavage site and before a conserved putative Kex2-type cleavage site (K-R or R-R), both preceding the conserved N-terminal arginine residue.

Expression of the gene *AoCO4* in the filamentous fungus *T. reesei*

The *A. oryzae* gene *AoCO4* was chosen as a representative of short putative tyrosinase proteins, and it was subjected to heterologous production and biochemical characterization. Two sequences from *A. oryzae* without a proper histidine pattern were found (number 3, Q2UCH2, and 4, Q2UFM6, in Online Resource 1). The protein AoCO4 contained the common central domain of tyrosinases (pfam00264; Marchler-Bauer et al. 2007) and had 14.7% of identity to the full-length *T. reesei* tyrosinase, 13.3% to tyrosinase from *N. crassa*, and 20.4% to *Streptomyces castaneoglobisporus* tyrosinase. AoCO4 was aligned to the well-characterized fungal tyrosinases from *T. reesei* and *A. bisporus* and the bacterial tyrosinase from *S. castaneoglobisporus*. The CuA histidine pattern had two possibilities for the first histidine residue (HA1): H111 or H127 matching the long and short tyrosinase histidine pattern (Fig. 1).

The *A. oryzae* genomic sequence coding for AoCO4 was cloned and eventually transformed into *T. reesei*. Transformants were first selected on plates containing L-tyrosine and secondly in liquid culture according to the activity detected on catechol in the culture medium, as no significant activity was detected with L-tyrosine, and a background activity was found with L-dopa in the parental strain.

AoCO4 was produced in a 10 L bioreactor in inducing medium at a pH of 5.5 in order to prevent inactivation of

the enzyme. The enzyme was harvested after 5 days cultivation, resulting in a 7-fold higher activity yield as compared to the shake flask cultivations (data not shown). Expression of AoCO4 in *T. reesei* resulted in good yields, corresponding to approximately 1.5 g L⁻¹ enzyme production.

Purification and biochemical characterization of AoCO4

The buffer-changed culture filtrate was applied to cation exchange chromatography in 20 mM sodium acetate buffer, pH 4.8. Catechol oxidase eluted in two fractions, the major activity in a NaCl concentration of 90 mM and also some activity of 60 mM. Both fractions were further purified in ResourceQ column. The overall recovery of the activity in the two-step purification procedure was 3.9% and 0.7% for pools 1 and 2, respectively (Table 2). As analyzed by SDS-PAGE, both pools appeared as a single band with an apparent molecular weight of 53 kDa. However, the MALDI-TOF MS analysis of purified pools resulted in a MW of 39,348.8 Da for AoCO4 pool 1 and a mixture of MW 39,348.8 and 40,482.64 for pool 2 AoCO4. The N-terminal sequence of the purified AoCO4 pool 1, as determined by Edman degradation, was GGCTLQNLRV, indicating that AoCO4 pool 1 was N-terminally processed after R69, and the first 51 residues after the putative 18 residues-signal sequence were thus cut off. We also performed N-terminal sequencing of the pool 2. The result indicated that this pool contained equimolar amount of a protein cleaved after Q25 and after R69. The specific activity of the pool 2, containing the two differently processed proteins, was slightly lower than the specific activity of the AoCO4 processed after R69, thus suggesting that N-terminal processing in these different positions does not have substantial effect on the enzyme activity. The sequence analysis of the *A. oryzae* catechol oxidase by ExPaSy tools predicted a signal peptide cleavage site between residues A18-F19 and five potential propeptide cleavage sites for Kex2/furin-type proteases. The cleavage takes place after a dibasic recognition site (K-R or R-R). Since the AoCO4 pool 1 was the predominant form of the produced catechol oxidase, we continued the characterization with that pool 1.

Isoelectric focusing of AoCO4 and subsequent staining with TBC showed a single band in the gel corresponding to pI5.2. The enzyme activity of the purified AoCO4 was relatively low on all tested mono- and diphenolic substrates. After 30 min of incubation at 25°C, AoCO4 showed activity on the diphenolic compounds catechol, 4-*tert*-butylcatechol, caffeic acid, and hydrocaffeic acid and on the monophenolic compounds aminophenol and guaiacol and also on the catechins, among all tested substrates. Furthermore, after 24 h of incubation, AoCO4 showed

Fig. 1 Alignment of *A. oryzae* (AoCo4) amino acid sequence with the tyrosinases of *T. reesei* (TrTyr2, tre45445), *A. bisporus* (AbTyr, CAA11562), *N. crassa* (NcTyr, EAA35696), *S. castaneoglobisporus* (ScTyr, AAP33665), and with the plant catechol oxidase of *I. batatas* (IbCO, CAC83609). Identical amino acids are indicated by asterisks and similar amino acids by dots. The conserved N-terminal arginine residue is in a box; the signal sequence and the C-terminal domains, when determined, are in italics. The copper-binding regions are in bold. Cysteine residues conserved in short tyrosinases are shaded, and the C-terminal tyrosine motifs are underlined. Phenylalanine 261 of IbCO is in a black background. The experimentally determined N-terminal amino acid of AoCo4 is circled, and the peptides realigned by mass finger printing are double-underlined

AbTyr	MSLIATVGTGGVKNRLNIVDFVKNE-	26
NcTyr	MSTDIKFAITGAVPLRRELRLDQNNYP	27
TrTyr2	MLLSASLSALALATVSLAQTTHIPVTGVFVSPGAAVPLRQINIDLAKSG-	50
ScTyr	MVTVEKNQATLTADEK	15
AoCo4	MVALQALSGLLLASQALAFPAASQQAATATLPTTASSSTAVASSQLDQLANFAYNVTTDS	60
IbCO	APIQAEIISKCVVPPADLPPGAVVNDCCPPVASNIVDYKIPVVVITMKVPLFAAHTMKDKDAI	60
AbTyr	KFFTLVYRSLLELLQAKE-----QHDISSFFQLAGIHGLPFFTEWAK	66
NcTyr	EQFNLYLLGLRDFQGLD-----EAKLDSYYQVAGIHGMFFKFWAG	67
TrTyr2	PQWDLYVQAMYNMSKMD-----SHDPYSFFQIAGIHGAPYIEYNK	90
ScTyr	RRFVAIVLELK-----RSGRYDEFVRTHNEFIMSDDT	47
AoCo4	VAGGESKRGGTLQNLRVTRDWRAFSKTKKDYINSVLQKLPSRTPPAHLAPGARTRY	120
IbCO	AKFAKAVELMKALPADD-----PRNFYQQALVHCAYCNGGYD	97
AbTyr	ERPSMNLRYKAG-----YCTHGQVLFPTWHRTYLSVLEQILQGAIEVAKKFTSN--	115
NcTyr	VPDSTHWSQPGSSGFGG-----YCTHSSILFITWHRPYLALYEQALYASVQAVAKQFVEVGG	124
TrTyr2	AGAKSGDGLG-----YCPHGEDLFLSWHRRPYVLLFEQALVSVAKGIANSYPPS--	139
ScTyr	SGERTG-----HRSPSFLPWHRFLLDQEQALQSDVSSVT--	82
AoCo4	DDFVAHINQTIHYTGTFLAWHRYFYEFEQALRDRSITGDYYPWNWG	171
IbCO	QVNFPPQE-----IQVHNSWLFPPFHRWLYFYERILGKLGIDPS--	137
AbTyr	-QTDWVQAQDLRQPYWDWG---FELMPPDEVIKNEEVNTNYDGKKISVKNPILRYHF	170
NcTyr	LRKRYVAAAKDFRAPPYFDWASQPPKGTALFPESLSRTIQVVDVVGKTKSINNPLHRTFT	184
TrTyr2	VRAKYQAAAAARAPYWDWAADSSVPAVTVPQTLKIN---VPSGSSSTKTVDYTNPLKTYFT	197
ScTyr	-----LPYWDS-----ADRTVRASLWAPDFLGGTRSDG-----	113
AoCo4	ADADNMEKSQVFDGSETSMGNG--EYIPNQGDILKLLGNYPALDLPSPGSGGGVTSFGF	229
IbCO	-----FGLPFWND-----NPGGMVLPDFLNDSTSSLYDSNRN-----	170
AbTyr	HPIDPSFKPYG-DFATWRTVRNPDNRNREDIPG-LIKKMRLEEGQIREKTYNMLKFNDA	228
NcTyr	HFVNPSPGDFSAAWSRYPSTVRYPNRLT GASRDRIAPILANELASLRNNVSLLLLSYKD	244
TrTyr2	PRMSLT- GSYG-EFTGGNDHTVRCAASKQSYPAIANSNLAAR--PYKSWIYDVLNHSQN	253
ScTyr	RVMDFGFAAFTGNWPINVRVDSRTYLRRSLGGSVAELPTRAEVESVLAISAYDLPPYNSA	173
AoCo4	KDYKLNLPAAALSPGGNMATAANPLTYNPRMKRSLITTELLORYNTFPKIVELILSDSD	289
IbCO	---QSHLPVVVLDLNGADTDVTDQRITDNLALMYKQMTNAGTAELELGLKAYRAGDA	227
AbTyr	WERFSNHGISDDQHAN---SLESVHDDIHVMVGYGKIEGHMDHFFFAFDPIFWLHHTNV	285
NcTyr	FDAFSYNRWDPNNTNPGDFGSLDVDHNEIHDTGG---NGHMSLSLEVSADFPLFWLHHVNV	301
TrTyr2	FADFASTSGPG-----INVEQIHNAIHWDCAGC---SQFLAPDYSGDFLFFMHHAQV	303
ScTyr	SEGFRNHLEGWRG-----VNLHNRVHVWVG---QMATGVSNDPFWFLHHAYV	219
AoCo4	IWDFQMTMQGVPGSGS-----IGVHGGHYSMGDD---PGRDVYVSPGDATFWLHHGMI	340
IbCO	PSPGAGSIETSP-----HIPIHRWVGDPRNTN--NEDMGNYSAGRDIAYFYCHHSNV	277
AbTyr	DRLLSLWKAINDV-VWTSGRNRDGTMGIAAPNAQINSETLEPFYQ-SGDKVWTSASLAD	343
NcTyr	DRLWSIWQDLNPNF-FMTPRPAPYSTFVAQEGESQSKSTPLEPFWDKSAANFWTSEQVKD	360
TrTyr2	DRMFAWEAIMPSSPLFTASYKQSRFNKSGSSTITPDSPLQPFYQ-ANGKFHTSNTVKS	362
ScTyr	DKLWAEWQRHHPDSAYVPTG-----GTPDVVDLNETMKPWNT-----VRPADLLDH	265
AoCo4	DRVWIIWQLDLRKRQNAISGTGTFMNPASPNTTLDTVIDLGYANGGPIAMRDLMSTT	399
IbCO	DRMWTIWQQLAGKP-----RKRDYTDSDLNATLFLFYDENGQAVKVRIGDSDL	325
AbTyr	TARLGSYPDFDKLVGGTKELIRDADLIDERYGSKPSSGARNTAFDILLADFKGITKEH	403
NcTyr	SITFGYAYPETQKWKYSVKEYQAAIRKSVTALYGSNVFANFVENVADRTPALKKQATG	420
TrTyr2	IQGMYSYQGIIEYWK-SQAQIKSSVTIINQLYGFN-SGKRNAPRDLSDIIVTVENL	420
ScTyr	TAYYTFDA-----	273
AoCo4	AGPFFYVYL-----	408
IbCO	LPWLDSKPVPTKKGKYASKAPFVASVPVTLDKVQVQVVARPKKRSRAEKEAEESI	397
AbTyr	KEDLKMWDWIHVAFKFKELKESFLFYFASDGGDQDQENCFVGSINAFRGTAPETCAN	463
NcTyr	EESKSTVAAAAHAVELSGAKKVAEKVHNVFQHAEEKAQK-----PVVPKDKTAEESTA	475
TrTyr2	IKTR--YFAKISVNVTEVTVRPAEINVYVGGQKAGSLIVMK-----LPAEGTVNGGFTI	472
IbCO	LLLEGVEVEIDRYAKFDVYVYLNDSDD-----PSGGDKKAEYAG	434
AbTyr	CQDNENLIQEGFIHLNHYLARDLESFEPQDVHFKLKEKGLSYKLYSRGDK--PLTSLSVK	521
NcTyr	AESLKYLPADG-----KYTDWIVNVRAQKHGLG-----QSFRVI	509
TrTyr2	DNPMQSILHGG-----LRNAVQAFTEIEVEILSKDQQAIPLETVPVSLIDLEVAVNT	525
IbCO	SSAHLPHKHKG-----MKKIRTLSLGLNEPLEDLGAEDDTIIIVALA	477
AbTyr	IEGRPLHPPGEHRPKYDHTQARVVFDDVAVHVIN- 556	
NcTyr	VFLGEFNPDPETWDEFNCVGRYCGRRAPPEQA- 542	
TrTyr2	LPSALDLPKYGQSRSHRAKAAQRGHRFAVPHIPL- 561	
IbCO	PKVGGGVSVSDNIKVVYGS- 496	

activity also on phenol, tyrosol, *p*-creosol, and aniline. No activity was detected on L-tyrosine and L-dopa in the tested conditions. The highest activity among the tested substrates was observed on catechol and TBC. TBC was used as substrate in further characterization of the enzyme. *A. oryzae* catechol oxidase was shown to be almost fully active within

a pH range of 5.0–7.0, with an optimum at pH 5.6 (Fig. 2a). Regarding the pH stability, AoCO4 showed good stability at alkaline and slightly acidic pH. Considerable activity losses started to take place below pH 5.0 (Fig. 2b). The enzyme was produced at pH 5.5, which can be considered as a safe pH for enzyme production. Temperature stability of *A.*

Table 2 Purification of the *A. oryzae* catechol oxidase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg ⁻¹)	Activity yield (%)	Purification factor
0 Culture filtrate	96	600	0.16	100	1
1 Cation exchange pool 1	14	27	0.50	10	3
Cation exchange pool 2	4.2	29	0.15	3	1
2 Anion exchange pool 1	5.4	4.8	1.10	3.9	9.4
Anion exchange pool 2	0.9	1.2	0.80	0.7	6.3

oryzae catechol oxidase was analyzed by incubating the enzyme in different temperatures at pH 7.0 and analyzing the residual activity. The enzyme showed half-lives of 20 and 2 h at 50°C and 60°C, respectively.

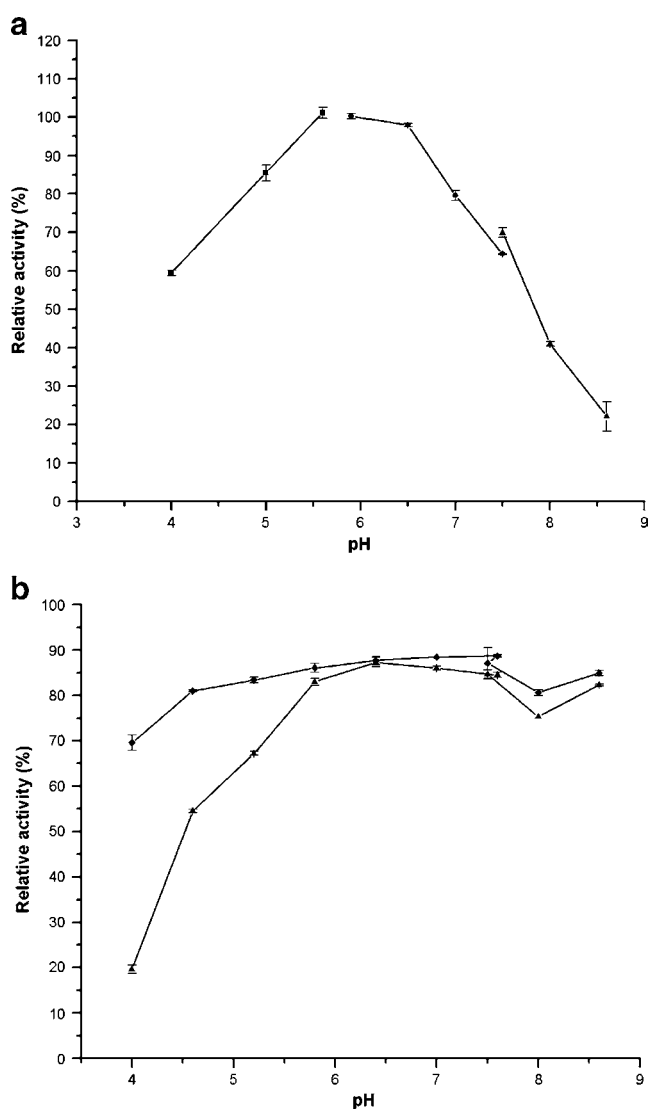


Fig. 2 **a** pH optimum of the purified *A. oryzae* catechol oxidase. The activity relative to the one measured on 0.1 M sodium phosphate pH 7 is reported for sodium acetate (squares), sodium phosphate (diamonds), and TRIS/HCl (triangles) buffers. **b** pH stability of the purified *A. oryzae* catechol oxidase. The relative activities were measured on TBC 15 mM after 1 (diamonds) and 48 (triangles) hours of incubation

The UV–visible absorption spectrum of purified AoCO4 showed an absorption maximum around 330 nm and verified the presence of the type 3 copper center (Fig. 3a). CD and fluorescence spectra of the purified *A. oryzae* catechol oxidase were recorded to obtain information on the secondary structure of the enzyme. The CD spectrum showed two minimum peaks at 200 and 220 nm (Fig. 4a),

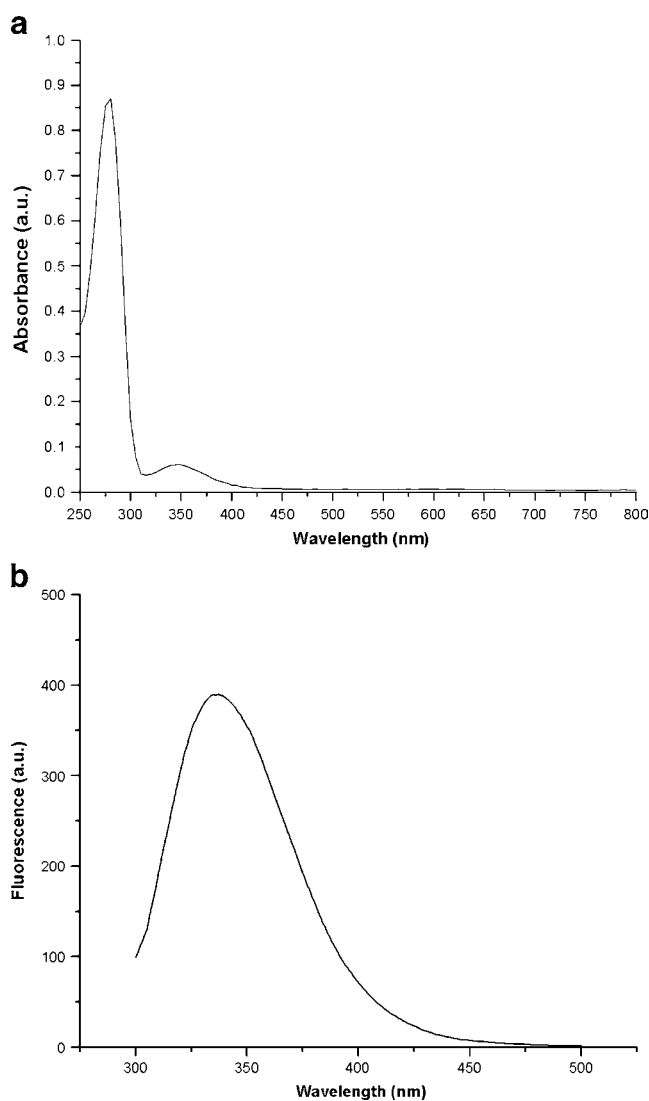


Fig. 3 UV–visible absorption spectrum (**a**) and fluorescence spectrum (**b**) of the purified *A. oryzae* catechol oxidase. The absorbance and fluorescence intensities are reported in arbitrary units (a.u.)

typical for α -helical structures. The sigmoidal unfolding curve for AoCO4 is indicative for a two-state denaturation model, with a clear folded–unfolded transition at 70°C (Fig. 4b), thus suggesting that AoCO4 has good thermostability properties as also confirmed by temperature stability data. By lowering the temperature to 40°C, it was not possible to recover the secondary structure of the enzyme after its thermal denaturation at 90°C. Upon excitation at 280 nm, the purified catechol oxidase exhibits fluorescence with maximum intensity near 330 nm (Fig. 3b).

