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Doctoral Dissertation

Characterization of oxidoreductases for biotechnological applications

submitted by

Verena BRAUNSCHMID, MSc

in partial fulfilment of the requirements for the academic degree

Doktorin der Bodenkultur (Dr.nat.techn.)

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Supervisor:
Univ.Prof. Dipl.-Ing. Dr.techn. Georg M. Gübitz
Institute of Environmental Biotechnology
Department of Agrobiotechnology, IFA-Tulln

Affidavit

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I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Tulln, 22.08.2022 Verena BRAUNSCHMID *(manu propria)*

This thesis is dedicated to my family

Do something with it first! If nothing works – you can still burn it!

George A. Olah, Nobel Prize Chemisty 1994

Supervisory team

Priv.-Doz. Mag. Dr. Doris Ribitsch

Priv.-Doz. Dr. Gibson Stephen Nyanhongo

Univ.Prof. Dipl.-Chem. Dr.rer.nat. Antje Potthast

Assoc. Prof. Dipl.-Ing. Dr. Roland Ludwig

Prof. Giovanna Cristina Varese

Preface

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List of publications

Publications that comprise the main part of this cumulative dissertation

1. Antonino Biundo, Verena Braunschmid, Matthias Pretzler, Ioannis Kampatsikas, Barbara Darnhofer, Ruth Birner-Gruenberger, Annette Rompel, Doris Ribitsch, and Georg M. Guebitz. 2020. “Polyphenol Oxidases Exhibit Promiscuous Proteolytic Activity.” Communications Chemistry 3 (1): 1–8. https://doi.org/10.1038/s42004-020-0305-2.

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   A.B. and V.B. performed the main experiments and analysed the data. M.P., I.K. and A.B. produced the enzymes. B.D. and R.B. performed MS analyses. D.R., A.B. and V.B. planned the experiments. A.B. and V.B. wrote the manuscript. D.R. and G.M.G. corrected the manuscript. A.R., D.R. and G.M.G. supervised the work. Funding was acquired by G.M.G., D.R., G.S.N. and A.R.. All authors discussed the presented data and commented on the manuscript. Authors have read and agreed to the published version of the manuscript.

2. Verena Braunschmid, Karin Binder, Sarah Fuerst, Raditya Subagia, Caroline Danner, Hedda Weber, Nikolaus Schwaiger, Gibson S. Nyanhongo, Doris Ribitsch, and Georg M. Guebitz. 2021. “Comparison of a Fungal and a Bacterial Laccase for Lignosulfonate Polymerization.” Process Biochemistry 109 (October): 207–13. https://doi.org/10.1016/J.PROCBIO.2021.07.001.

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   V.B. and K.B. expressed the enzymes. V.B., K.B., S.F., C.D. and R.S. performed the experiments and analyzed the data. D.R. and V.B. planned the experiments. V.B. wrote the manuscript and G.M.G. and D.R. corrected it. V.B. supervised the work of K.B., S.F. and C.D.. G.M.G., D.R. and G.S.N. supervised the overall work. Funding was acquired by G.M.G., D.R. and G.S.N. Authors discussed and commented on the presented data and the manuscript. Authors have read and agreed to the published version of the manuscript.

3. Verena Braunschmid, Sarah Fuerst, Veronika Perz, Sabine Zitzenbacher, Javier Hoyo, Cesar Fernandez-Sanchez, Tzanko Tzanov, et al. 2020. “A Fungal Ascorbate Oxidase with Unexpected Laccase Activity.” International Journal of Molecular Sciences 21 (16): 5754. https://doi.org/10.3390/ijms21165754.

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   V.B., S.F., V.P. and S.Z. expressed the enzyme, performed the majority of the experiments and analysed the data. J.H. and C.F.-S. under the supervision of T.T. performed all experiments concerning redox potential. G.S. modelled the enzyme under the supervision of K.G. D.R., S.Z., V.P. and V.B. planned the experiments. V.B. wrote the manuscript and G.M.G. and D.R. corrected it. V.B. supervised the work of S.F.. G.M.G., D.R. and G.S.N. supervised the overall work. Funding was acquired by G.M.G., D.R. and G.S.N. Authors discussed and commented on the presented data and the manuscript. Authors have read and agreed to the published version of the manuscript.