Discussion

Tyrosinases characterized so far are typically intracellular enzymes. An apoenzyme contains approximately 600

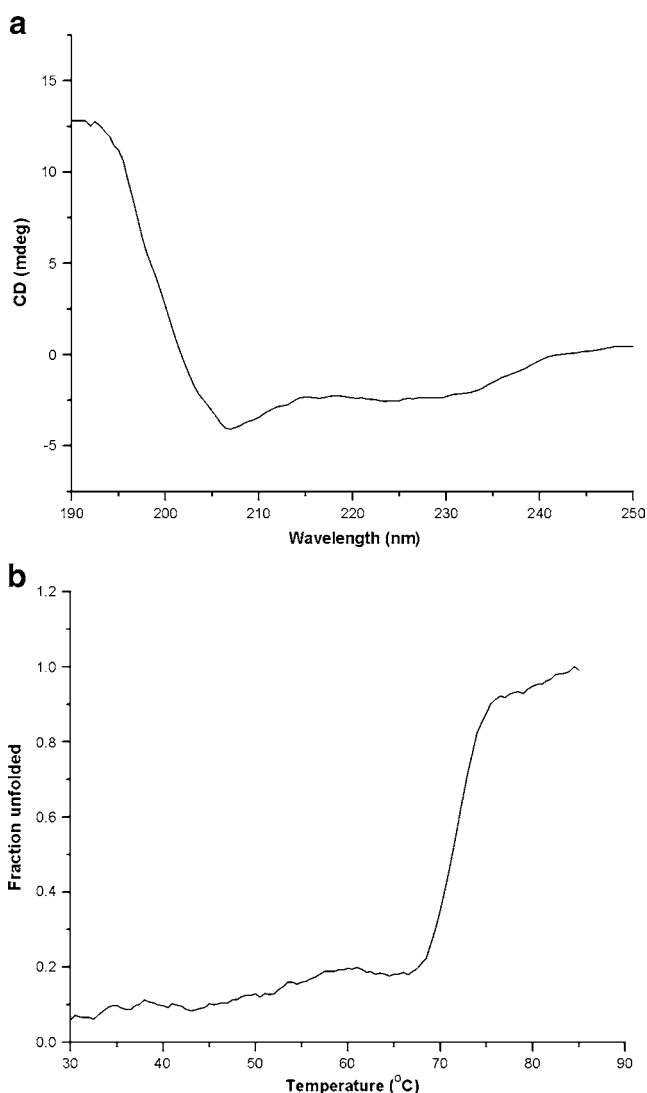


Fig. 4 **a** CD spectrum of the purified *A. oryzae* catechol oxidase in 0.1 M sodium phosphate buffer pH=7.0. **b** Thermal denaturation of the purified *A. oryzae* catechol oxidase by circular dichroism

amino acids residues and two domains: a catalytic domain and a C-terminal domain connected to the globular core by a linker region. Production of the enzyme in an active form requires the cleavage of the C-terminal domain or a harsh chemical treatment with, for example with SDS, to modify the conformation. The first extracellular fungal tyrosinase was reported from the filamentous fungus *T. reesei* in 2006, and the mature enzyme was lacking the C-terminal domain (Selinheimo et al. 2006).

A homology search for novel secreted tyrosinases to known fungal tyrosinases in the public fungal genomes retrieved a new class of short tyrosinase-like proteins lacking the entire linker region and the C-terminal domain. Compared to a model protein, in the tyrosinase from *T. reesei*, the level of similarity was generally 30% and identity around 20%. A different histidine pattern was found at the first copper-binding site, CuA, where only seven residues were between the first two conserved histidines instead of the 23 found in common fungal tyrosinases (Table 1). In contrast to long tyrosinases, six cysteine residues were conserved in short tyrosinase-like sequences, possibly improving the protein stability by forming disulphide bonds. Accordingly, the representative enzyme AoCO4 was found to be quite thermostable, having a half-life of 20 h at 50°C, when compared to the long tyrosinase from *T. reesei* that showed a half-life of 15 min at 50°C (Selinheimo et al. 2006).

Long and short secreted tyrosinase-like sequences subjected to phylogenetic analysis grouped in separate clades with a common origin (Online Resource 1). The length variation seen in the tyrosinase family members could be largely explained by diversification into two main groups after other fungi were separated from Chytridiomycota, rather than by random noise due to errors in gene models or incorrect detection of protein domains. The phylogenetic analysis suggested that the short tyrosinase-like sequences originated from an ancestral tyrosinase version possessing a C-terminal region following the catalytic domain. The evolution process thus leads to the loss of the C-terminal region without affecting the conservancy of important residues for structure and activity and comprised in the structural core described by Flurkey and Inlow (2008).

We produced heterologously AoCO4, the *A. oryzae* type 3 copper protein, which falls into a short tyrosinase-like enzyme group, lacking the C-terminal domain. The purified enzyme had activity on diphenolic compounds, but no activity on tyrosine was detected in the assay conditions. The conditions in selection of the transformants were, however, very different as compared to activity assay conditions. When transformants were selected, the enzyme activity was recorded on agar plates for 7 days, which is long enough to show even minor activities. The absorption maximum at around 330 nm in the UV–visible absorption

spectrum confirmed the presence of a type 3 copper center in the purified enzyme. The CD spectrum indicated that the secondary structure of AoCO4 consists of mainly α -helical structures, as also reported for the tyrosinase from *A. bisporus* and from *S. castaneoglobisporus* (Saboury et al. 2004; Matoba et al. 2006). The spectroscopic analysis thus suggested that the folding of the protein was correct. Furthermore, upon excitation at 280 nm, AoCO4 exhibits fluorescence with maximum intensity near 330 nm, which is in accordance with the reported values for the tyrosinases purified from *A. bisporus* and *N. crassa* (Beltramini and Lerch 1982; Karbassi et al. 2003). The spectroscopy data suggested that the absence of the C-terminal domain does not affect either the copper incorporation or the correct folding of the protein. According to literature, the role for the C-terminal domain in tyrosinases is analog to the caddie protein ORF378 in *S. castaneoglobisporus* tyrosinase, and it is supposed to modulate the activity of the enzyme by covering the active site and thus preventing substrate binding (Matoba et al. 2006).

To our knowledge, no tyrosinase activity has been reported from enzymes encoded by short sequences of tyrosinases. Three genes encoding tyrosinases have been isolated from *A. oryzae* and partially characterized: *MelB* (Obata et al. 2004), *MelD* (Masayuki et al. 2004), and *MelO* (Fujita et al. 1995). All these tyrosinases contain a C-terminal domain, which is cleaved from the mature protein. *A. oryzae* cultures are applied in the fermentation process for production of sake where tyrosinases are involved in the melanin formation (Te Biesebeke and Record 2008). Two tyrosinases from *A. oryzae*, *MelO* and *MelB*, were produced respectively in submerged culture and in solid-state culture. Regarding characterization of *A. oryzae* tyrosinases, very few data are available. Both *MelO* and *MelB* show activity on L-tyrosine and L-dopa; however, the activity of *A. oryzae* tyrosinases on other substrates has not been reported (Obata et al. 2004; Fujita et al. 1995). Contrary to the *A. oryzae* tyrosinases, AoCO4 did not show activity on L-tyrosine and L-dopa in the test conditions. *MelB* and *MelD* tyrosinases have the maximum activity at pH 5.6 (Masayuki et al. 2004), similar to AoCO4.

Flurkey and Inlow (2008) collected from literature data on the N-terminal and/or C-terminal residues of tyrosinases and catechol oxidases and showed that in all cases the N-terminus of the protein is located prior to a conserved arginine residue, while the C-terminus contains the conserved tyrosine motif. In all the three structures of type 3 proteins so far resolved, these residues are located in two adjacent parallel β -sheets that essentially bring together the N- and C-terminal ends of the polypeptide chain and form a compact globular protein. Proteolysis after the conserved arginine and/or before the tyrosine residue would eliminate the β -sheet and pi-cation interaction, and the overall

structure would likely be destabilized with loss of enzymatic activity (Flurkey and Inlow 2008). When the sequence of AoCO4 was aligned with other fungal tyrosinases, it was possible to identify the N-terminal conserved arginine residue and the tyrosine motif, being R80 and (Y-V-Y) 405–407 (Fig. 1). Despite of the N-terminal processing of the first 51 amino acids from the putative N-terminus, the conserved arginine is still retained in the mature protein. The vicinity of the Kex2/furin-type protease site (K68-R69) to the conserved arginine residue could play a key role in the N-terminal processing of the enzyme. It has been reported that Kex2/furin-type proteases are involved in the maturation of proteins in the Golgi apparatus of *T. reesei* (Goller et al. 1998). Most probably the pi-cation interaction of the arginine and the tyrosine motif occurs before the processing of the protein, thus determining the eventual cleavage sites in the N- and/or C- termini.

Decker et al. (2006) analyzed the structures of tyrosinases, catechol oxidases, and hemocyanins. The CuA site in *I. batatas* catechol oxidase is shielded by a phenylalanine residue (F261), while it is not masked by any bulky residue in the tyrosinase from *S. castaneoglobisporus*. This could indicate that accessibility to the CuA site is necessary for the binding and oxidation of monophenols to *o*-quinones, providing a possible explanation for the functional difference between catechol oxidase and tyrosinase. Analysis of AoCO4 sequence identified a valine residue (V324), which corresponds to the phenylalanine residue (F261) of *I. batatas* catechol oxidase. A smaller steric hindrance of the valine residue could allow some activity on monophenols as well. We observed activity on monophenolic compounds, as aminophenol and guaiacol. The purified AoCO4 did not show activity on L-tyrosine, although transformants were selected based on their activity on L-tyrosine, on agar plate.

We have cloned and expressed a short tyrosinase-like enzyme from *A. oryzae* lacking a C-terminal domain. This protein was not classified as a tyrosinase, but as a catechol oxidase, since it was lacking activity on tyrosine. The suggested role for the C-terminal domain in tyrosinases is to keep the enzyme inactive in the secretory pathway. The loss of the C-terminal domain in AoCO4 might be related to the evolution process of this and perhaps other similar enzymes. Because the enzyme is not capable of oxidizing tyrosine, it might not be required that the enzyme is inactive during secretion. The lack of activity on L-tyrosine and L-dopa may indicate that the role of AoCO4 is not related to melanin biosynthesis. The detected activity on aminophenol might, on the other hand, suggest a role of AoCO4 in detoxification; also the extracellular nature of the protein would be in agreement with this possible role. More studies of short tyrosinase-like enzymes are needed in order to obtain information on their properties and possible role.

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PUBLICATION II

**Secreted fungal sulfhydryl oxidases:
sequence analysis and characterisation of a
representative flavin-dependent enzyme
from *Aspergillus oryzae***

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RESEARCH ARTICLE

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Secreted fungal sulfhydryl oxidases: sequence analysis and characterisation of a representative flavin-dependent enzyme from *Aspergillus oryzae*

Greta Faccio*, Kristiina Kruus, Johanna Buchert, Markku Saloheimo

Abstract

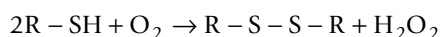
Background: Sulfhydryl oxidases are flavin-dependent enzymes that catalyse the formation of de novo disulfide bonds from free thiol groups, with the reduction of molecular oxygen to hydrogen peroxide. Sulfhydryl oxidases have been investigated in the food industry to remove the burnt flavour of ultraheat-treated milk and are currently studied as potential crosslinking enzymes, aiming at strengthening wheat dough and improving the overall bread quality.

Results: In the present study, potential sulfhydryl oxidases were identified in the publicly available fungal genome sequences and their sequence characteristics were studied. A representative sulfhydryl oxidase from *Aspergillus oryzae*, AoSOX1, was expressed in the fungus *Trichoderma reesei*. AoSOX1 was produced in relatively good yields and was purified and biochemically characterised. The enzyme catalysed the oxidation of thiol-containing compounds like glutathione, D/L-cysteine, beta-mercaptoethanol and DTT. The enzyme had a melting temperature of 57°C, a pH optimum of 7.5 and its enzymatic activity was completely inhibited in the presence of 1 mM ZnSO₄.

Conclusions: Eighteen potentially secreted sulfhydryl oxidases were detected in the publicly available fungal genomes analysed and a novel proline-tryptophan dipeptide in the characteristic motif CXXC, where X is any amino acid, was found. A representative protein, AoSOX1 from *A. oryzae*, was produced in *T. reesei* in an active form and had the characteristics of sulfhydryl oxidases. Further testing of the activity on thiol groups within larger peptides and on protein level will be needed to assess the application potential of this enzyme.

Background

Disulfide bonds are essential for the stability and function of intracellular and secreted proteins. The subject of this work are sulfhydryl oxidases, SOX, and in particular glutathione oxidases (E.C. 1.8.3.3), enzymes catalysing the formation of *de novo* disulfide bonds between thiol groups with the subsequent reduction of oxygen to hydrogen peroxide (equation 1).



The name sulfhydryl oxidase is sometimes also referred to thiol oxidases (EC 1.8.3.2), enzymes that also oxidise thiol groups using oxygen as electron acceptor but reducing it to water. Thiol oxidases have been

isolated from fungi, e.g. *Mycothecium* [1], *Piricularia* and *Polyporus* [2].

The first secreted fungal enzyme containing FAD and able to oxidise glutathione and several sulfhydryl compounds was reported in 1982 from *Penicillium sp.* K-6-5. This enzyme had negligible activity on cysteines in proteins. Furthermore, it was not effective on the reactivation of reduced RNase A [3]. In 1987, a secreted sulfhydryl oxidase active on protein associated thiol groups was isolated in *Aspergillus niger* culture filtrates [4]. The enzyme was found to be homodimeric, and each subunit was binding tightly but non-covalently a FAD molecule. *A. niger* sulfhydryl oxidase was active on glutathione and in a lesser extent on homocysteine, DTT, cysteine, a g-glu-cys dipeptide characterised by a carboxylamide bond [5] and its presence increased the rate of reactivation of reduced ribonuclease A [4]. The sulfhydryl oxidase from *A. niger* and *Penicillium* have a different

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evolutionary origin than the well-characterised intracellular sulphhydryl oxidases, of the Erv family, and are thought to be more related to thioredoxin reductases and pyridine nucleotide flavin disulfide oxidoreductases [3,4,6]. Their physiological role is however still unclear. Metallo-sulphhydryl oxidases containing iron [7] or copper [8] have also been reported.

The action of sulphhydryl oxidases on small thiol-containing compounds and the production of hydrogen peroxide, similarly to the well-known glucose oxidase [9], make sulphhydryl oxidase very attractive for the food industry. Sulphhydryl oxidases can be a valid alternative to the use of chemical additives, such as potassium bromate or ascorbic acid, for the improvement of the strength and handling properties of wheat dough in the baking industry.

The aim of this work was to analyze the putative secreted sulphhydryl oxidases in the publicly available fungal genomes and to produce and biochemically characterise one of the identified enzymes, i.e. AoSOX1 from *Aspergillus oryzae*.

Results and Discussion

Analysis of secreted fungal sulphhydryl oxidases

The search for secreted proteins carrying a predicted disulphide oxidoreductases domain of class II and, in particular, FAD-dependent ones (see Materials and method section) retrieved among 398 proteins 48 with a signal sequence, no ER retention signal and no putative transmembrane segments, and thus are highly likely to be secreted (Table 1). The only characterized protein found among them is the sulphhydryl oxidase from *A. niger* (AnSOX, [NCBI:CAK40401]) [4]. Numerous retrieved proteins have been found in the *Aspergillus spp.* and *Neosartoria fischeri*, a close relative of the *Aspergilli*. Alignment of the sequences identified allowed the selection of 18 proteins possessing the CXXC motif characteristic of thiol:disulfide oxidoreductases like sulphhydryl oxidases (Figure 1). Three main deletions can be identified from the alignment in Figure 1, e.g. protein [Swiss-Prot: Q2H2X8] lacks residues in position 57-83 and 302-318, and protein TRIRE0077288 (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>) in position 190-211 (residues numbered according to the alignment).

Sequence features typical of FAD dependent pyridine nucleotide disulphide oxidoreductases (IPR013027) were reflected in three regions. Firstly, a conserved motif characteristic of the Rossmann fold (V/I)(V/I)GXGXXGXXXA/L, where X is any residue, is found in the N-terminal region of the sequences (residues 38-49 in the alignment in Figure 1) suggesting that the proteins bind to a nucleotide cofactor such as FAD or NAD(P), with a $\beta\alpha\beta$ -fold and possibly function as oxidoreductases [10]. The second conserved region is

located in the middle of the protein sequence and contains the two conserved cysteine residues of the CXXC motif (residues 177-180 in Figure 1) that is characteristic of the thiol:disulfide oxidoreductases, including sulphhydryl oxidases and, in general, oxidoreductases of the thioredoxin fold family [11]. Many conserved residues are found in this region and the following pattern can be identified RKHHL(A/G)TGXXDX₆GXX(E/D)XY(G/A)XGXYYCXXC(D/H)GYE (X is any amino acid, H a hydrophobic amino acid and Y an aromatic residue). Here is located the ATG motif common to FAD and NADPH-binding domains and reported to be located at the end of the fourth β -strand interacting with the cofactor [12].

The dipeptide comprised between the cysteine residues has been shown to affect the redox properties of proteins with a CXXC motif [13]. The proline-tryptophan dipeptide found in AoSOX1 and another eleven of the sulphhydryl oxidases studied here has never been characterized, but the N-terminal proline is thought to positively affect the local conformation in DsbA, a protein required for disulfide bond formation in *E. coli* [14]. Quan and co-authors also showed a positive correlation between the presence of an aromatic residue in the C-terminal position and a higher catalytic efficiency. The third conserved region is in the C-terminus and includes firstly, the GD motif (TXHXGHY(A/G)HGD, residues 340-350 in Figure 1) that is involved in the binding of the ribityl moiety of FAD in most flavoproteins with two-dinucleotide binding domains but not common in single FAD-binding domain proteins, e.g. absent in cholesterol oxidase and glucose oxidase, and secondly, the G-helix (AHXXG, residues 362-366 in Figure 1)[12,15]. The final stretch is rich in glutamic acid residues and is predicted to have intrinsic disorder, e.g., after residue E350 in AnSOX and after K349 in AoSOX1 [16].

An overall lower level of conservation is observed in sequence [Swiss-Prot:Q4W4QJ0] from *Aspergillus fumigatus* and [Swiss-Prot:Q55JR2] from *C. neoformans*. Four of the sequences retrieved (at the top of the alignment in Figure 1) clearly group separately and have a different dipeptide (LF) between the cysteine residues of the active site, e.g. [Swiss-Prot:A4QYP9] from *Magnaporthe grisea*, [Swiss-Prot:A1DN23] from *N. fischeri*, [Swiss-Prot:Q0CME9] from *Aspergillus terreus* and [Swiss-Prot:Q5MBU7] from *A. fumigatus*. The three last-named sequences have an unusually long predicted signal peptide, e.g. 28 residues for [Swiss-Prot:Q0CME9] and [Swiss-Prot:A1DN23], 27 residues for [Swiss-Prot:Q5MBU7].

Production of AoSOX1 in *Trichoderma reesei*

A representative protein, AoSOX1 [NCBI:BAE61582], with 64.7% level of identity to *A. niger* sulphhydryl oxidase

Table 1 Secreted fungal sulfhydryl oxidases listed by organism and their key features

Family	Organism	Name	Status	Length	Signal peptide length	Identity to AnSOX (%)	CXXC motif
Chaetomiaceae	<i>C. globosum</i>	[Swiss-Prot:Q2H2I0]]	putative	517	26	14.4	–
Chaetomiaceae	<i>C. globosum</i>	[Swiss-Prot:Q2H2X8]	putative	356	18	37.9	CPWC
Hypocreaceae	<i>T. reesei</i>	TRIRE0077288 ¹	predicted	382	19	37.1	CPWC
Magnaporthaceae	<i>M. grisea</i>	[Swiss-Prot:A4QYP9]	putative	325	20	24.2	CLFC
Magnaporthaceae	<i>M. grisea</i>	[Swiss-Prot:A4R670]	predicted	398	19	40.1	CPWC
Onygenales	<i>C. immitis</i>	[Swiss-Prot:Q1DQ56]	predicted	400	20	10.5	–
Phaeosphaeriaceae	<i>P. nodorum</i>	[Swiss-Prot:Q0ULD1]	putative	425	18	16.5	–
Phaeosphaeriaceae	<i>P. nodorum</i>	[Swiss-Prot:Q0V1M0]	predicted	392	22	18.0	–
Saccharomycetaceae	<i>P. stipitis</i>	[Swiss-Prot:A3LTU5]	predicted	370	20	19.5	–
Saccharomycetaceae	<i>L. elongisporua</i>	[Swiss-Prot:A5DYY0]	uncharacterised	393	32	14.8	–
Saccharomycetaceae	<i>S. cerevisiae</i>	[Swiss-Prot:P52923]	evidence at protein level	378	21	17.5	–
Saccharomycetaceae	<i>A. gossypii</i>	[Swiss-Prot:Q759I7]	predicted	2195	24	3.5	–
Sordariaceae	<i>N. crassa</i>	[Swiss-Prot:Q75A02]	predicted	627	22	12.7	–
Sordariaceae	<i>N. crassa</i>	[Swiss-Prot:Q75A31]	putative	538	27	12.3	–
Sordariaceae	<i>N. crassa</i>	[Swiss-Prot:Q75AD4]	predicted	589	19	12.9	–
Tremellaceae	<i>C. neoformans</i>	[Swiss-Prot:Q55JR2]	putative	346	21	23.3	CIFC
Trichomaceae	<i>A. clavatus</i>	[Swiss-Prot:A1C4Y4]	putative	388	19	61.8	CPWC
Trichomaceae	<i>A. clavatus</i>	[Swiss-Prot:A1CE06]	predicted	599	19	9.4	–
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1CZW3]	putative	386	19	62.8	CPWC
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1DD16]	predicted	609	20	10.3	–
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1DJI6]	putative	382	23	14.8	–
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1DKE0]	putative	515	20	11.0	–
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1DN23]	putative	334	28	25.8	CLFC
Trichomaceae	<i>N. fischeri</i>	[Swiss-Prot:A1DPI7]	putative	387	23	46.2	CPWC
Trichomaceae	<i>A. niger</i>	[Swiss-Prot:A2QUK3]	AnSOX	392	19	100.0	CPWC
Trichomaceae	<i>A. niger</i>	[Swiss-Prot:A2R6C0]	predicted	440	23	9.0	–
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0C958]	predicted	360	28	19.4	–
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0CBT8]	predicted	381	21	14.4	–
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0CE26]	predicted	387	19	65.4	CPWC
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0CME9]	putative	335	28	25.8	CLFC
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0CVW0]	predicted	453	21	14.9	–
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0CZE7]	putative	519	48	10.8	–
Trichomaceae	<i>A. terreus</i>	[Swiss-Prot:Q0D021]	putative	388	18	55.3	CPWC
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2TXY9]	predicted	518	41	10.1	–
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2U4P3]	predicted	389	23	47.3	CPWC
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2U5L3]	predicted	478	20	14.0	–
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2UA33]	AoSOX1	384	19	64.7	CPWC
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2UCA6]	predicted	422	17	9.0	–
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2UE40]	predicted	625	28	14.7	–
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2UTD4]	predicted	502	28	12.5	–
Trichomaceae	<i>A. oryzae</i>	[Swiss-Prot:Q2UV04]	predicted	513	28	11.8	–
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q4WEM5]	predicted	386	19	62.3	CPWC
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q4WF68]	putative	515	20	11.3	–
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q4WFR5]	putative	382	23	15.8	–
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q4WLA0]	predicted	508	34	10.1	–
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q4WQJ0]	inferred from homology	392	29	21.8	CAVC
Trichomaceae	<i>A. fumigatus</i>	[Swiss-Prot:Q5MBU7]	predicted	334	27	26.2	CLFC
Ustilaginaceae	<i>U. maydis</i>	[Swiss-Prot:Q4PCL0]	predicted	583	29	15.0	–

¹ *T. reesei* genome, <http://genome.jgi-psf.org/Trire2/Trire2.home.html>

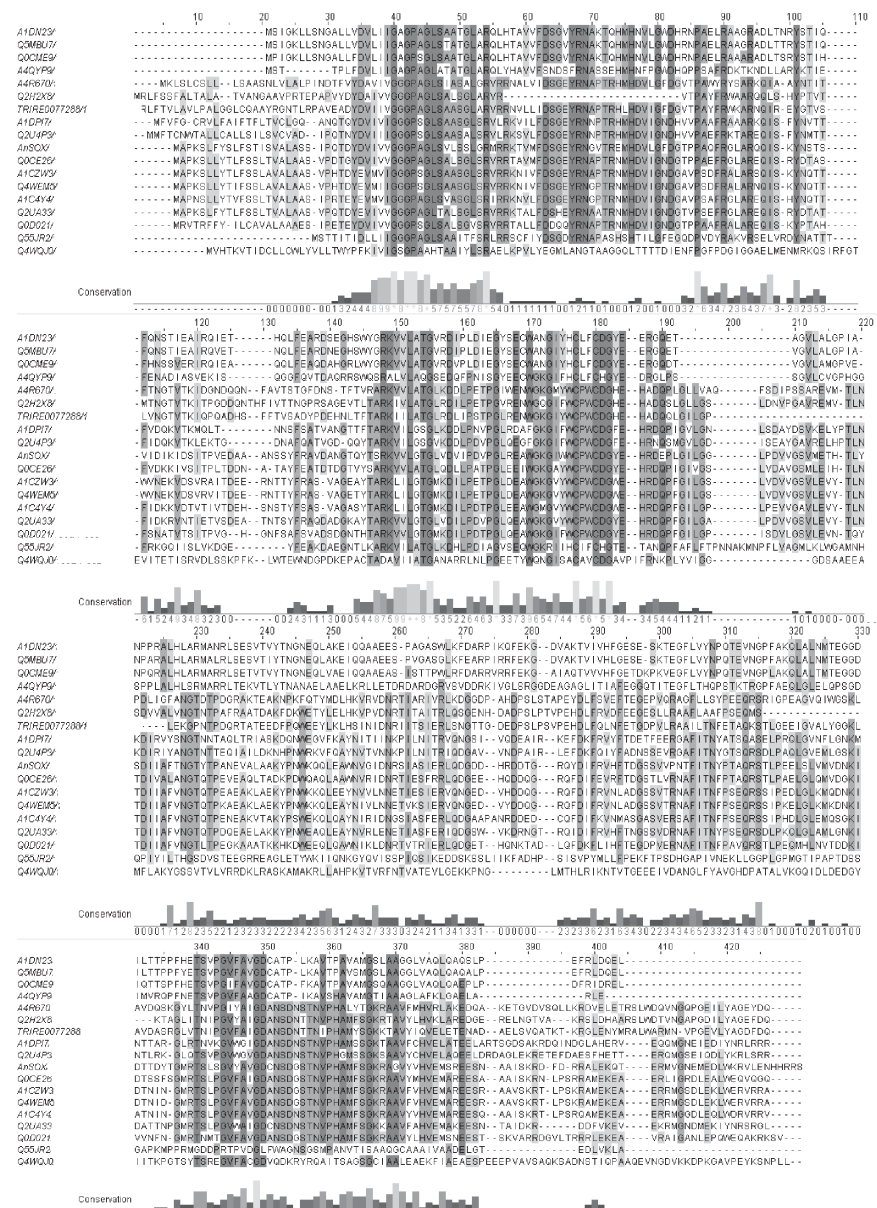


Figure 1 Sequence alignment. Sequence alignment of the candidate secreted SOX proteins containing the CXXC motif to the well-known enzyme *A. niger* SOX, [Swiss-Prot:A2QUK3]. Residues background intensity accords to the level of identity and the level of conservation of each residue is indicated by the histogram below and by a 0 to 5 value. The alignment was performed with ClustalW2 and visualised by JalView (http://www.jalview.org). Sequences: [Swiss-Prot:A1DN23] from *N. fischeri*, [Swiss-Prot:Q5M8U7] from *A. fumigatus*, [Swiss-Prot:QOCME9] from *A. terreus*, [Swiss-Prot:A4QYP9] from *M. grisea*, [Swiss-Prot:A4R670] from *M. grisea*, [Swiss-Prot:Q2H2X8] from *C. globosum*, TRIRE0077288 from *T. reesei*, [Swiss-Prot:A1DPI7] from *N. fischeri*, [Swiss-Prot:Q2U4P3] from *A. oryzae*, AnSOX [Swiss-Prot:A2QUK3] from *A. niger*, [Swiss-Prot:Q0CE26] from *A. terreus*, [Swiss-Prot:A1C2W3] from *N. fischeri*, [Swiss-Prot:Q4WEM5] from *A. fumigatus*, [Swiss-Prot:A1C4Y4] from *A. clavatus*, AoSOX1 [Swiss-Prot:Q2UA33] from *A. oryzae*, [Swiss-Prot:Q0D021] from *A. terreus*, [Swiss-Prot:Q55JR2] from *C. neoformans* and [Swiss-Prot:Q4WQJ0] from *A. fumigatus*.

was chosen for expression in *T. reesei* and subsequent biochemical characterization. The AoSOX1 protein consists of 384 amino acids including a predicted signal sequence of 19 residues. The closest homologs to AoSOX1 are three putative thioredoxin reductases from *Aspergillus flavus* (99% sequence identity, [NCBI:

EED47993]), *Neosartorya fischeri* (70%, [NCBI: XP_001266180]) and *A. fumigatus* (68%, [NCBI: XP_747990]). AoSOX1 gene is predicted to be interrupted by two introns. The gene coding for AoSOX1 (1304 bp) was amplified by PCR from the genomic DNA of *A. oryzae* and cloned

by Gateway recombination into a *T. reesei* expression vector under the control of the strong inducible *cbh1* promoter. A C-terminal six-histidine tag was added to the expression construct. The expression plasmid was transformed into *T. reesei* and, after screening, one clone, producing the highest activity on glutathione, was selected for enzyme production. The highest production level in shake flask was reached after 5 days of cultivation and was adequate for purification; no fermentor cultures were thus needed. The protein content of the medium at the end of the cultivation was 1.4 g/l and AoSOX1 production level of 70 mg/l accounted for about 5% of the total secreted proteins, as estimated using the specific activity value. The production of AoSOX1 in an active form showed that the two introns were correctly spliced from the transcript. The secretion of AoSOX1 when its gene is expressed in *T. reesei* suggested its extracellular production also in the native strain, *A. oryzae*.

Enzyme purification

Attempts to purify AoSOX1 with the help of the histidine tag were unsuccessful because of the protein did not bind to copper-chelated Chelating Sepharose Fast Flow resin. Besides, Western blotting analysis of samples containing AoSOX1 with primary anti-Histag antibodies showed no recognition. Furthermore, the C-terminal peptide containing the six-histidine tag was never detected after tryptic digestion and peptide analysis. These findings suggest that possibly the histidine tag was cleaved off the recombinant enzyme by a host protease.

The AoSOX1 enzyme was purified by anion-exchange in a Hitrap DEAE FF column (elution at 166 mM NaCl concentration) and size-exclusion chromatography on a Superdex 75 HR 10/30 column (elution volume 11.3 ml). Results of a typical AoSOX1 purification are summarised in Table 2. AoSOX1 purification by anion exchange and size-exclusion chromatography lead to an activity yield of 68% and a purification factor of 21. The second purification step did not increase the purity but was crucial for the removal of brown-coloured compounds probably derived from the medium used in the cultivations.

Molecular characterization

AoSOX1 in the purified form migrates in SDS PAGE in two bands at a molecular weight around 45000 and the MW determined by mass spectrometry was 43959. The difference in molecular mass can be ascribed to N-glycosylation considering that six asparagine residues in the protein are potential N-glycosylation sites and a net mass reduction is observed by SDS PAGE after treatment with PNGase F (Figure 2a, b). Deglycosylation resolved the two bands corresponding to AoSOX1 into a single one of apparently 40000. This suggests that the double band is due to heterogeneity of the N-glycans.

AoSOX1 in the culture medium and in the purified form was identified by peptide mass fingerprinting in the NCBI sequence database (<http://www.ncbi.nlm.nih.gov>) with a maximum of 24.5% sequence coverage (Figure 2b). Peptides containing putative N-glycosylation sites were not detected suggesting that at least some of the N-glycosylation sites would be occupied by glycans.

Biochemical characterization

Substrate specificity determination and inhibition studies

AoSOX1 showed activity both on small thiol compounds such as cysteine and also on larger molecules such as the tripeptide glutathione (Table 3). The highest activity was registered with glutathione, similarly to the glutathione oxidase from *Penicillium sp.* K-6-5 [3], and the second highest with DTT. A Michaelis-Menten behaviour was observed with all the compounds tested and the highest activity was detected with glutathione as substrate. Glutathione is however an improbable physiological substrate for secreted sulfhydryl oxidases and, even though their role is still unclear, their action on cell-wall or secreted proteins, in the formation of extracellular matrix [17] or for the maturation of peptides produced non-ribosomally [18] cannot be excluded.

AoSOX1 activity was only slightly affected by the presence of the chelating compound EDTA, the salts $MgSO_4$ and $MnSO_4$ and the denaturing agent urea (Table 4). Similarly to glutathione oxidase from *Penicillium* [3], the activity of AoSOX1 was drastically reduced in presence of $ZnSO_4$. This latter result was probably due to the interaction of the zinc ion with cysteine residues necessary for the catalytic activity of AoSOX1.

Table 2 Purification of AoSOX1 from the *T. reesei* culture medium

	Volume (ml)	Total activity ^b (10^6 nkat)	Total protein (mg)	Specific activity (10^6 nkat mg^{-1})	Purification factor	Activity yield (%)
Cell free culture medium ^a	60	311	324	0.96	1	100
Hitrap DEAE FF	108	261	13	20.20	21	84
Superdex 75 HR	8	210	11	19.97	21	68

^a The cell-free culture medium (220 ml) was concentrated and buffer exchanged.

^b Enzymatic activity was measured with the HVA-peroxidase coupled assay.

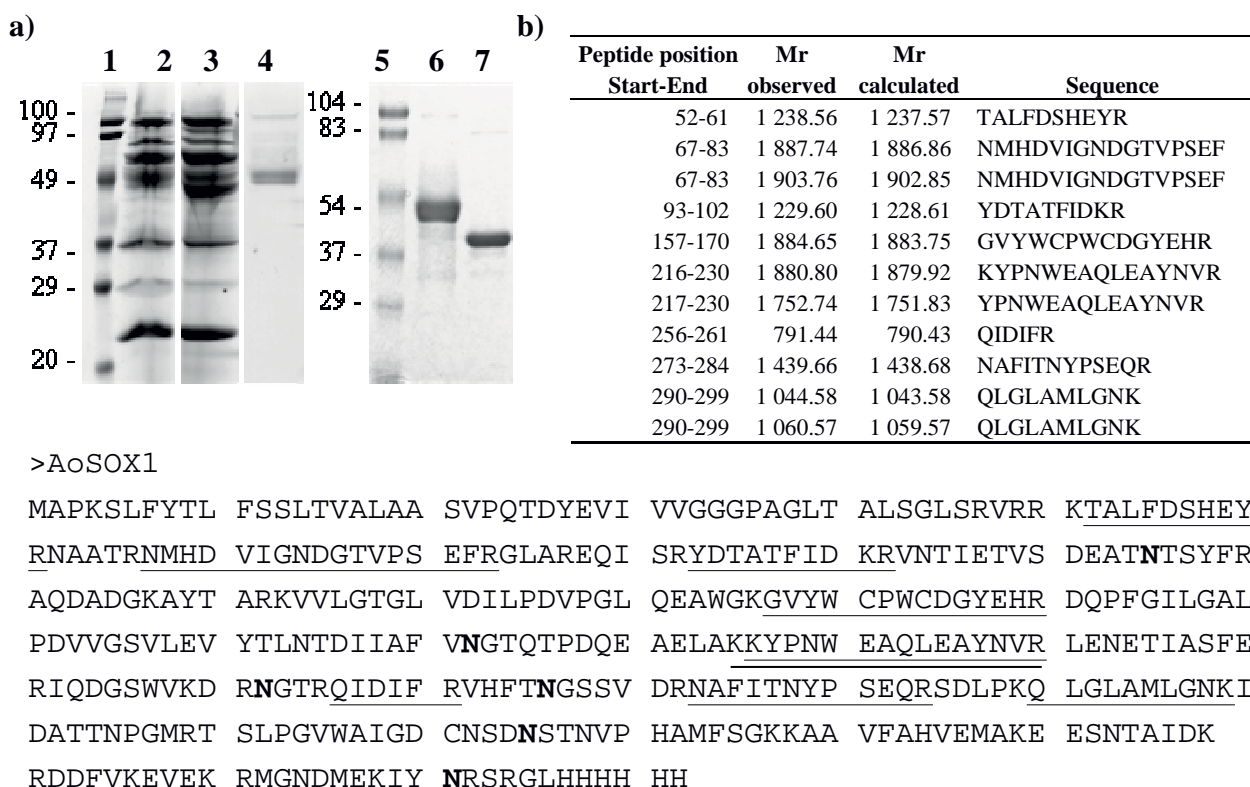


Figure 2 AoSOX1 molecular characterisation. (a) SDS PAGE analysis (12%, Tris/HCl) showing the *T. reesei* culture medium of the parental strain (lane 1, 80 µg), of the AoSOX1 expressing strain (lane 2, 80 µg), purified native protein (5 µg and 10 µg, lane 4 and 6, respectively) and the deglycosylated protein (10 µg, lane 7). Lanes 1 and 5 contain molecular weight markers. (b) Identification by peptide mass fingerprinting of the purified AoSOX1 after in-gel digestion with trypsin. The sequences and masses of the peptides matching with the AoSOX1 sequence are listed and underlined in the protein sequence. Asparagine residues belonging to potential N-glycosylation sites are in bold.

Considering the cysteine content of AoSOX1, e.g. 3 cysteines, there is a high probability that the reactive cysteines are the C177 and C180 forming the CXXC motif.

pH and temperature behaviour

AoSOX1 showed a significant pH stability retaining more than 80% of the initial activity when incubated in a pH range of 4 to 8 after 24 hours (data not shown). AoSOX1 and AnSOX showed a similar pH stability; the pH optimum value of 8, measured for AoSOX1 (Figure 3a) was higher than the value (5.5) reported for AnSOX [4,5] and similar to the ones for glutathione oxidase from *Penicillium* (7-7.8). AoSOX1 showed good

temperature stability at 30 and 40°C retaining more than 70% of the initial activity after 24 hours (Figure 3b). The activity of AoSOX1 was reduced to 40% and 8% respectively, after one and 24 hours incubation at 50°C. No activity was detected after 30 minutes of incubation at 60 and 70°C (Figure 3b).