Abstract

Enzymes are tools of every living cell and have become valuable tools in biotechnology. Their usage has become more widespread, as knowledge increased and costs decreased. Especially oxidoreductases, which perform vital redox reactions in nature, are often employed and of high interest for biotechnological applications. Increasing our understanding of these tools is a first necessary step for successful application. This thesis is aimed at characterizing novel oxidoreductases and their reactions. Therefore, different oxidoreductases were produced through heterologous expression and characterized biochemically. One group of oxidoreductases are polyphenol oxidases, for which we describe a novel reaction, not previously known for polyphenol oxidases, wherein polyphenol oxidases perform a cleavage reaction of another enzyme, namely an esterase. Thereby the activity of the esterase is increased, especially on the plastic PET. This increased activity might be interesting for PET degradation or functionalization. Another group of oxidoreductases are multicopper oxidases. Most prominent in this group is the laccase family. Laccases are already employed for various applications, such as polymerization of lignin. In our study we produced and characterized a fungal and a bacterial laccase. The fungal laccase from *Melanocarpus albomyces* had characteristics typical for laccases, but polymerized lignin in a surprisingly alkaline environment. The bacterial laccase from *Streptomyces rochei* was most active at an unusually high temperature and was relatively stable. These characteristics are very interesting for various application. Ascorbate oxidases are closely related to laccases, but much less explored. Finally, in our study we characterized a novel multicopper oxidase from *Aspergillus flavus* that turned out to be an extraordinary ascorbate oxidase. Hence, this thesis contributes to a better understanding of enzymes in general and of certain oxidoreductases specifically.

Kurzfassung

Enzyme sind Werkzeuge jeder lebenden Zelle und haben sich zu wertvollen Werkzeugen der Biotechnologie entwickelt. Ihre Nutzung erhöhte sich mit zunehmendem Wissen und sinkenden Kosten. Besonders Oxidoreduktasen, die in der Natur wichtige Redoxreaktionen durchführen, werden oft eingesetzt. Die Erforschung dieser Werkzeuge ist ein erster wichtiger Schritt für eine erfolgreiche Anwendung. Ziel dieser Arbeit war es, neue Oxidoreduktasen und ihre Reaktionen zu charakterisieren. Dazu wurden mehrere Enzyme durch heterologe Expression hergestellt und biochemisch charakterisiert. Polyphenoloxidasen sind Oxidoreduktasen, für welche wir in unserer Studie eine für Polyohenoloxidasen unbekannte Reaktion beschreiben, bei der Polyphenoloxidasen ein anderes Enzym, eine Esterase, spalteten. Dadurch wurde die Aktivität der Esterase erhöht, insbesondere auf dem Kunststoff PET. Diese erhöhte Aktivität könnte für den Abbau oder die Funktionalisierung von PET interessant sein. Eine weitere Gruppe von Oxidoreduktasen sind Multikupferoxidasen. Am bekanntesten ist die Laccase-Familie. Laccasen werden bereits für viele Anwendungen, zum Beispiel für die Polymerisation von Lignin, eingesetzt. In unserer Studie haben wir eine pilzliche von *Melanocarpus albomyces* und eine bakterielle Laccase von *Streptomyces rochei* hergestellt und charakterisiert. Die pilzliche Laccase hatte typische Eigenschaften, polymerisierte jedoch Lignin in einer überraschend sauren Umgebung. Die bakterielle Laccase war bei einer ungewöhnlich hohen Temperatur am aktivsten und relativ stabil. Diese Eigenschaften sind für viele Anwendungen sehr interessant. Ascorbatoxidasen sind mit Laccasen verwandt, aber viel weniger erforscht. Wir haben in unserer Studie eine neuartige Multikupferoxidase von *Aspergillus flavus* charakterisiert, die sich als außergewöhnliche Ascorbatoxidase herausstellte. Somit trägt diese Arbeit zu einem besseren Verständnis von Enzymen im Allgemeinen und von bestimmten Oxidoreduktasen im Besonderen bei.