Molecular properties of AoSOX1

The absorption spectrum of AoSOX1 showed three peaks at 275, 370 and 440 nm and a shoulder at 365 nm, like for the glutathione oxidase from *Penicillium* [3], revealing its flavoenzymatic nature (Figure 4). The cofactor was spectrophotometrically identified to be

Table 3 Kinetic constants of AoSOX1 on five different substrates

Substrate	K_m (mM)	V_{max} (nkat ml ⁻¹)	V_{max}/K_m (s ⁻¹)	k_{cat} (s ⁻¹)	Efficacy number (M ⁻¹ s ⁻¹)
Glutathione	2.78 ± 0.56	(15.3 ± 2.89) × 10 ⁷	55 010	3.60 × 10 ⁶	1.29 × 10 ⁹
L-Cys	6.11 ± 0.13	(22.2 ± 0.57) × 10 ⁵	364	5.23 × 10 ⁴	8.56 × 10 ⁶
D-Cys	1.55 ± 0.03	(25.3 ± 0.02) × 10 ⁴	163	5.95 × 10 ³	3.84 × 10 ⁶
DTT [thiols]	2.41 ± 0.13	(13.1 ± 0.22) × 10 ⁶	5 451	3.09 × 10 ⁵	1.28 × 10 ⁸
β-mercaptoethanol	9.73 ± 0.08	(21.3 ± 0.05) × 10 ⁵	219	5.01 × 10 ⁴	5.15 × 10 ⁶

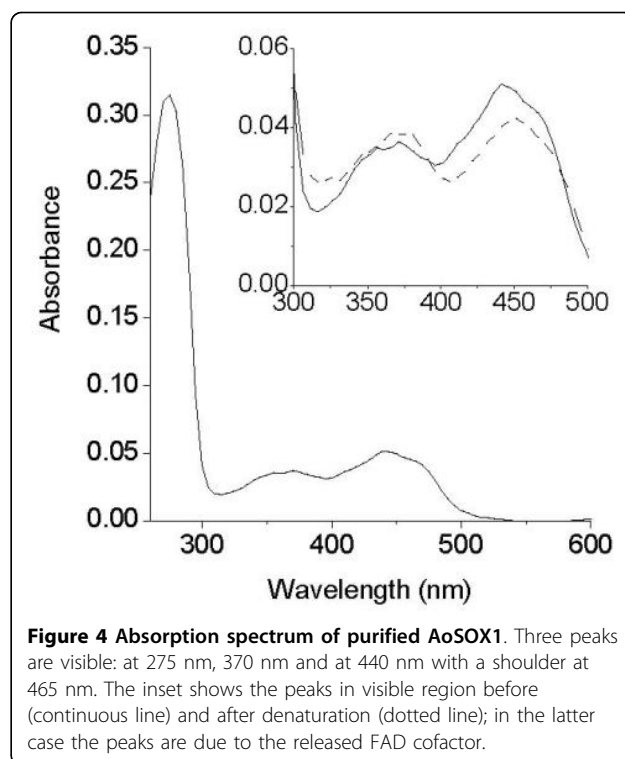
Table 4 Inhibition of AoSOX1 by various compounds

Inhibitor concentration	Residual activity ^a (%)
EDTA	1 mM 85 ± 5 10 mM 91 ± 7
KI	1 mM 65 ± 6 10 mM 48 ± 11
MgSO ₄	1 mM 55 ± 7 10 mM 80 ± 6
MnSO ₄	1 mM 81 ± 11 10 mM 94 ± 4
Na ₂ SO ₄	1 mM 61 ± 10 10 mM 70 ± 5
ZnSO ₄	1 mM 1 ± 0 10 mM 3 ± 1
SDS	1 mM 58 ± 10 10 mM 64 ± 11
NaCl	1 mM 91 ± 12 10 mM 74 ± 13
Urea	1 mM 80 ± 6 10 mM 79 ± 11

^a Values are expressed as means ± standard deviations.

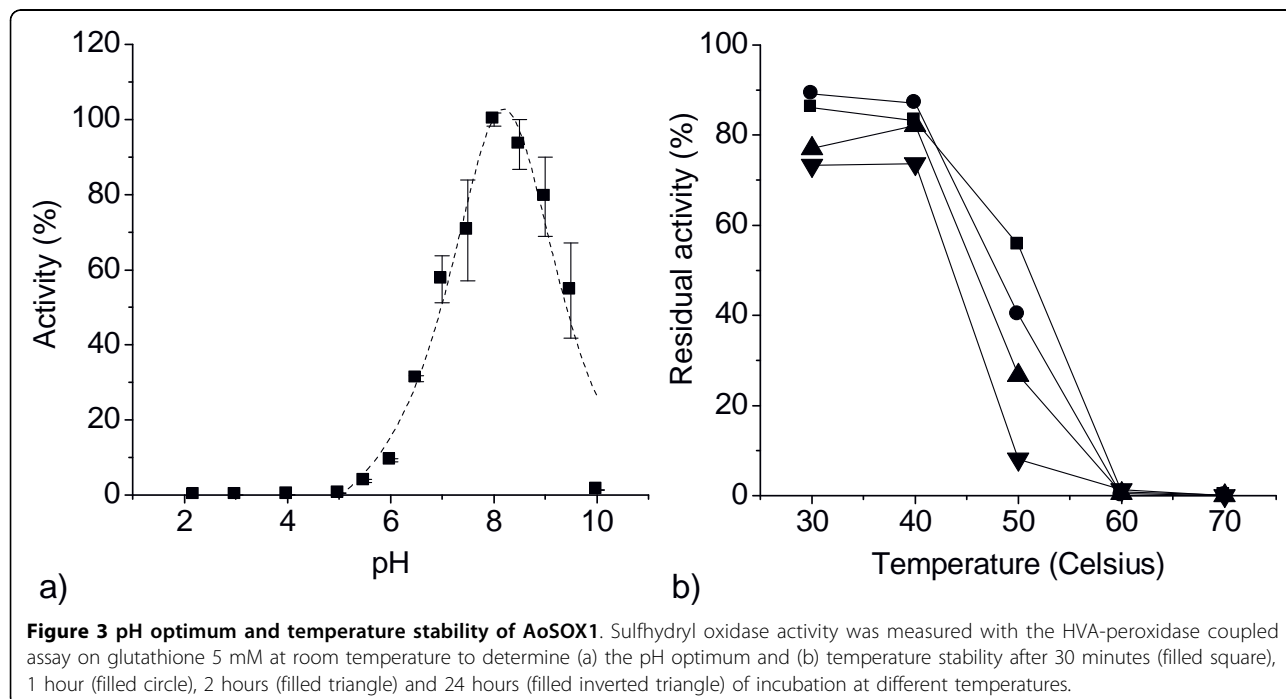
FAD after the cofactor was released from the enzyme by thermal and chemical denaturation (Figure 4, inset).

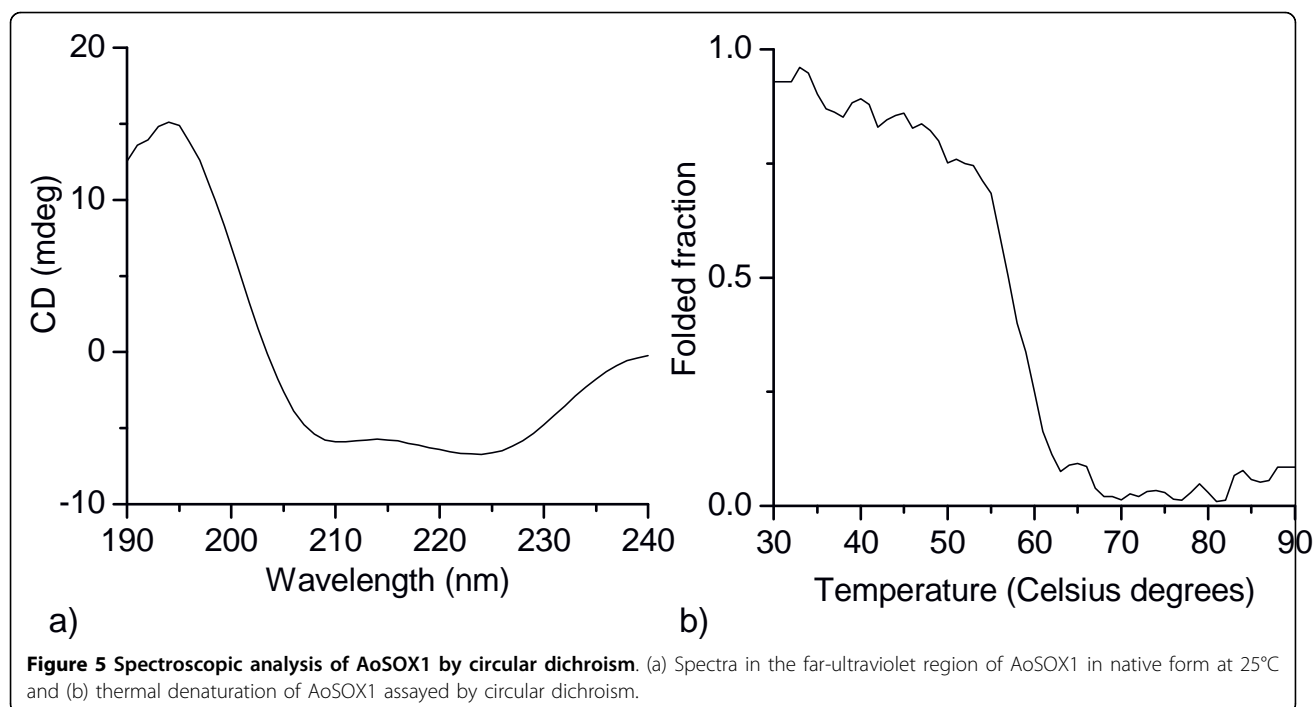
The secondary structure of AoSOX1 presented alpha-helical elements, as evidenced by the two negative peaks at 210 and 225 nm in the CD spectrum (Figure 5a). Thermal denaturation of AoSOX1 revealed a melting temperature of 57°C (Figure 5b). The available three-dimensional structures are of intracellular sulfhydryl



oxidases and it would be interesting to see how the structure of a secreted enzyme like AoSOX1 compares with these structures.

The stability of AoSOX1 against chaotropic denaturants, e.g. guanidinium hydrochloride, was studied by





equilibrium unfolding measurements (Figure 6). The exposure of the tryptophan side chains to a more polar environment, as a result of protein unfolding was reflected in a red shift of the fluorescence peak from 334 to 355 nm and was completed at a 3.5 M denaturant concentration. Tryptophan and FAD fluorescence had both a marked increase between 2 and 2.5 M denaturant concentration that can be due to the presence of three of the six tryptophan residues of AoSOX1 (W154, W160 and W163) in close proximity of the catalytically active di-cysteine pair and thus possibly to the

isoalloxazinic ring of the FAD cofactor. A drastic loss of activity was observed at a denaturant concentration above 1 M.

Conclusions

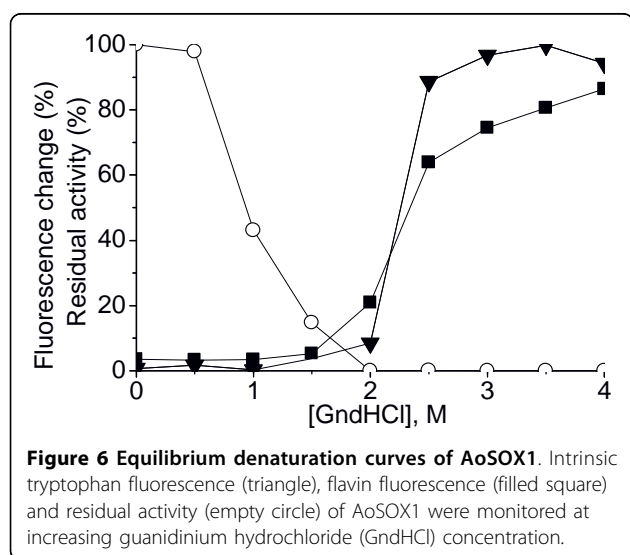
In the present study the sequences of potentially secreted fungal sulfhydryl oxidases were analyzed and a novel representative enzyme, AoSOX1 from *A. oryzae*, was produced in *T. reesei* in an active form. The characteristics of the enzymes are typical for sulfhydryl oxidases: the enzyme is capable of oxidizing small molecular compounds like glutathione, DTT and cysteine and the enzyme has a non-covalently bound FAD as a cofactor. Further testing of the activity on thiol groups within larger peptides and on protein level will be needed to assess the application potential of this enzyme.

Methods

All chemicals were purchased from Sigma-Aldrich but D-cysteine hydrochloride monohydrate that was purchased from Fluka. All columns for protein purification were purchased from GE Healthcare and all the purification steps were carried out with an Äkta purifier system (Amersham Biosciences).

SOX sequence analysis

An in-house database containing 28 public fungal genomes [19] was analysed by InterProScan program [20] for the presence of a pyridine nucleotide disulphide



oxidoreductases domain of class II and, in particular, FAD-dependent ones (IPR000103 and IPR013027 respectively). Presence of a target peptide for the secretory pathway was considered and determined with TargetP [21]. Multiple sequence alignments were produced with the software ClustalW [22] and pairwise alignments at EMBL-EBI (<http://www.ebi.ac.uk/Tools/emboss/align/index.html>).

Isolation and expression of AoSOX1 gene in *Trichoderma reesei*

The gene coding for AoSOX1 was amplified by PCR from the genomic DNA of *A. oryzae*, strain VTT-D-88348, and with Dynazyme EXT polymerase (Finnzymes, Helsinki, Finland). The PCR program included an initial denaturation step of 3 min at 98°C, followed by 25 cycles of 30 s at 98°C, 30 s at 60°C and 45 s at 72°C. This was followed by a final elongation step of 10 min at 72°C. Cloning was done with the Gateway technology (Invitrogen) and the primers were designed to incorporate *attB* sites in the PCR product to allow the insertion, via BP reaction, into the pDONR221 cloning vector (5' GGGGACAAGT TTGTACAAA AAGCAGGCTA TCATGGCTCC TAAGTCTCTT TTCTAC3', 3'GGGG ACCACT TTGTACAAGA AAGCTGGGTT CAGTGG TGGT GGTGGTGGTG CAGGCCTCTA GACCGAT-TAT A5'). Primers were also designed to introduce a C-terminal tag of six histidines to the gene product. The subsequent LR recombination reaction transferred AoSOX1 gene into the pMS186 expression vector producing the plasmids pGF008. The pMS186 contained the Gateway reading frame cassette C between the *cbh1* (cellobiohydrolase 1) promoter and terminator, and a hygromycin resistance cassette. The recombinant plasmid was transformed as described [23] into a variant of the *T. reesei* strain VTT-D-00775 [24]. Transformants were streaked twice consecutively on plates containing hygromycin B (125 µg/ml) and then screened with PCR for the presence of the expression construct. Positive transformants were purified to single spore cultures and grown in shake flasks for 9 days at 28°C in 50 ml of *Trichoderma* minimal medium supplemented with 4% lactose, 2% spent grains and 100 mM piperazine-N,N'-Bis (3-propanesulfonic acid) pH 5.5. SOX activity was assayed after 5, 7 and 9 days on reduced L-glutathione. The transformant giving the highest activity was grown in 250 ml culture medium in a 2 L flask at 28°C for 5 days for routine protein production and subsequent characterisation.

Activity measurements

Oxygen consumption assay

The reaction was initiated by the addition of the enzyme to 1.8 ml of buffered substrate solution in a

fully filled-in vial and the oxidation rate ($\text{nmol l}^{-1} \text{s}^{-1}$) was calculated from the linear part of the oxygen consumption curve. OXY-10 mini-multi-channel oxygen meter (PreSens Precision Sensing GmbH, Germany) was used in the measurements.

HVA-peroxidase coupled assay

SOX activity was generally measured in a 96 well microtiter plate with a coupled assay modified from Raju [25] where 10 µl of enzyme solution was added to 95 µl of a 1:1 mixture of 1.4 µM peroxidase type II and 1 mM homovanillic acid (HVA); the reaction was started adding 55 µl of substrate, e.g. 5 mM reduced L-glutathione. Reagents were dissolved in 50 mM potassium phosphate buffer, 0.3 mM EDTA pH 7.5. The production of the fluorescent HVA dimer was followed at excitation wavelength 320 nm and emission wavelength 420 nm. The activity as variation of fluorescence was calculated in arbitrary units (AU) per minute. Activity measurements were performed in a black 96-well microtiter plate and fluorescence was measured using a Varioskan spectral scanning multimode reader (Thermo Electron Co., Vantaa, Finland).

The conversion of activity values in AU to kat ml^{-1} was possible with a calibration curve (Figure 7). Reaction mixtures containing different amounts of monomeric HVA were monitored to completion and the final fluorescence value was plotted against the number of moles of HVA dimer theoretically produced, and stoichiometrically equivalent to the molecules of oxygen consumed. In example, a standard reaction mixture for activity measurement contained 0.3 mM monomeric

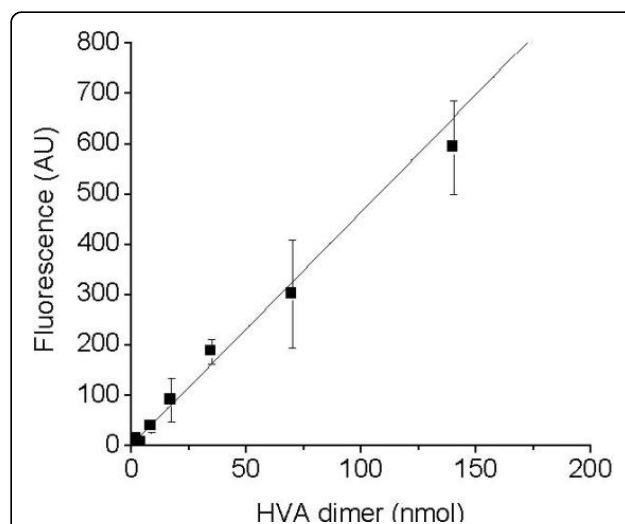


Figure 7 Conversion of activity values expressed in arbitrary units per minute per ml to katal per ml. Linear correlation between the moles of HVA-dimer theoretically formed in the reactions at completion and the final fluorescence values measured in arbitrary units ($y = 4.63 \times +1.40$, $R = 0.988$).

HVA that during a standard activity assay could lead to the formation of a maximum of 24 nmoles in dimeric form, in a 160 μ l reaction volume. Three identical reaction mixtures were measured, and a conversion factor of 8.6 was obtained for converting the activity values $\text{AU ml}^{-1} \text{ min}^{-1}$ to nkat ml^{-1} .

The final reaction mixtures were analysed similarly to [26] by HPLC (Hypersil BDS C-18 5 μ m, 4.6 \times 150 mm, Agilent, operated by a Waters 600 E system controller). The retention time of residual HVA monomer in the reaction mixtures was determined by comparison to solutions of HVA and measuring the absorbance of the eluate at 280 nm (Waters 2996 Photodiode Array detector). The HVA dimer elution was monitored by measuring the fluorescence ($\lambda_{\text{ex}} = 315 \text{ nm}$, $\lambda_{\text{em}} = 425 \text{ nm}$, Waters474 Scanning Fluorescence detector). The retention time of HVA in the monomeric form was 2.6 minutes while it was 3.2 minutes for the dimeric form. Dimerization of HVA in the reaction mixtures was confirmed by the appearance of a major fluorescent peak corresponding to dimeric HVA in all reactions.

Assay with Ellman's reagent

Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), was used in a qualitative assay for the detection of SOX activity in the fractions of the purification. The enzyme sample of 10 μ l was incubated with 30 μ l of 13.3 mM glutathione for 1 hour and the reaction was stopped by adding 300 μ l of 0.1 mM DTNB. Reagents were dissolved in PBS buffer (75 mM KH_2PO_4 , 68 mM NaCl pH 7.5).

Protein purification

Cell-free medium containing AoSOX1 (220 ml) was concentrated by ultrafiltration (Amicon) using a 10000 cut-off ultrafiltration membrane (Millipore, Espoo, Finland), and was exchanged by dialysis into 20 mM Tris-HCl pH 7 at 4°C. Proteins were separated by anion exchange chromatography (column Hitrap DEAE FF) and eluted in 25 column volumes with a linear 0-300 mM NaCl gradient. Fractions showing a significant activity were then pooled and AoSOX1 was further purified by size-exclusion chromatography (column Superdex 75 HR 10/30, 0.5 ml min^{-1} flow) in 20 mM Tris-HCl pH 7, 150 mM NaCl.

N-glycans of AoSOX1 were removed from the denatured protein with a PNGase F treatment according to the manufacturer's instructions (Calbiochem, Merck KGaA Darmstadt, Germany) and the result was visualized by SDS PAGE. The protein content was determined with the Bio-Rad DC (Bio-Rad, Richmond, CA, USA) protein assay kit. The purity was estimated to be higher than 95% by electrophoresis in a 12% SDS PAGE [27], using a Pre-stained SDS PAGE Standard (GE Healthcare, Uppsala, Sweden) and Coomassie Brilliant

Blue (Pharmacia Biotech, St. Albans, UK). Purified AoSOX1 protein concentration was determined using the extinction coefficient $12160 \text{ M}^{-1} \text{ cm}^{-1}$ at 450 nm of the cofactor that was experimentally determined based on the amount of released FAD after SDS (0.2%) and heat treatment (10-30 minutes at 95°C in the dark).

Spectroscopic methods (circular dichroism, UV/visible and fluorescence spectroscopy)

Absorption spectra were measured in 20 mM Tris-HCl pH 7.0 at 25°C using a Cary Varian 100 Bio UV-Vis spectrophotometer. Circular dichroism (CD) spectra were recorded on a JASCO model J-720 CD spectrometer equipped with a Peltier PTC-38WI thermally controlled cuvette holder. Far-UV CD measurements (190-240 nm) were performed with 2.5 μ M purified enzyme in 0.1 M sodium phosphate buffer pH 7 at 25°C, using a 1 mm cell and bandwidth of 1 nm. Spectra were accumulated four times and the values were corrected for buffer contributions. Thermally induced denaturation was followed as the change in a signal at 222 nm in a 30-90°C temperature range with a 2°C min^{-1} heating rate. Fluorescence spectra were recorded on a Varian Cary Eclipse Fluorescence Spectrophotometer using a 50 μ M AoSOX1 solution in 20 mM Tris pH 7 at 20°C. Protein unfolding was done by the addition of amounts of a 6 M guanidinium hydrochloride solution to get a 0 to 5.5 M denaturant concentration in the sample. An incubation time of 15 minutes was allowed before spectrum recording. Excitation wavelength of 290 nm for the tryptophan residues and 450 nm for the flavin cofactor were used, and the fluorescence was recorded in a 300-450 nm and 450-600 nm wavelength intervals, respectively.

Peptide fingerprinting and molecular weight determination

Bands in the gel (SDS PAGE, 12% acrylamide) were excised and the protein was treated essentially as described by Rosenfeld [28] for peptide mass fingerprinting experiments. The molecular weight of AoSOX1 was determined by MALDI TOF-MS after the enzyme was transferred to distilled water, sinapinic acid was used as matrix in the mass spectrometry. MALDI TOF-MS experiments were carried out in a TOF mass spectrometer Autoflex II (Bruker Daltonik, Bremen, Germany) equipped with laser (160 pulse width, 50 Hz repetition rate) as described [29].

Determination of pH optimum and stability measurements

The enzymatic activity was measured with the HVA-peroxidase coupled assay; to reduce the influence of pH on the substrate and the reagents of the assay, all

solutions and the enzyme were used in a 10-fold higher concentration in assay buffer and diluted to the final concentration in buffer at the proper pH directly in the well of the microtiter plate, e.g. McIlvaine buffer (pH 2.2-8.0), 20 mM Tris/HCl (pH 8.5) and 100 mM CAPSO (pH 8.5-10). AoSOX1 pH stability was determined by incubating the enzyme in McIlvaine buffer (pH 2.2-8.0) and 20 mM Tris/HCl (pH 8.5) and measuring the residual activity after 1 and 24 hours. Temperature stability was assayed incubating aliquots of enzyme solution at 30, 40, 50, 60 and 70°C and measuring the activity in withdrawn samples after 1 and 24 hours with the HVA-peroxidase coupled assay.

Inhibition studies

Sulfhydryl oxidase activity was measured on glutathione (5 mM) by oxygen consumption assay at room temperature in the presence of inhibitors (EDTA, KI, NaCl, MgSO₄, MnSO₄, Na₂SO₄, ZnSO₄, SDS and urea) at 1 and 10 mM concentrations in 50 mM potassium phosphate buffer pH 7.5.

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Authors' contributions

GF carried out the genome mining study, the experimental work and drafted the manuscript. MS and KK participated in the design and conceived of the study and helped to draft the manuscript. JB participated in the design and coordination of the study. All authors read and approved the final manuscript.

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PUBLICATION III

**Production and characterisation of AoSOX2
from *Aspergillus oryzae*, a novel flavin-
dependent sulfhydryl oxidase with good pH
and temperature stability**

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Production and characterisation of AoSOX2 from *Aspergillus oryzae*, a novel flavin-dependent sulfhydryl oxidase with good pH and temperature stability

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Abstract Sulfhydryl oxidases have found application in the improvement of both dairy and baking products due to their ability to oxidise thiol groups in small molecules and cysteine residues in proteins. A genome mining study of the available fungal genomes had previously been performed by our group in order to identify novel sulfhydryl oxidases suitable for industrial applications and a representative enzyme was produced, AoSOX1 from *Aspergillus oryzae* (Faccio et al. BMC Biochem 11:31, 2010). As a result of the study, a second gene coding for a potentially secreted sulfhydryl oxidase, AoSOX2, was identified in the genome of *A. oryzae*. The protein AoSOX2 was heterologously expressed in *Trichoderma reesei* and characterised with regard to both biochemical properties as well as preliminary structural analysis. AoSOX2 showed activity on dithiothreitol and glutathione, and to a lesser extent on D/L-cysteine and beta-mercaptoethanol. AoSOX2 was a homodimeric flavin-dependent protein of approximately 78 kDa (monomer 42412 Da) and its secondary structure presents alpha-helical elements. *A. oryzae* AoSOX2 showed a significant stability to pH and temperature.

Keywords Sulfhydryl oxidase · Secreted · Fungal · Flavoenzyme · Production · Characterization

Introduction

Flavin-dependent sulfhydryl oxidases (E.C. 1.8.3.3, SOXs) catalyse the oxidation of thiol groups to form disulfide

bonds, between either the cysteine residues of a polypeptide chain or small molecules, with the reduction of molecular oxygen to hydrogen peroxide. Sulfhydryl oxidases are characterised by a redox-active di-cysteine motif CXXC (where X is any amino acid) that transfers the electrons from the substrate to the flavin cofactor and eventually to the final acceptor, molecular oxygen.

The enzymes with sulfhydryl oxidase activity studied in detail so far have been isolated from mammalian, plant and bacterial sources (Ostrowski and Kistler 1980; Hooper et al. 1996; Hooper 1999; Hooper et al. 1999; Bardwell 1991; Farrell and Thorpe 2005; Janolino and Swaisgood 1975; Jaje et al. 2007; Levitan et al. 2004; Wu et al. 2003; Lisowsky 2001; Neufeld et al. 1958; Gerber et al. 2001; Alejandro et al. 2007; Ito and Inaba 2008). SOX activity has been detected also in the culture medium of many fungal species (Kusakabe et al. 1983) and secreted sulfhydryl oxidases have been isolated from *Aspergillus* (Vignaud et al. 2002; de la Motte and Wagner 1987) and *Penicillium* cultures (Kusakabe et al. 1982).

The first industrial application of sulfhydryl oxidases was reported in 1977 when Swaisgood discovered the ability of SOXs to oxidise the thiol group exposed in beta-lactoglobulin during the ultrahigh temperature treatment of milk and thus to remove its unpleasant burnt flavour (Swaisgood 1977). Sulfhydryl oxidases showed positive effects also when applied to bread production, as strengthening the dough network was due to the formation of disulfide bonds from the thiol groups of the cysteine residues present (Belton 1999; Shewry et al. 2002; Shewry 2000). The oxidation of thiol groups can be promoted by the addition of additives such as potassium bromate and ascorbic acid, in many industrial bakeries, however sulfhydryl oxidases can constitute a valuable alternative. Unlike bovine sulfhydryl oxidase (Kaufman and Fennema 1987),

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the fungal enzyme isolated from *Aspergillus niger* cultures has been reported to improve the rheological properties of wheat dough (Haarasilta and Vaisanen 1989; Haarasilta et al. 1991) though few studies have tried to clarify the mechanism.

Additionally, the few reported fungal extracellular sulfhydryl oxidases, like the one from *A. niger*, are structurally related to thioredoxin reductases and pyridine nucleotide flavin disulfide oxidoreductases (Coppock and Thorpe 2006; Heckler et al. 2008) and little is known about their structure and mechanism of action.

Aiming at the production of a novel sulfhydryl oxidase with potential industrial application, a genome mining study of the publicly available fungal genomes was recently carried out by our group and led to identification of numerous potentially secreted sulfhydryl oxidases. This same study that successfully led to the production and biochemical characterisation of the secreted sulfhydryl oxidase AoSOX1 from *Aspergillus oryzae* (Faccio et al. 2010), identified in the genome of the fungus *A. oryzae* a second predicted protein, AoSOX2, with sequence features typical of sulfhydryl oxidases. In this study, protein AoSOX2 was heterologously produced in the filamentous fungus *Trichoderma reesei* to be biochemically and briefly structurally characterised.

Materials and methods

Protein purification was carried out with an ÄKTA purifier system and chromatography columns from Amersham Biosciences, Uppsala, Sweden. Spectroscopic measurements were done with a Cary 100/300 spectrophotometer (Varian Inc., The Netherlands) and a JASCO model J-720 CD spectrometer equipped with a Peltier PTC-38WI thermally controlled cuvette holder. Fluorescence was measured at 20 °C with a Cary Eclipse Fluorescence Spectrophotometer (Varian Inc., The Netherlands).

Microorganisms

The genomic DNA of *A. oryzae* VTT-D-88348 strain was used as a donor of the target gene (GenBank accession no. Q2U4P3). For the production of AoSOX2 a cellobiohydrolase I-disruptant strain VTT-D-00775 of *T. reesei* was used as a host (Kontkanen et al. 2009). All the strains used in the study are available at the VTT Culture Collection (Technical Research Centre of Finland, Finland).

Protein production and purification

AoSOX2 gene was amplified from the genomic DNA of *A. oryzae* by polymerase chain reaction (PCR) with Dyna-

zyme EXT polymerase (Finnzymes, Espoo, Finland) and the following primers: GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TCA TGA TGT TCA CCT GCA ACT GGA CT primer forward and GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAG TGG TGG TGG TGG TGG TGA CGA CGG CTG AGT CTC TTG TA primer reverse. AoSOX2 gene was cloned with the Gateway technology (Invitrogen) between the cellobiohydrolase I (*cbhI*) promoter and terminator of the expression vector pMS186 that carries and hygromycin resistance cassette. The construct obtained was transformed into an industrial *T. reesei* strain VTT-D-00775 (Kontkanen et al. 2009; Penttila et al. 1987). Transformants were streaked twice consequently on antibiotic resistance and the positive ones further controlled with a PCR-based method. Transformants were selected to uninucleate clones and grown in shake flasks (volume 50 ml) for 9 days at 28 °C in *Trichoderma* minimal medium supplemented with 4% lactose, 2% spent grains and 100 mM piperazine-N,N'-Bis(3-propanesulfonic acid) pH 5.5. The transformant giving the highest activity in the medium with L-reduced glutathione as substrate was selected. Shake flask cultivations for routine protein production were carried out in 250 ml of the same medium at pH 5.5 in a 2 l flasks for 5 days at 28 °C.

Proteins from AoSOX2-containing culture medium were concentrated and transferred to 10 mM potassium phosphate buffer pH 7.5 by ultrafiltration using a 10,000-cutoff membrane (Amicon stirred cell 8400, Millipore, Espoo, Finland) at 4 °C. Proteins were separated by ion exchange chromatography on a HiPrep Q FF column (volume 20 ml, GEHealthcare) in a linear 0–150 mM NaCl gradient. Fractions showing activity on glutathione were pooled, concentrated, and AoSOX2 was further purified by gel filtration on a Superdex 200 HR 10/30 column in 10 mM potassium phosphate pH 7.5, 150 mM NaCl. Protein concentration was generally measured with the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA, USA). Purified protein was quantified using the extinction coefficient at 450 nm of the FAD cofactor ($\epsilon_{\text{FAD}}=11,300 \text{ M}^{-1} \text{ cm}^{-1}$; Macheroux 1999).

Activity assays

Sulfhydryl oxidase activity was measured following the oxygen consumption after addition of the enzyme to a substrate solution placed in a 1.8 ml full vial at 20 °C (OXY-10 mini-multi-channel oxygen metre, PreSens Precision Sensing GmbH, Regensburg, Germany). Sulfhydryl oxidase activity was also assayed with a peroxidase-homovanillic acid coupled assay modified from Raje et al. (2002) as described by Faccio et al. (2010). Fractions obtained from the purification steps were assayed for activity on glutathione with Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid)) as reported (Faccio et al. 2010).

Protein identification and molecular weight determination

The AoSOX2 protein was identified by peptide mass fingerprinting with Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI TOF-MS) after in-gel trypsin digestion of the corresponding band in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE; Rosenfeld et al. 1992). Molecular weight was determined by MALDI TOF-MS Autoflex II (Bruker Daltonik, Bremen, Germany) equipped with laser (160 pulse width, 50 Hz repetition rate) and by size-exclusion chromatography with a Superdex 200 HR 10/30 column (10 mM potassium phosphate buffer pH 7.5, 150 mM NaCl) calibrated with high and low molecular weight standards from the gel filtration calibration kit by Pharmacia (Uppsala, Sweden).

Spectroscopic analysis

Absorption spectra of the purified protein were recorded in 10 mM potassium phosphate pH 7.5 at 20 °C. Reactivity with sodium sulphite was tested with an initial protein concentration of 28.7 μM (FAD content, extinction coefficient at 450 nm $11,300 \text{ M}^{-1} \text{ cm}^{-1}$) and spectra were recorded 15 min after the addition of a small volume (1–15 μl) of a 10 mM or 1 M sodium sulphite in 50 mM potassium phosphate buffer, pH 7.5 at 20°C. Circular dichroism spectra were measured in 10 mM sodium phosphate (pH 7.0) at 25 °C with a 1 mm optical path using a JASCO model J-720 spectrometer equipped with a Peltier PTC-38WI thermally controlled cuvette holder. Protein concentration was 1.7 μM in the circular dichroism analyses.

Kinetic measurements

Activity was measured with the peroxidase-homovanillic acid coupled assay (Faccio et al. 2010) in presence of different substrate concentrations and the kinetic parameters K_m and V_{\max} were calculated by linear regression with the Hanes plot (Hanes 1932) using the graphing software Origin 7.5 SRO (OriginLab Corporation, Northampton, MA, USA). All substrates were dissolved in 50 mM potassium phosphate buffer, 0.3 mM EDTA pH 7.5. The inhibition by different compounds at 1 mM concentration (EDTA, KI, MgSO_4 , MnSO_4 , Na_2SO_4 , SDS, NaCl, urea, and ZnSO_4) was assayed with 5 mM glutathione in 50 mM potassium phosphate buffer pH 7.5 measuring activities with and without inhibitor by the oxygen consumption method with a OXY-10 mini-multichannel oxygen metre (PreSens Precision Sensing GmbH, Germany). Activities were calculated from the linear part of the oxygen consumption curve ($\text{nmol l}^{-1} \text{ s}^{-1}$).

pH and temperature behaviour

The optimum pH was determined assaying the sulfhydryl oxidase activity with glutathione in McIlvaine buffer in a pH range 2.2–8.0, 20 mM Tris/HCl pH 8.5 and 100 mM CAPSO range pH 8.5–10. AoSOX2 stability towards pH was measured as residual activity after 1- and 24-h incubation of the enzyme in McIlvaine citrate/phosphate buffer in a pH range 2.2–8.0 and 20 mM Tris/HCl pH 8.5. Thermal stability was assayed incubating aliquots of enzyme at 30, 40, 50, 60, and 70 °C and measuring the activity after 0.5 and 1 and after 24 h.

Unfolding studies

Guanidinium-induced unfolding was monitored by following the fluorescence of the tryptophan residues ($\lambda_{\text{ex}} = 290 \text{ nm}$) and of the flavin cofactor ($\lambda_{\text{ex}} = 450 \text{ nm}$). The protein concentration was 9.8 μM in 10 mM potassium phosphate buffer pH 7.5 and the spectrum was recorded 15 min after the addition of the denaturant. Residual activity of AoSOX2 incubated in analogous conditions was measured with glutathione and using the HVA-peroxidase coupled assay. Circular dichroism was used to monitor the loss of secondary structure as signal change at 222 nm in a 30–90 °C temperature interval, with a $2 \text{ }^\circ\text{C min}^{-1}$ heating rate.

Results

A genome mining study for secreted fungal sulfhydryl oxidases was previously conducted and yet lead to the identification of the secreted enzyme AoSOX1 from *A. oryzae* (Faccio et al. 2010). Within the same study, a second candidate protein from *A. oryzae*, AoSOX2, was identified and selected for production in the filamentous fungus *T. reesei*. Protein AoSOX2 was predicted to be 389 amino acid long with an 18 amino acids long signal peptide starting with two methionine residues. AoSOX2 sequence presented the di-cysteine motif $\text{C}_{158}\text{XXC}_{161}$ characteristic of not only sulfhydryl oxidases of the Erv family and *Escherichia coli* thioredoxin reductases but also many oxidoreductases with a thioredoxin fold. Sequence analysis of AoSOX2 suggested a conserved thioredoxin reductase fold. AoSOX2 sequence was 98% identical to a predicted thioredoxin reductases from *Aspergillus flavus* (ABY86217), 67% identical to an uncharacterised protein from *Penicillium chrysogenum* (Pc12g03690) and 65% identical to a putative cytoplasmic thioredoxin reductase from *Neosartorya fischeri* (XP_001258605). The highest degrees of similarity to characterised proteins were to the sulfhydryl oxidase from *A. niger* (47.3% identity; de la Motte and Wagner 1987) and

AoSOX1 from *A. oryzae* (50.6% identity; Faccio et al. 2010).

Cloning and protein expression

The gene *Q2U4P3* coding for protein AoSOX2 was amplified by PCR from the genomic DNA of *A. oryzae*, cloned and inserted between the *cbh1* inducible promoter and terminator in a *T. reesei* expression vector. The resulting expression plasmid pGF009 was then transformed into *T. reesei*. Cloning was designed to add a C-terminal six-histidine tag to AoSOX2. Transformants positive to both antibiotic selection and PCR screening were selected for small-scale shake flask cultivations with inducing medium and the strain giving the highest activity on glutathione in the medium was selected for routine protein production. A maximum production level of AoSOX2 of 180 mg/l in shake flask culture was achieved after 5 days cultivation.

Enzyme purification and molecular characterisation

AoSOX2 enzyme was purified in a two-step process including first anion exchange chromatography, where AoSOX2 eluted at a 166 mM salt concentration, and secondly size-exclusion chromatography, where AoSOX2 eluted as a single peak after 15.3 ml. The loss of activity detected during the purification that had a yield of 24% (Table 1), was possibly due to an inhibitory effect of the salts used on the enzymatic activity. The solution containing purified AoSOX2 had a bright yellow colour and migrated as a major protein band around 45,000 Da in SDS PAGE (Fig. 1a). Purification by affinity chromatography and detection by Western blot analysis with anti-histidine tag-specific antibodies were unsuccessful, possibly due to unpredictable cleavage of the tag by *T. reesei* proteases, e.g., considering the presence of potential recognition sites for Kex2-like proteases immediately before the tag (K₃₈₄R₃₈₅ and R₃₈₈R₃₈₉).