1 Introduction

The need for sustainable energy production, materials and waste treatment is driving the research and application of biocatalysts, although they have been already employed in traditional applications for a long time such as in cheese production or later as washing agents [1,2]. Recent years have seen a rise in enzyme application in new fields and new substrates, such as synthetic polymers [3] and extensive research, as well as large-scale industrial production, has dropped the costs of enzymes, making them affordable for a broader range of applications.

In living cells, almost all reactions are catalyzed by enzymes, which speed up reactions up to 10^{17} times, compared to uncatalyzed reactions [4]. They are as versatile as the functions they must perform, even though the number of different enzymes is limited. Therefore, some enzymes can fulfill various functions [5]. Multifunctionality of enzymes is very useful in developing activity on new substrates and catalyzing new reactions. This becomes particularly interesting when there is a need to clean up novel man-made chemical pollution, or in new industrial processes.

Enzymes are classified by the International Union of Biochemistry and Molecular Biology (IUBMB) according to the reactions they catalyze and are assigned an enzyme commission (EC) number accordingly. This classification disregards the evolutionary and structural relations between enzymes. The enzyme class with the number 1 assigned is the oxidoreductase family. Oxidoreductases perform oxidations of a substrate while reducing another substrate. Usually, the substrate which does not fulfil the main biological function is labelled co-factor [6].

Enzymes discussed in this thesis are oxidoreductases of different families. They were grouped according to their structure, homology, substrates, and reaction mechanism, into polyphenol oxidases (PPOs) and multi-copper oxidases (MCOs). This classification differs from the EC-classification but is frequently found in literature [7–9]. Both enzyme families use copper for the redox reaction and electron transport inside their structure. The co-factor of MCOs and PPOs is dioxygen, which is reduced to water. A prominent difference between PPOs and MCOs is the number of copper ions bound in their structure. While PPOs have two copper ions in their structures, MCOs have four [10,11].

1.1 Polyphenol oxidases

Polyphenol oxidases (PPOs) contain a dinuclear copper site and exhibit catecholase activity (EC 1.10.3.1) or analogous activities [12]. The PPO family includes three enzyme groups, namely tyrosinases (EC 1.14.18.1 and EC 1.10.3.1), catechol oxidases (EC 1.10.3.1) and aurone synthases (EC 1.21.3.6) (Figure 1 a,b,c)[12]. Catechol oxidases perform the oxidation

of *o*-diphenols to *o*-quinones, which is the catecholase activity. Tyrosinases exhibit two different enzyme activities, cresolase activity (EC 1.14.18.1) followed by catecholase activity [12]. With their cresolase activity tyrosinases catalyze the *ortho*-hydroxylation of monophenols to *o*-diphenols. This reaction is followed by the catecholase activity, which is the same oxidation as performed by catechol oxidases. The catalytic mechanism of aurone synthases is analogue to the reaction mechanism of tyrosinases. It performs a hydroxylation, followed by an oxidation to react chalcones to aurones, forming a yellow pigment [12]. All the oxidation reactions are coupled to a reduction of oxygen to water. The reaction proceeds via an intermediate state (*met* form), where oxygen is reduced to hydroxide. Oxygen is bound by two copper ions in the active center, via a bridge formation (*oxy* form). The *oxy* form can attack a substrate directly [7].