The purified AoSOX2 and the corresponding band in SDS PAGE analysis of AoSOX2-containing culture medium were identified as the correct gene product with a maximum of 23.1% of sequence coverage, by peptide mass fingerprinting

after in-gel trypsin digestion. Seven peptides positively matched the predicted ones from AoSOX2 (R₅₄-N₆₄, K₅₅-N₆₄, R₇₀-F₈₆, K₁₁₂-T₁₂₉ and K₁₃₁-G₁₅₃ and K₂₁₄-K₂₁₉), among them was the tryptic peptide containing the di-cysteine motif CXXC (K₁₅₃-GIFWCPWCDGFEHR-N₁₆₇).

Molecular weight of the purified enzyme (42,412 Da) was determined by MALDI TOF-MS and it corresponded to the mature form of AoSOX2 (predicted mass 41512, lacking the 23 amino acids long signal peptide) binding a FAD molecule. The molecular weight in the native form was determined by size-exclusion chromatography concomitantly with the second purification step. The elution of partially purified AoSOX2 in size-exclusion chromatography coincided with a single peak having activity on glutathione and to an increase in absorbance both at 280 nm and 440 nm. AoSOX2 elution volume corresponded to an approximate molecular weight of 78 kDa suggesting a dimeric form of the enzyme.

The visible spectrum of AoSOX2 had three peaks at 272, 354 and 445 nm with a shoulder visible at 470 nm which is in accordance with the enzyme being a flavoprotein (Fig. 1a). Unlike in other flavoproteins, the flavin cofactor of oxidases, and in particular of proteins with a positive charge near the flavin N(1) locus (Ghanem and Gadda 2006), reacts with sodium sulphite to form a covalent reversible bleached complex (Massey et al. 1969). Incubation of AoSOX2 in presence of sodium sulphite was reflected in a decrease of absorbance of the visible peak of flavin (Fig. 1b). The maximum change in absorbance was registered at 445 nm and was reached at a sulphite concentration equal or higher than 2.5 mM (Fig. 1b inset). Considering curve 8 (Fig. 1b) as a full conversion of AoSOX2 to the complex with sulphite and that sulphite was in excess, k_d values of 0.41×10^{-3} and 0.39×10^{-3} were calculated for curves 2 and 3.

Substrate specificity and stability measurements

The substrate specificity of AoSOX2 was tested on five substrates using the peroxidase-homovanillic acid coupled assay and in all cases a Michaelis–Menten behaviour was found. The smallest Michaelis constant was detected with L-cysteine and second smallest with glutathione while the

Table 1 Purification of the enzyme AoSOX2 from *A. oryzae* by anion-exchange and size-exclusion chromatography

	Volume (ml)	Protein content (mg/ml)	Activity (10 ⁶ nkat/ml)	Specific activity (10 ⁶ nkat/ml)	Purification factor	Yield (%)
Culture medium	240	2.20	1.16	0.53	1	100
Hiprep Q FF	370	0.05	0.64	11.62	21.9	84
Superdex 200 HR 10/30	9.5	1.10	7.08	6.41	12.1	24

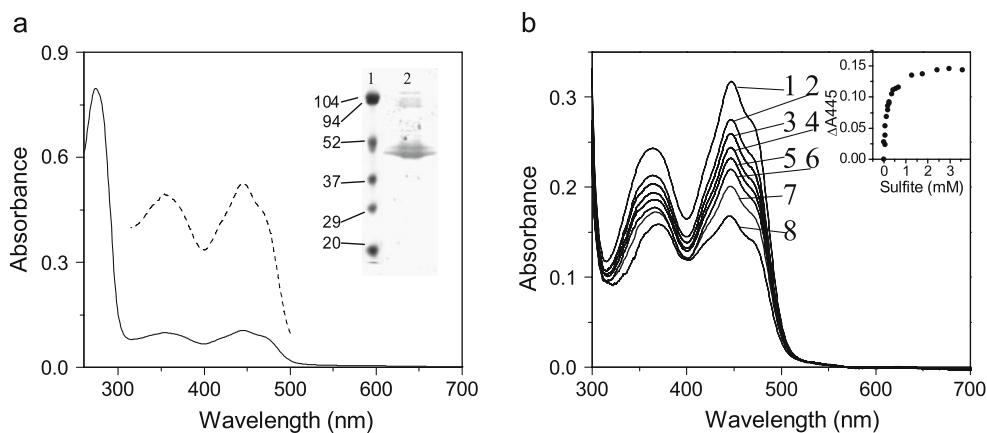


Fig. 1 UV-visible spectrum and binding of sulphite to the purified enzyme AoSOX2 from *A. oryzae*. The flavin cofactor bound to AoSOX2 is indicated in the absorbance spectrum by two peaks at 354 and 445 nm, with a shoulder at 470 nm (values 5 \times , dashed line). Inset SDS PAGE analysis of purified AoSOX2 (2, 4 μ g) and molecular weight standards (1), expressed in kDa (a). Purified enzyme was

titrated with sulphite in 50 mM potassium phosphate, pH 7.5 at 20°C. Curves 1–8 correspond to a sulphite concentration of 0, 0.03, 0.06, 0.11, 0.20, 0.23, 0.4, 3.56 mM, respectively. Inset absorbance variation registered at 445 nm with increasing sulphite concentration (b)

highest in presence of the D-isomer of cysteine evidencing a certain degree of stereoselectivity. The highest turnover number (k_{cat}) and enzyme efficiency ($k_{\text{cat}}/K_{\text{m}}$) was instead found for the substrate dithiothreitol and in a lesser extent for glutathione (Table 2). Activity of AoSOX2 was not significantly affected by the inhibitors tested at a concentration of 1 mM except for zinc sulphate that caused a loss of 95% of the initial activity. Two denaturants were tested, urea and SDS, and only with the latter one the initial activity was reduced to its 57%. The presence of magnesium and sodium sulphate caused a reduction of AoSOX2 activity to approximately 55% and 66% of the initial value.

AoSOX2 had a pH optimum of 8 (Fig. 2a) and showed a good pH stability retaining more than 80% of the initial activity in a pH range 5–8.5 within 24 h (Fig. 2b). The enzyme showed also a significant stability at high temperatures, as indicated by the melting temperature around 75 °C measured by circular dichroism at 222 nm (Fig. 3a). Furthermore, 68% of activity was retained after incubation at 60 °C for 1 h and 42% residual activity was detected after 24 h at 50 °C (Fig. 3b).

Structural characterisation

Structural stability of AoSOX2 towards chemical and thermal unfolding was investigated by fluorescence and by circular dichroism, respectively. The secondary structure was investigated by circular dichroism. AoSOX2 presented alpha-helical elements as evidenced by the two negative peaks at 210 and 220 nm in the spectrum (Fig. 4). Mature AoSOX2 protein sequence is characterised by the presence of 15 phenylalanine, 11 tyrosine and 4 tryptophan residues. Equilibrium unfolding of AoSOX2 was monitored as a variation of intrinsic fluorescence due to the presence of aromatic residues, e.g. tryptophan, after addition of the denaturant guanidinium hydrochloride. At the same time, the increase of fluorescence upon excitation at 450 nm, due to the exposure of the flavin cofactor, was recorded. Both the intrinsic protein fluorescence and the flavin fluorescence increased significantly at a denaturant concentration higher than 4 M (Fig. 5). The exposure of the tryptophan residues from the hydrophobic protein environment to the aqueous solution was reflected in a shift of the peak of

Table 2 Kinetic parameters of the purified enzyme AoSOX2 from *A. oryzae* with five substrates

Substrate	K_{m}^{a} (mM)	$V_{\text{max}}^{\text{a}}$ (10^6 nkat/ml)	$V_{\text{max}}/K_{\text{m}}$ (s^{-1})	k_{cat} (10^4 s^{-1})	$k_{\text{cat}}/K_{\text{m}}$ (10^6 M^{-1} s^{-1})
Glutathione	3.7 \pm 0.1	1.36 \pm 0.01	370	9.75	26.50
L-Cysteine	0.9 \pm 0.1	0.09 \pm 0.00	106	0.68	7.60
D-Cysteine	13.1 \pm 3.0	1.53 \pm 0.16	116	7.86	5.98
Dithiothreitol (thiols)	4.9 \pm 0.4	6.55 \pm 0.02	1,342	33.78	69.23
Beta-mercaptoethanol	7.9 \pm 0.0	1.08 \pm 0.00	136	5.54	7.00

^a Values are reported as average \pm standard deviation ($N=3$)

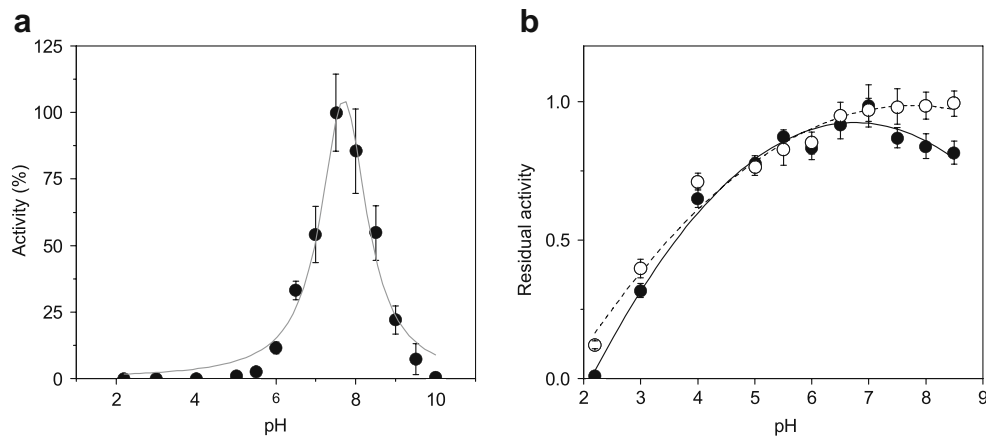


Fig. 2 pH optimum and stability to pH of the purified enzyme AoSOX2 from *A. oryzae*. The catalytic activity of AoSOX2 was tested in different pH conditions to determine the pH optimum (a). The pH stability of AoSOX2 was measured as residual after 1 h (empty circles) and after 24 h (filled circles) incubation of the enzyme

solution in different pH conditions (b). In both cases, the activity was measured with glutathione as a substrate and the peroxidase-homovanillic acid coupled assay (values as average±standard deviation, $N=3$)

maximum emission from 337 to 358 nm (Fig. 5). Two of the four tryptophan residues (W_{157} and W_{160} , not W_{218} and W_{313}) of the mature form of AoSOX2, i.e. lacking the residue W_7 present in the signal sequence, are in close proximity to the redox-active cysteine pair $C_{158}XXC_{161}$: this suggested that they are possibly near to the FAD binding site and explains the similar behaviour observed for intrinsic and flavin fluorescence (Fig. 5). Enzymatic activity was assayed in analogous conditions. The activity was gradually lost with the increase in denaturant concentration. However, the enzyme retained more than 50% of the initial activity at a denaturant concentration lower than 3 M and was completely inactivated at a 6 M guanidinium hydrochloride concentration.

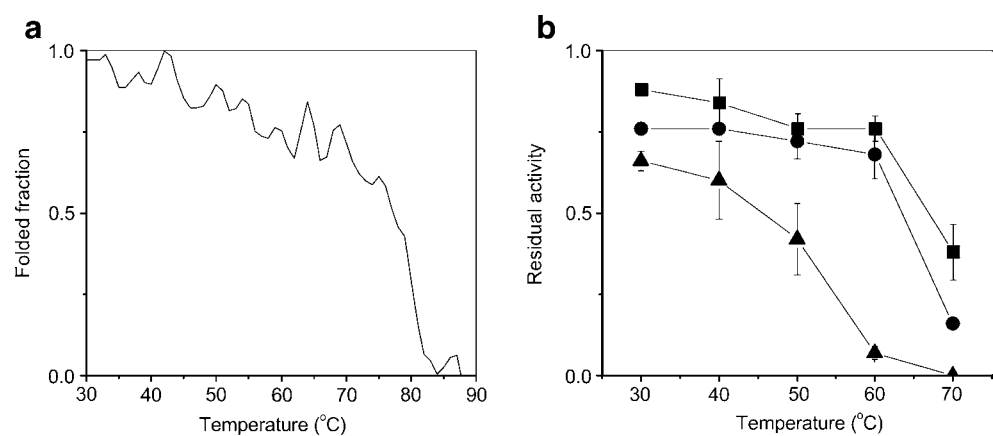
Discussion

The use of enzymes for the improvement of food processing and properties is currently common since

enzymes have many advantages such as specificity of action, milder treatment conditions, higher product quality, lower manufacturing costs, lower energy consumption and reduced waste management. The industry demands for its application enzymes that are robust and efficient. As these characteristics are generally ascribed to extracellular enzymes, we produced a secreted sulfhydryl oxidase to be evaluated as a potential tool for food processing.

The work presented here is the continuation of a previous genome mining study concerning fungal secreted sulfhydryl oxidases that lead yet to the production and biochemical characterisation of the sulfhydryl oxidase AoSOX1 from *A. oryzae*, recently characterised by our group (Faccio et al. 2010). The sulfhydryl oxidase characterised in this work, AoSOX2, showed to be more robust with regard to pH and temperature behaviour than AoSOX1, even though AoSOX1 and AoSOX2 were derived from the same mesophilic fungus *A. oryzae* and in particular, from a strain that was isolated from a food

Fig. 3 Stability to temperature of the purified enzyme AoSOX2 from *A. oryzae*. Denaturation of AoSOX2 by temperature was monitored as loss of secondary structure at 222 nm by circular dichroism (a). The stability at different temperatures was measured as residual activity after 30 min (square), 60 min (circle) and after 24 h (triangle) using glutathione as substrate and the HVA-peroxidase activity assay (b)



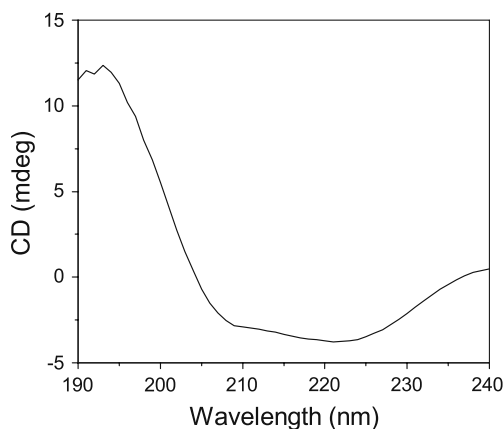


Fig. 4 Circular dichroism spectrum of purified AoSOX2 from *A. oryzae*. The circular dichroism spectrum of AoSOX2 presents two negative peaks at 210 and 220 nm indicating the presence of alpha helical elements in its secondary structure

fermentation plant and had an optimal growth temperature of 25 °C.

The enzyme AoSOX2 was more resistant to chemical denaturation than the previously characterised AoSOX1 from *A. oryzae* (Faccio et al. 2010) and QSOX from milk

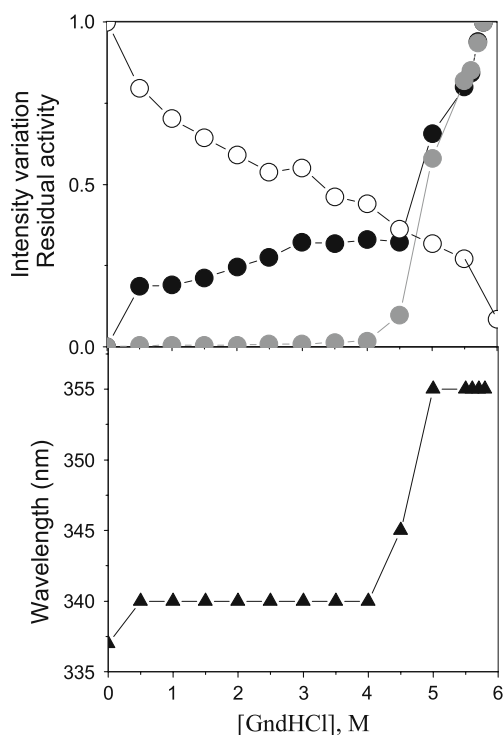


Fig. 5 Unfolding study of the purified enzyme AoSOX2 from *A. oryzae*. Protein unfolding was reported as variation of intensity of the intrinsic fluorescence (filled circle, upper panel) and of the flavin cofactor fluorescence (grey circle, upper panel). The residual activity of AoSOX2 (empty circle, upper panel) and the wavelength of the maximum tryptophan fluorescence (triangle, lower panel) are reported for the different denaturant concentration tested

(Janolino et al. 1980). AoSOX2 started to unfold at a concentration of guanidinium chloride (4.5 M) higher than the one reported for AoSOX1 (2 M) and QSOX (2 M; Faccio et al. 2010; Janolino et al. 1980). An early increase in the tryptophan fluorescence of AoSOX2 was observed at a denaturant concentration of 0.5 M, probably due to the exposure of the distal residue W₃₁₃ thus suggesting the loose interaction of the C-terminal region with the surface of the molecule.

In addition, AoSOX2 showed a higher temperature stability than AoSOX1 since retained activity after 1 h of incubation at 60 °C. Moreover, the melting temperature of AoSOX2 around 75 °C was significantly higher than the one reported for AoSOX1 from *A. oryzae* (55 °C; Faccio et al. 2010) and the homodimeric enzyme Erv2 from yeast (59.2 °C; Vala et al. 2005).

Gel filtration done in this work indicated that AoSOX2 is a dimeric enzyme. This is not unusual among sulfhydryl oxidases and similar results have been obtained for the secreted SOX from *A. niger* (de la Motte and Wagner 1987) and the enzyme AoSOX1 from *A. oryzae* (data not shown). A dimeric form has also been reported for the sulfhydryl oxidase from the yeast *Saccharomyces cerevisiae* (Lee 2000) and the plant *Arabidopsis thaliana* (Levitani et al. 2004).

The secondary structure preliminary characterisation by circular dichroism suggested alpha helical elements, thus supporting the predicted thioredoxin fold. Considering that the thioredoxin fold allows the insertion between secondary structure elements (Martin 1995), the higher molecular weight of AoSOX2 compared to thioredoxins, typically 12–15 kDa, may not exclude this fold. The three-dimensional structure of any sulfhydryl oxidases related to thioredoxin reductases has yet to be resolved.

The physiological role of secreted sulfhydryl oxidases has not been clarified yet although few enzymes have been reported and, from *A. oryzae* in particular, two enzymes AoSOX1 (Faccio et al. 2010) and AoSOX2 have been characterised. The activity of AoSOX1 and AoSOX2 on small compounds such as glutathione and dithiothreitol, and their extracellular localisation possibly excludes their participation to the maturation of newly synthesised proteins. However, enzymes with sulfhydryl oxidase activity isolated from various sources have been suggested of being involved in the formation of extracellular matrix (Hooper 1999; Tury et al. 2006), in the production of hydrogen peroxide with antimicrobial purposes (Ostrowski and Kistler 1980) and in the maturation of bioactive non-ribosomal peptides (Wang et al. 2009).

In conclusion, a novel sulfhydryl oxidase enzyme from *A. oryzae* with interesting biochemical properties has been cloned, produced and characterised in this work. Future

studies will be conducted to assess the activity of AoSOX2 on protein-associated sulfhydryl groups and to evaluate its crosslinking activity aiming at industrial food and non-food applications.

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PUBLICATION IV

**Sulfhydryl oxidase enhances the effects of
ascorbic acid in wheat dough**

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Sulfhydryl oxidase enhances the effects of ascorbic acid in wheat dough

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Keywords

sulfhydryl oxidase, wheat, *Aspergillus oryzae*, ascorbic acid.

Abstract

Various enzyme families such as sulfhydryl oxidase have been successfully applied to bread production although their mechanism of action has not been fully described yet. In this study we investigated the effects of the recently characterized fungal sulfhydryl oxidase AoSOX1 in fresh and frozen dough alone and in combination with ascorbic acid. The addition of AoSOX1 to an additive-free dough resulted in a weaker and more extensible dough while opposite effects were detected in the presence of ascorbic acid. The hardening of the doughs registered upon the combined use of AoSOX1 and ascorbic acid was dependent on the amount of enzyme used and not on the amount of ascorbic acid. The ability of the sulfhydryl oxidase to enhance the effects of the ascorbic acid system suggests their combined use as a valuable tool to stabilize the structure of fresh and frozen dough.

Introduction

Frozen doughs are increasingly used by consumers and retailers. However, the freezing process significantly reduces the quality of the dough, especially in cases of prolonged frozen storage (Ribotta et al., 2001). The reduction in dough performance after freezing is reported to be caused by (a) yeast damage, with the subsequent release of reducing compounds such as glutathione (Ribotta et al., 2003), (b) formation and growth of ice crystals and (c) enzymatic and chemical reactions in the unfrozen dough phase during frozen storage (Berglund et al., 1991; Kulp et al., 1995). The growth of ice crystals may result in a less continuous and more ruptured gluten matrix (Ribotta et al., 2001; Berglund et al., 1991), observed as depolymerization of high molecular weight glutenins during frozen storage (Ribotta et al., 2001). Modification of the freezing conditions (Yi and Kerr, 2009), an increase of yeast cryoresistance (Pepe et al., 2005) or on the use of additives and improvers (Selomulyo and Zhou, 2007) have been attempted to overcome the negative effects caused by the frozen storage.

In bread production, improvers of both chemical and enzymatic nature are commonly used to enhance the technological properties of the dough and sensory quality of bread, and to guarantee an industrial production with constant quality irrespective of variations of the properties of the raw materials. The addition of ascorbic acid, also known as vitamin C, has a well-studied strengthening effect on dough that leads to a higher rising of the dough (Meredith, 1965; Sarwin et al., 1993; Every, 1999b; Jorgensen, 1939; Grosch and Wieser, 1999; Koehler, 2003). A generally accepted mechanism of the action of ascorbic acid in dough has been proposed (Table 1) (Sarwin et al., 1993; Grosch and Wieser, 1999; Hahn and Grosch, 1998).

Ascorbic acid is firstly oxidized, either by the endogenous ascorbic acid oxidase (E.C. 1.10.3.3) (Every, 1999a) or atmospheric oxygen (Meredith, 1965), to its major stable oxidized product dehydroascorbic acid (eq. 1 Table 1), which is reported to be the actual dough improver (Every et

al., 1996). Mixing conditions and oxygen incorporation into the dough are known to affect this reaction. In the presence of reduced glutathione, the dehydroascorbic acid is reduced to ascorbic acid by the endogenous glutathione dehydrogenase (E.C. 1.8.5.1, eq. 2 Table 1) (Kaid et al., 1997; Walther and Grosch, 1987; Boeck and Grosch, 1976) or spontaneously by the glutathione present in the flour (Winkler, 1992). This reaction subtracts reduced glutathione to possible weakening thiol/disulfide-exchange reactions causing the slow depolymerization of gluten proteins by opening the disulfide bonds of the protein network (eq. 3 Table 1) (Ribotta et al., 2003). Additionally, the oxidized glutathione produced is able to block free protein-associated thiol groups (eq. 4 Table 1), e.g. glutenins, preventing them to take part to thiol-disulfide exchange reactions (Grosch and Wieser, 1999; Hahn and Grosch, 1998; Boeck and Grosch, 1976). When combined with potassium bromate, the ability of ascorbic acid to inhibit freezing damage is more pronounced (El-Hady et al., 1999). Since potassium bromate is prohibited as a flour improver by numerous countries, the use of oxidative enzymes together with ascorbic acid could provide an alternative to enhance the strength of the gluten matrix. Sulfhydryl oxidases have been tested for baking applications. Sulfhydryl oxidase catalyses the oxidation of thiol groups from small compounds and from cysteine residues within a polypeptide chain. Concomitantly, a molecule of oxygen is reduced to hydrogen peroxide (eq.5 Table 1).

Table 1 - Reaction sequence proposed by Grosch in 1999 to explain the improver effect of ascorbic acid in dough and reaction catalyzed by sulfhydryl oxidase (EC. 1.8.3.3).

Equation	Reaction ^a	Catalyst
<i>Ascorbic acid mechanism of action</i>		
1	$AA + \frac{1}{2}O_2 \rightarrow dhAA + H_2O$	Ascorbate oxidase
2	$dhAA + 2GSH \rightarrow AA + GSSG$	Glutathione dehydrogenase
3	$GSSG + P-SH \rightarrow GSSP + GSH$	not enzymatic
4	$GSH + PSSP \rightarrow GSSP + PSH$	not enzymatic
<i>Sulfhydryl oxidase</i>		
5	$GSH + O_2 \rightarrow GSSG + H_2O_2$	AoSOX1 from <i>Aspergillus oryzae</i>

^a AA L-ascorbic acid, dhAA L-dehydroascorbic acid, GSH reduced glutathione, GSSG oxidized glutathione, P-SH protein-associated thiol group, P-SS-P inter-protein disulfide bond, GSSP protein-bound glutathione.

While no effect was detected with the sulfhydryl oxidase isolated from milk (Kaufman and Fennema, 1987), positive results have been obtained with the fungal sulfhydryl oxidase from *Aspergillus niger* when used in combination with glucose oxidase or hemicellulase (Soupe, 2000; Vaisanen et al., 1996). However, only few studies have tried to elucidate the mechanism of sulfhydryl oxidase in dough (Kaufman and Fennema, 1987).

The sulfhydryl oxidase AoSOX1 was obtained from *Aspergillus oryzae*, recently characterized and heterologously expressed in the filamentous fungus *Trichoderma reesei* (Faccio et al., 2010).

AoSOX1 is a flavin-dependent enzyme with a molecular mass of 42 kDa and active on glutathione, dithiotheithrol and in a lesser extent D- and L-cysteine (Faccio et al., 2010).

The aim of this work was to investigate the impact of a fungal sulfhydryl oxidase, AoSOX1, on wheat dough and to elucidate the possible interaction of the reaction catalyzed by sulfhydryl oxidase with ascorbic acid.

1. Experimental

1.1. Materials

The wheat flours used were commercial white wheat flours: 'Kruunu' (Raisio plc, Finland) containing approx. 42 ppm ascorbic acid and 'Wienerjauho' (Raisio plc, Finland) without ascorbic acid.

Compressed yeast was Suomen Hiiva Oy, Finland. L-Ascorbic acid was purchased from Merck and L-reduced glutathione from Sigma-Aldrich. The sulfhydryl oxidase AoSOX1 from *Aspergillus oryzae* was heterologously produced in the filamentous fungus *Trichoderma reesei* and purified as described in (Faccio et al., 2010).

1.2. Dough fermentation studies

The two flours used in the study were analyzed according to standard methods as described in Table 1. Doughs were prepared by mixing the ingredients (flour, water, 2.8% fresh yeast and 1.5% salt) in a 2-step kneading process with a spiral kneader (Diosna SP 12 F, Dierks and Sohne, GmbH, Osnabruck, Germany) for 2 min at a low speed (100 rpm) and then for 3 min at high speed (200 rpm). Mixing was performed at room temperature combining flour, water and the yeast whereas the addition of salt was delayed of 30 seconds. The dough was divided into three portions of 300 g, one portion was assayed immediately while the other two were placed in plastic bags and frozen in a blast freezer (Porkka Finland Oy, Finland) for two days monitoring the internal temperature with a Digital Precision Thermometer GTH 175 / Pt (Greisinger electronics GmbH, Regenstauf, Germany) and then transferred to a conventional freezer at -20°C for 1 to 6 weeks. Frozen doughs (300 g) were placed at 0°C for approx. 15 hours until thawed and incubated at 30°C for 30 minutes (final internal temperature 20±1°C) before being assayed. Dough development was studied with a Rheofermentometer F3 (Chopin group, Tripette and Renaud, France) at 42°C for 2-3 hours with a

weight load of 2 kg. Results were analyzed for statistical difference by one-way analysis of variance (ANOVA) repeated measures (software Origin, OriginLAB, Northampton, MA, USA).

1.3. Rheological studies

Dough was prepared mixing 100 g of flour to the corresponding amount of water, or aqueous solution containing the enzyme preparation, in a Kenwood Chef mixer with a K-beater attached (KM300, Kenwood Limited, New Lane, Havant, Hampshire, UK) for two minutes at speed two at room temperature (24°C). The flour to be enriched with ascorbic acid was pre-mixed for 1 hour at 24°C with ascorbic acid. Water amount was determined according to the water absorption of the farinograph (Brabender, Duisburg, Germany) at a 500 BU consistency (Supplementary Data 1). The dough was molded and kept pressed during the relaxation time (20-50 minutes) at 24 °C. Three doughs were prepared separately and at least five dough strips were assayed per condition tested by using the Kieffer dough and gluten extensibility rig fitted onto a TA.XT2 texture analyzer (STable Micro Systems, Ltd., U.K.) equipped with a 5 kg load cell as described by Kieffer (Kieffer et al., 1998).

1.4. Enzyme activity measurements

Dehydroascorbic acid was prepared in ultrapure water essentially as described previously (Kaid et al., 1997). Ascorbic acid formation from dehydroascorbic acid was monitored as absorbance at 265 nm with a Varioskan spectral scanning multimode reader (Thermo Electron Co., Vantaa, Finland). Different reaction mixtures were prepared with whether 2 mM dehydroascorbic acid or 0.2 mM ascorbic acid, 2 mM glutathione and amounts of AoSOX1 varying from 0 to 20000 U. Activity measurements were carried out in a total volume of 0.3 ml in 96-well microtiter plates using sodium phosphate buffer at pH 6 to minimize the spontaneous reduction of dehydroascorbic acid by

glutathione. Reactions were started by the addition of glutathione. Reaction rates were calculated by linear regression over 1 min interval using the software provided by the manufacturer.

2. Results

2.1. Effect of sulfhydryl oxidase on frozen dough stability

The effect of sulfhydryl oxidase was evaluated with respect to the development of fresh and frozen dough. The dough was prepared in a zero-time process. Part of the dough was freshly assayed and part frozen in a blast freezer for 2 days.

Freezing was first carried out with a refrigeration rate of $-29.63^{\circ}\text{C}/\text{hour}$ until the zero temperature was reached thereafter a fifteen folds slower rate of $-2^{\circ}\text{C}/\text{hour}$ was used (Supplementary data 2).

After the blast freezer, the dough was transferred to a conventional freezer.

The dough fermentation was monitored with a Rheofermentometer F3. Before starting the fermentation test, frozen loaves were thawed at 0°C overnight and subsequent incubation at 30°C for 30 minutes to reach dough inner temperature of $20 \pm 0.5^{\circ}\text{C}$.

The development curves of fresh dough prepared with the commercial flour preparation containing ascorbic acid were not affected by the addition of sulfhydryl oxidase but the enzyme decreased the maximum dough height, H_m , and time of maximum development T_1 and T_2 after 6 weeks of frozen storage (Supplementary data 3 and Figure 1 A, B and E). Gas retention properties and dough tolerance were not affected (Figure 1 C, D, F) although the onset of the latter was anticipated due to the decrease in T_1 and T_2 values.

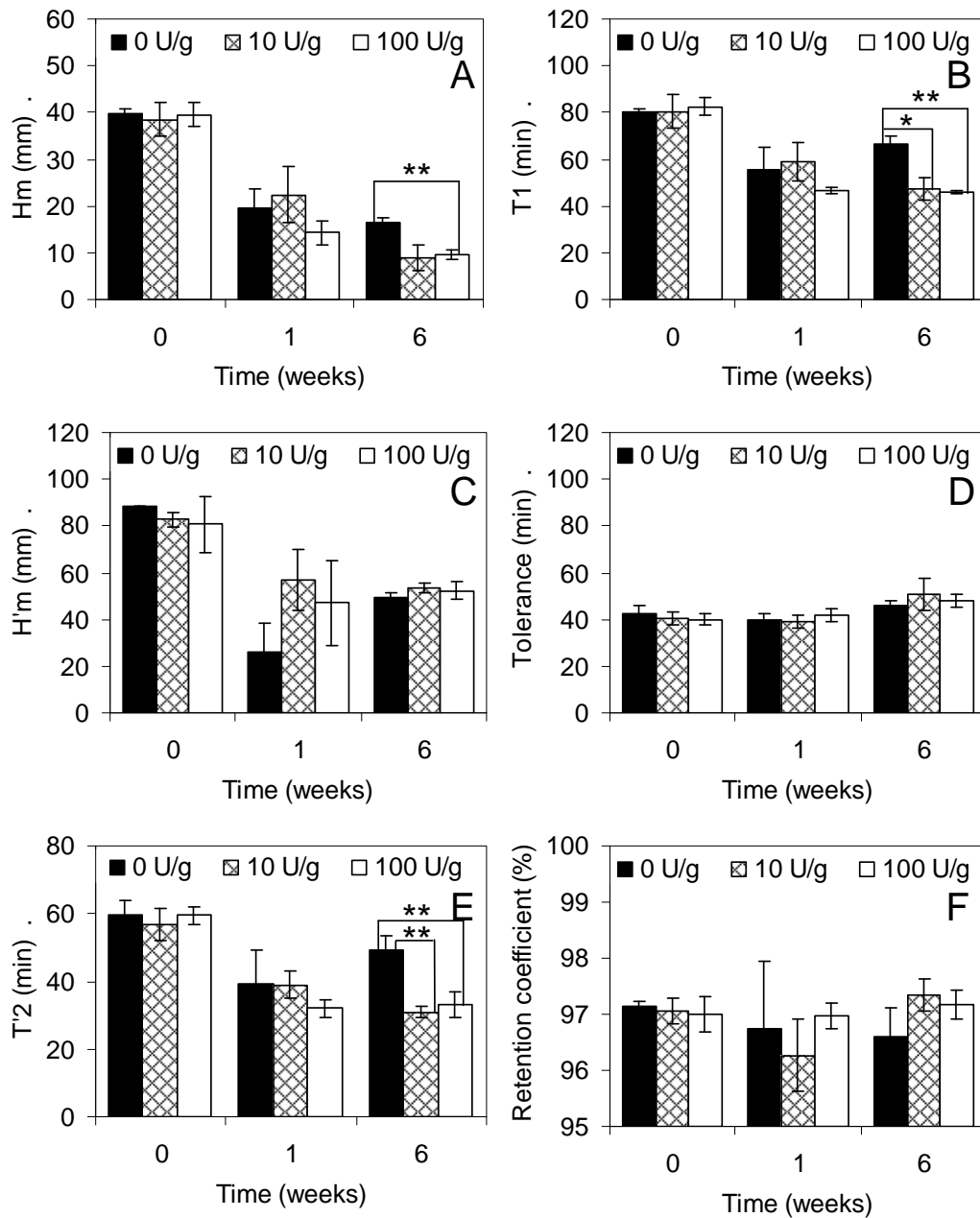


Figure 1 - Fermentation features of doughs prepared with a commercial flour containing ascorbic acid and different amounts of sulfhydryl oxidase. The development of doughs containing 0, 10 and 100 U/g of sulfhydryl oxidase was characterized in terms of maximum dough development (Hm, A), time of maximum dough development (T1, B), maximum gas production (H'm, C), tolerance (D), time to reach the 88% of maximum dough development (T'2, E) and retention coefficient (R, F). Values are reported as average \pm standard deviation (n = 3, significant difference between some samples is represented by: * p-value < 0.05 and ** p-value < 0.01).

To exclude the possible interference of the oxidants (e.g. 42 ppm ascorbic acid) present in the commercial flour preparation that was used, the experiment was performed also using ascorbic acid-free flour. Similarly, AoSOX1 had no significant effect on fresh dough (Figure 2). However, frozen doughs without ascorbic acid and containing the sulfhydryl oxidase were significantly softer than the control dough. After one week of frozen storage, the presence of 100 U/g of AoSOX1 in the dough aggravated the damaging effects of freezing, e.g. giving a reduction in the maximum of gas retention ($H'm$, Figure 2 C) and total volume (Figure 2 D) comparable to 4 weeks of frozen storage of the control dough. However, this did not affect dough tolerance (data not shown). After 4 weeks of frozen storage, the gas retention coefficient (R %) of the dough containing 1 and 10 U/g of AoSOX1 was higher than in the control dough (Figure 2 F).

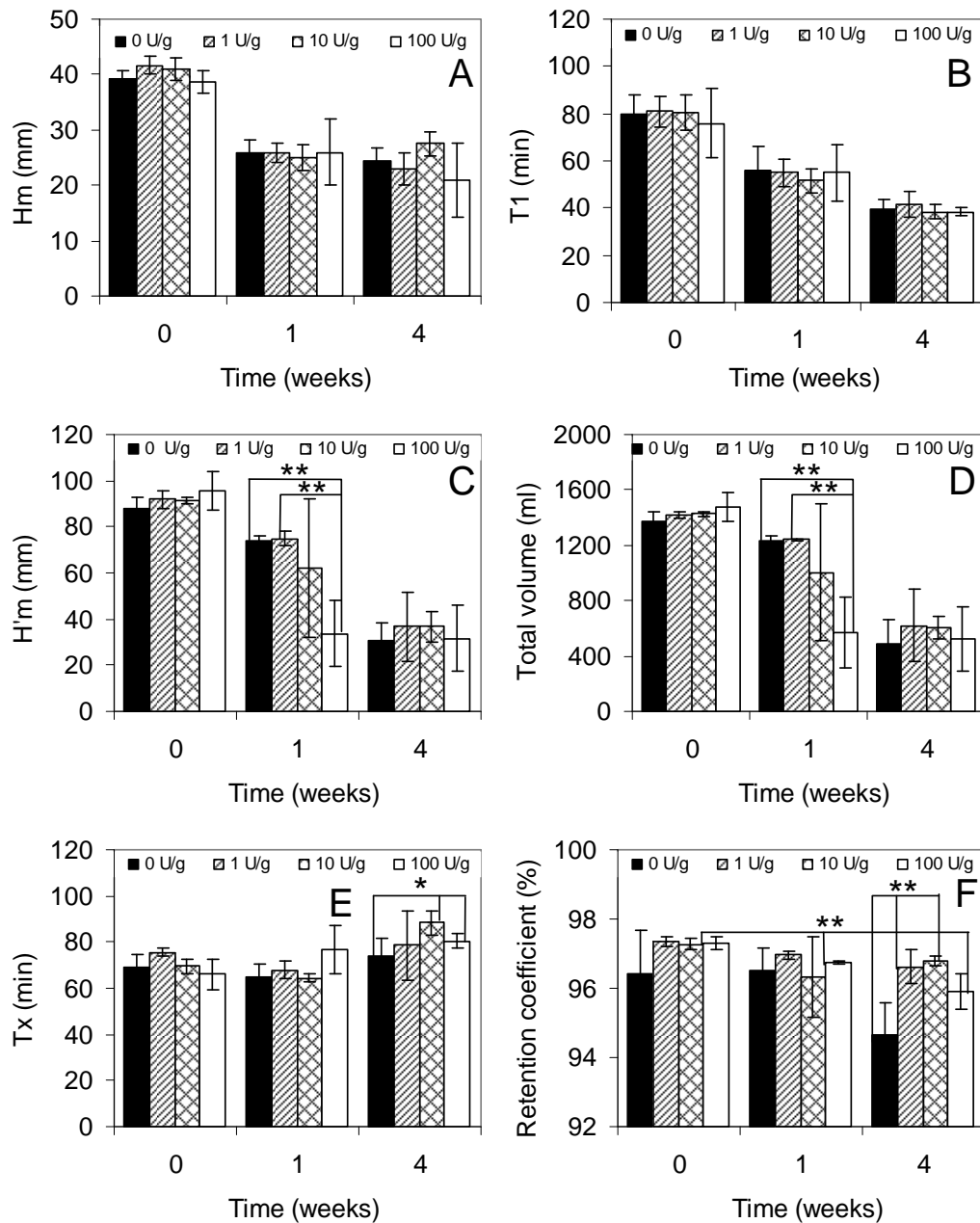


Figure 2 - Fermentation features of doughs prepared with ascorbic acid-free flour. The development of doughs containing 0, 1, 10 and 100 U/g of sulfhydryl oxidase was characterized in terms of maximum height (Hm, A), time of maximum development (T1, B), maximum gas production (H'm, C), total volume (D), time of appearance of dough porosity (Tx, E) and retention coefficient (R %, F). Values are reported as average \pm standard deviation (n = 3, significant difference between some samples is represented by: * p-value < 0.05 and ** p-value < 0.01).