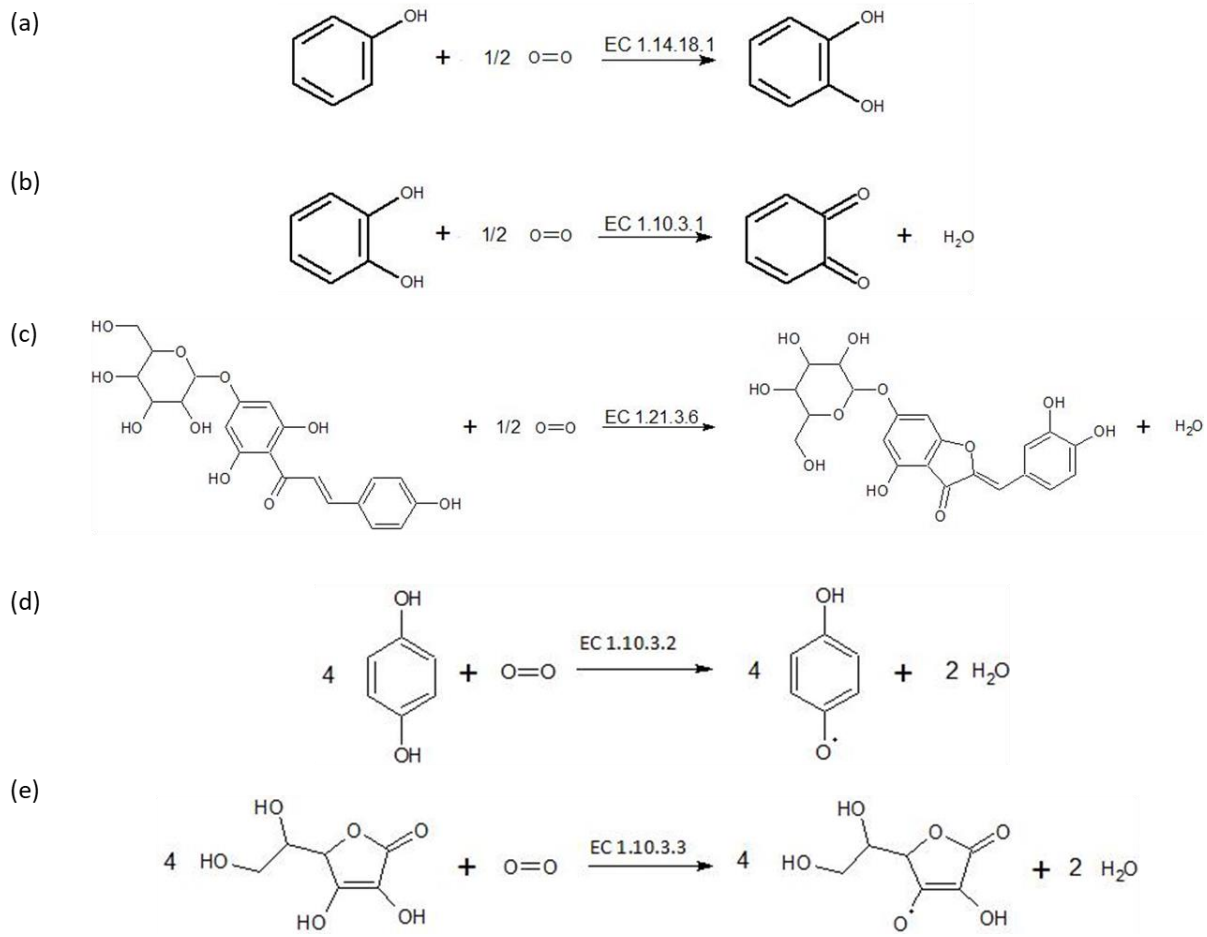

Figure 1: Reaction scheme of cresolases (EC 1.14.18.1) (a), catecholases (EC 1.10.3.1) (b)[13], aurone synthase (EC 1.21.3.6) (c), laccases (EC 1.10.3.2) (d) and ascorbate oxidases (EC 1.10.3.3) (e) modified from BRENDA [14].

The structure of the active site of PPOs is conserved. Four α-helices form a bundle that surrounds two type 3 copper ions, CuA and CuB. The copper ions are coordinated by three histidine residues each [15]. While these features are largely conserved for PPOs,

considerable variation is found in the residues binding the substrate. The substrate binding residues are responsible for the substrate specificity [7].