2.2. Effect of sulfhydryl oxidase on the rheology of fresh dough

The effect of sulfhydryl oxidase on dough properties was further investigated by using an ascorbic acid-free flour and a non-yeasted system. The effects due to sulfhydryl oxidase addition and ascorbic acid were fast and visible after 20 minutes of relaxation time. When increasing dosages of sulfhydryl oxidase were used a loss of strength and an increase in dough elasticity was observed (Figure 3 A). With a sulfhydryl oxidase dosage of 100 U/g the strength was reduced by 22% and the elasticity increased by 23% after 20 minutes of relaxation time.

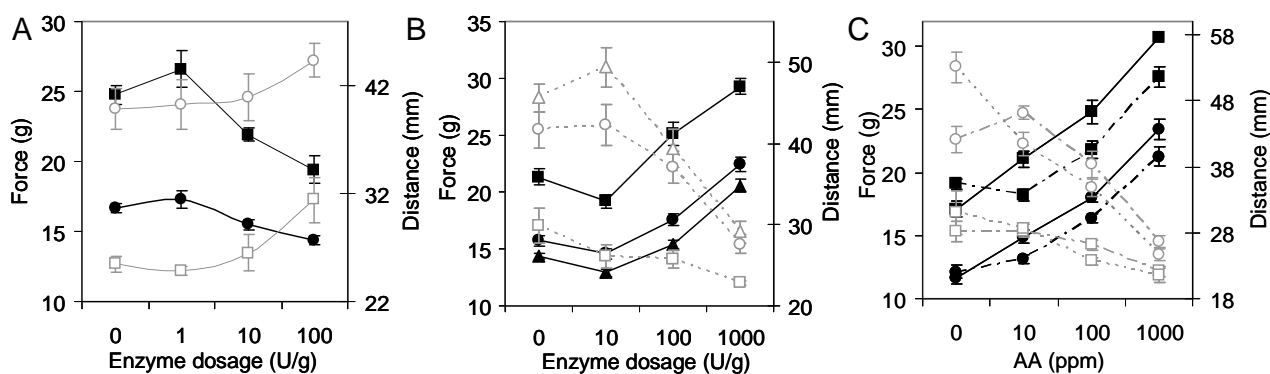


Figure 3 - Effect of different dosages of sulfhydryl oxidase on the properties of non-yeasted dough without (A) and with 100 ppm of ascorbic acid (B), and effect of 1000 U/g of sulfhydryl oxidase on the properties of dough containing increasing concentrations of ascorbic acid (C). The effects were assayed after 20 (square), 40 (circle) and 50 minutes (triangle) in terms of strength (black line) and elasticity (grey line). In panel C, control doughs are indicated by a point-dash line and doughs containing sulfhydryl oxidase by a continuous line.

When the flour was enriched with ascorbic acid (100 ppm), a higher resistance to elongation and lower extensibility were detected upon enzyme addition (Figure 3 B). At a concentration of 100 and 1000 U/g, sulfhydryl oxidase increased dough strength of 7 and 43% and reduced the extensibility by 15 and 57% after 50 minutes, respectively.

The interaction of sulfhydryl oxidase with the ascorbic acid system in the dough was further investigated by testing whether the effect of the sulfhydryl oxidase was dependent on the ascorbic

acid concentration. Sulphydryl oxidase (1000 U/g) treatment resulted in about 10-15% increase in strength after 20 and 40 minutes of relaxation time, regardless the concentration of ascorbic acid (Figure 3 C). About 10% reduction of extensibility was observed after 40 minutes in doughs containing ascorbic acid (Figure 3 C). Furthermore, the addition of 10 ppm of ascorbic acid had minimal effect on dough properties, e.g. 8% strength increase after 40 minutes relaxation time, while the combination with 1000 U/g of enzyme gave a 23% strength increase (Figure 3 C).

Effect of sulphydryl oxidase on the oxidation of ascorbic acid

The impact of the reaction catalyzed by the sulphydryl oxidase AoSOX1 on the conversion of dehydroascorbic acid to ascorbic acid was measured by following spectrophotometrically the increase in absorbance at 265 nm using different amounts of sulphydryl oxidase in the assay (Figure 4 A). The rate of ascorbic acid production, obtained after subtracting the contribution of the sole oxidation of glutathione catalyzed by the sulphydryl oxidase, was decreased in the presence of higher enzyme concentrations (dotted line in Figure 4 A). This result suggested that the enzymatic reaction produced reactive species such as hydrogen peroxide able to oxidize the ascorbic acid, spontaneously produced by the reaction between dehydroascorbic acid and glutathione back to the UV-transparent dehydroascorbic acid. This decrease in ascorbic acid was also detected by a lower absorbance value at 265 nm after 2 minutes (Figure 4A, inset).

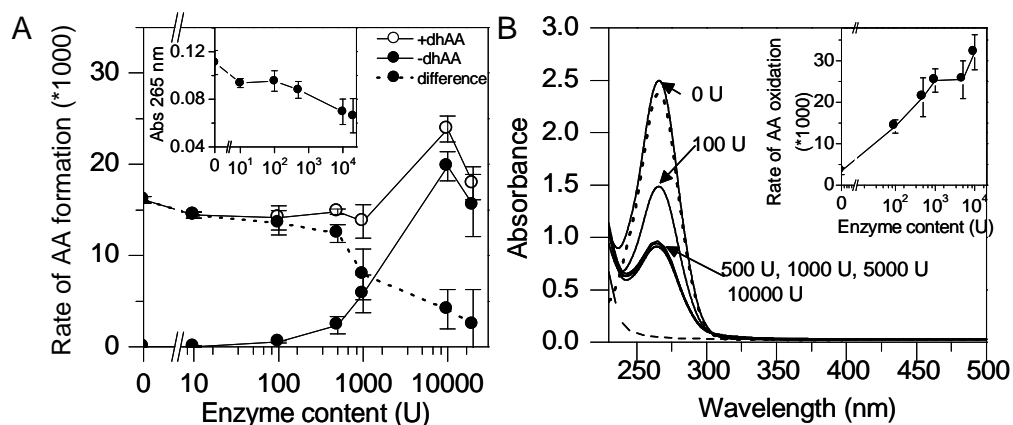


Figure 4 - Effect of the reaction catalysed by sulfhydryl oxidase on the reduction of dehydroascorbic acid (A) and on the oxidation of ascorbic acid (B). The reaction mixture contained 2 mM glutathione and 2 mM dehydroascorbic acid or 0.2 mM ascorbic acid in presence of different amounts of sulfhydryl oxidase. In panel A, the rate of ascorbic acid formation (dotted line) is reported as difference of the rate measured in mixtures containing dehydroascorbic acid, sulfhydryl oxidase and glutathione (empty circle) and control mixtures lacking the dehydroascorbic acid (filled circle, continuous line). As indication of the final ascorbic acid concentration, the final absorbance value at 265 nm is reported (A inset). In panel B, the oxidation rate of ascorbic acid to dehydroascorbic acid caused by the hydrogen peroxide produced by the reaction catalyzed by the sulfhydryl oxidase (A); the absorbance spectra of the reaction mixtures after two hours of reaction time (controls: glutathione, dashed line, and ascorbic acid, dotted line, B inset).

In order to evaluate the influence of the hydrogen peroxide produced by the enzymatic reaction on the ascorbic acid oxidation, different reaction mixtures containing increasing amounts of sulfhydryl oxidase were prepared in the presence of ascorbic acid. The sulfhydryl oxidase AoSOX1 had no activity on ascorbic acid. The conversion of ascorbic acid to dehydroascorbic acid was monitored as decrease in absorbance at 265 nm over a two hour period of time. A constant decrease in ascorbic acid was detected and the rate of the conversion increased with the amount of catalyst present in the reaction (Figure 4 B, inset). UV-Vis spectra of the final reaction mixtures showed a marked decrease in the peak corresponding to a lower concentration of ascorbic acid (Figure 4 B).

3. Discussion

Sulfhydryl oxidases have been reported to have a positive effect on dough properties especially in combination with other enzymes such as hemicellulases and glucose oxidase (Soupe, 2000; Vaisanen et al., 1996; Haarasilta et al., 1991).

Most have described the use of *Aspergillus niger* sulfhydryl oxidase (de la Motte and Wagner, 1987; Vignaud et al., 2002), an enzyme with a pH optimum of 5.5, very similar to the conditions found in the dough (pH 6). The enzyme utilized in our study, AoSOX1, had a pH optimum around 8 yet it retains 10% of (de la Motte and Wagner, 1987) activity in the conditions tested (Faccio et al., 2010) and was able to modify the characteristics of the dough already with an enzyme dosage of 10 U/g of flour corresponding to nanograms of enzyme.

Sulfhydryl oxidase AoSOX1 was tested on two similar flour preparations except for the presence or absence of ascorbic acid, e.g. similar sulfhydryl content but differing in the presence of ascorbic acid. Sulfhydryl oxidase AoSOX1 showed no effect on the development of fresh yeasted dough in the conditions tested. However, the effects of sulfhydryl oxidase could be detected after the dough had been weakened, e.g. damaged by frozen storage, and in a simplified yeast-free system, e.g. flour-water dough.

As evidenced by the rheological studies, the presence of sulfhydryl oxidase in the dough without yeast and ascorbic acid resulted in a weaker and more extensible dough. The weakening effect seemed to be due to the increased presence of oxidized glutathione, whose disulfide form has been shown to promote the softening of the dough (Hahn and Grosch, 1998), by promoting thiol/disulfide exchange reactions (Koehler, 2003) and to the formation of hydrogen peroxide, which has been reported to increase the elasticity of the dough (Liao et al., 1998).

The effect of sulfhydryl oxidase in a simpler system such as water-flour dough was thus tested. The combination of sulfhydryl oxidase and ascorbic acid resulted in an enhancement of dough strength.

By using 100 ppm of ascorbic acid and 1000 U/g flour of sulfhydryl oxidase about 37% strength increase was obtained; by using 1000 ppm ascorbic acid the strength increase was 44%. Thus, about the same effect could be obtained with ten-fold lower ascorbic acid amount when sulfhydryl oxidase was combined to the treatment.

Studies of both yeasted and not-yeasted doughs clearly revealed that the weakening effect of AoSOX1 was reversed whenever ascorbic acid was present in the system and a positive interaction between the sulfhydryl oxidase and the ascorbic acid was evident.

Two hypotheses were postulated concerning whether AoSOX1 participated directly in the ascorbic acid system, acting as an additional glutathione dehydrogenase and promoting ascorbic acid formation, or indirectly, producing the hydrogen peroxide able to oxidize ascorbic acid to the reactive species dehydroascorbic acid. Sulfhydryl oxidase had no activity on ascorbic acid and dehydroascorbic acid and was thus not directly involved in the reaction mechanism. Studies in model solution system showed a competitive effect of the sulfhydryl oxidase-catalyzed reaction on the non enzymatic oxidation of ascorbic acid. The boosting effect that sulfhydryl oxidase has on the ascorbic acid system can be explained in two ways (Figure 5).

Firstly, sulfhydryl oxidase, similarly to ascorbic acid, might contribute to the removal of reduced glutathione (Figure 5 A), thus positively affecting the ascorbic acid system, and secondly, the hydrogen peroxide produced by the reaction catalyzed by the sulfhydryl oxidase is able to oxidize the ascorbic acid to dehydroascorbic acid (Figure 5 B), generally recognized as responsible for the improving effect (Joye et al., 2009). Additionally, the results clearly indicate that the removal of free thiol compounds, e.g. glutathione, and concomitant hydrogen peroxide production by sulfhydryl oxidase are not enough to confer a hardening effect on the dough, unless ascorbic acid was present.

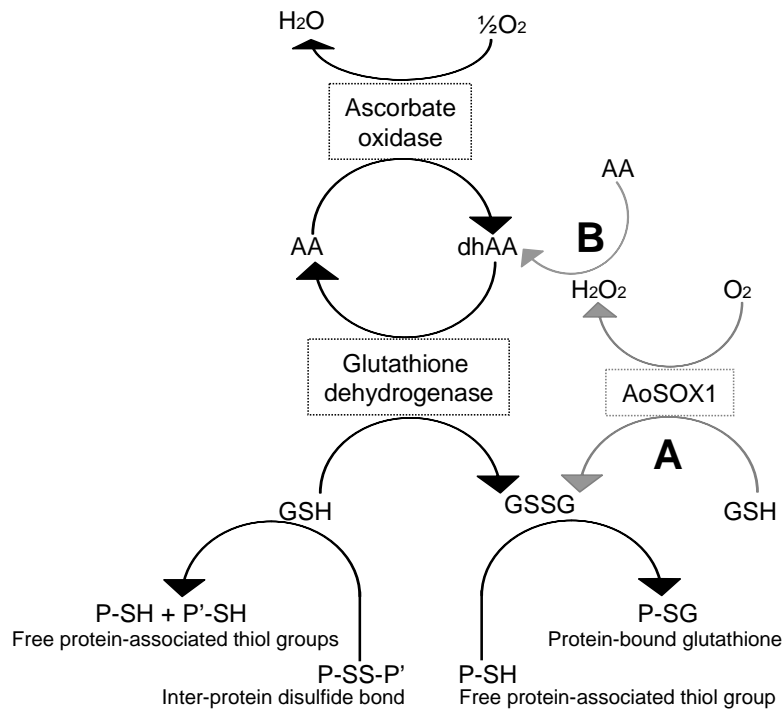


Figure 5 - Interaction between the ascorbic acid system and the reaction catalyzed by the sulfhydryl oxidase. The sulfhydryl oxidase contributes to the removal of reduced glutathione (A) and the production of oxidized glutathione able to block free protein thiol groups; it also produces the hydrogen peroxide able to oxidize the ascorbic acid to the reactive species dehydroascorbate (B).

On the other hand, the cooperative effect of AoSOX1 with the ascorbic acid system hardened the dough significantly, as seen from the rheological measurements. The increased resistance to extension and reduced extensibility was seen as a poor fermentation capacity of doughs after 6 weeks of frozen storage. The combination of ascorbic acid and AoSOX1 at a dosage of 10 and 100 U/g showed smaller maximal rise during shorter fermentation time than the control dough. The formation of excessive disulfide bonds in gluten matrix may have led to more rigid dough without the ability to stretch enough during the growth of gas bubbles or the formation of hydrogen peroxide may have had a deleterious effect on yeast activity.

Combined use of sulfhydryl oxidase and low ascorbic acid levels could improve both fresh and frozen dough baking. Future experiments will be addressed to evaluate the effects of the positive interaction between the sulfhydryl oxidase and ascorbic acid in the baked product and to determine the amounts of ascorbic acid and sulfhydryl oxidase required for an optimal dough development.

4. Acknowledgements

GF was funded by a personal grant by Finnish Cultural Foundation. The skilful assistance of Martina Lille, Leena Pulkki, Arja Viljamaa and Outi Santala is acknowledged.

5. Abbreviations

AA L-ascorbic acid, dhAA L-dehydroascorbic acid, GSH reduced glutathione, GSSG oxidized glutathione, P-SH protein-associated thiol group, P-SS-P inter-protein disulfide bond, GSSP protein-bound glutathione.

6. Acknowledgements

GF was funded by a personal grant by Finnish Cultural Foundation. The skilful assistance of Martina Lille, Leena Pulkki, Arja Viljamaa and Outi Santala is acknowledged.

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Supplementary data

Supplementary Data 1 - Flour analysis

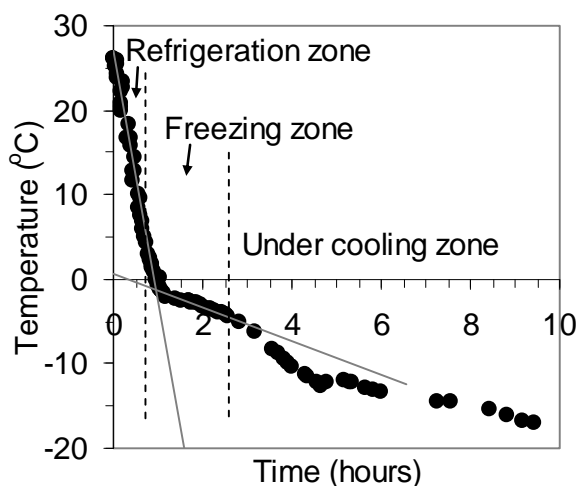
Property	Method	Flour	
		commercial with AA	without AA
Moisture (% dry matter)	130°C 60min (AACC 62-05) ^a	13.4	11.7
Water absorption (corrected for 500 FU, moisture 13.4%)	Farinograph (AACC 54-21) ^a	60.5	63.7
Protein content (% dry matter, Nx5.7 for wheat)	Kjeldahl method (EBC) ^b	13.2	12.1
Glutathione content (μmoles/g)	Andrews, 1995 ^c	3.52	4.2

^a AACC International (2009). Approved Methods of Analysis, 11th Ed. Method 44-15.02 Moisture—Air Oven Methods and Method 62-05.01 Preparation of Sample: Bread Approved November 3, 1999. AACC International, St. Paul, MN, USA.

^b European Brewery Convention (EBC) Analytica, 1998. Analytica EBC. Verlag Hans Carl Getranke-Fachverlag, Nurnberg, Germany.

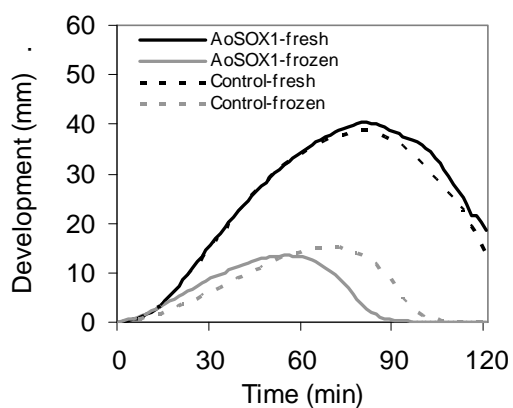
^c Andrews, D.C.; Caldwell, R.A.; Quail, K.J. Sulfhydryl analysis. I. determination of free sulfhydryls in wheat flour doughs. *Cereal Chem.* 1995;72(3):326-9.

Supplementary Data 2 - Temperature variation during the freezing process as measured at the centre of a dough (300 g) placed in the blast freezer.



Supplementary Data 3 - Fermentation of doughs made from commercial flour containing ascorbic acid with and without addition of sulfhydryl oxidase.

Fermentation was monitored by Rheofermentometer. Development curves were obtained for fresh (black lines) and frozen dough (six weeks of frozen storage, grey lines) with 100 U/g of SOX (continuous line) and without (dashed line).



Author(s) Greta Faccio		
Title Discovery of oxidative enzymes for food engineering Tyrosinase and sulfhydryl oxidase		
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 <i>Trichoderma reesei</i> 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 <i>Aspergillus oryzae</i> , was produced in <i>T. reesei</i> 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- <i>tert</i> -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 <i>A. oryzae</i> . These two novel sulfhydryl oxidases were produced in <i>T. reesei</i> 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.		
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Name of project		Commissioned by
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