Generally, PPOs consist of two domains, a catalytically active domain and a C-terminal domain (Figure 2) [16]. Plant PPOs have a third domain at the N-terminus, probably functioning as a signal peptide. In some bacterial PPOs the C-terminal domain is missing [17]. If present, the C-terminal domain shields the active site by inhibiting the access of substrates and thereby lowers the activity. In the active form of the enzyme, the C-terminal domain is cleaved off. The domain is, however, necessary for proper folding and might have further unknown functions [7].

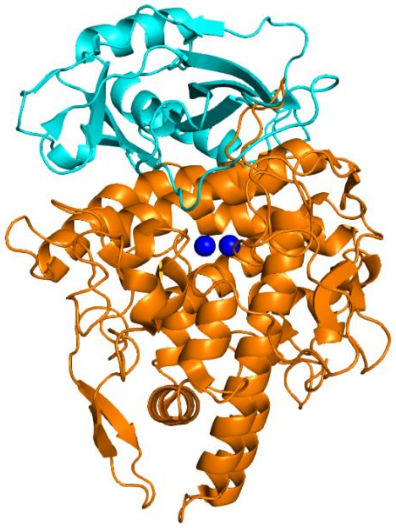

Figure 2: PPO Structure by the example of AbPPO4 (PDB 4OUA): C-terminal domain in turquoise, catalytically active domain in orange [18].

PPOs are widespread in nature, occurring in almost all lifeforms, from procaryotes to mammals. They have been extensively described in plants [19–22], owing mostly to their role in fruit and vegetable browning. The browning effect has received a lot of attention, because it deteriorates nutritional quality and thereby reduces longevity and transportability of fruits and vegetables [12]. The natural functions of PPOs are mostly considered to be protectory. In plants and insects, PPOs are involved in wound healing and protection against microorganism. They are furthermore important for pigmentation synthesis in mammals. Tyrosinases catalyze three separate reactions in the mammalian melanin biosynthesis. Melanin is also a protection mechanism against the damage of ultraviolet light [23].

For a long time, PPOs have been studied only for their effect in browning of foodstuffs, but their potential for diverse applications is high. Diphenols produced by tyrosinases could be applied for pharmaceuticals, for example as antioxidants or, in the case of L-3,4-dihydroxyphenylalanine, as drug against Parkinson's disease [24,25]. The broad substrate range of PPOs is advantageous for bioremediation of phenolic contaminants, such as dyes.

Furthermore, PPOs can be employed to detect phenolic compounds in food, water or pharmaceutical samples [24,26].

Four PPOs were employed in this thesis, PPOs from *Agaricus bisporus* (*Ab*PPO4), from apple (*Md*PPO2), from walnut (*Jr*TYR) and from *Croreopsis grandiflora* (*Cg*AUS1) [13]. Tyrosinases of the common mushroom *A. bisporus* are of the best studied and most employed tyrosinases [27]. Mushroom tyrosinases are commercially readily available, although their purity is usually questionable, especially because *A. bisporus* contains six isoforms [28]. The fourth isoform of *A. bisporus* PPO (*Ab*PPO4) can be produced in a pure form in *Escherichia coli* [29]. PPOs from apple (*Md*PPO2) and walnut (*Jr*Tyr) are some of the few plant PPOs that have been heterologously expressed and studied in more detail *in vitro* [22,30]. Both enzymes are active on monophenolic, as well as, diphenolic substrates and are categorized as tyrosinases [22,30]. A PPO from *Coreopsis grandiflora* (*Cg*AUS1) was shown to have hydroxylase activity towards its natural, monophenolic substrate, as well as catecholase activity. It was therefore identified as aurone synthase [31,32]. In our study we found a specific proteolytic activity of two of the aforementioned PPOs. This activity was not described in PPOs before and increased the activity of the cleaved protein, a carboxylesterase [13].

1.2 Multicopper oxidases

Multicopper oxidases (MCOs) are a highly versatile group of enzymes. As their name suggests, MCOs oxidize their substrates with the use of copper atoms. The oxidation reaction is coupled with a reduction of molecular oxygen to water [33]. Four electrons are subsequently stripped from four substrates and transferred to one di-oxygen molecule, which is reduced to two water molecules. Almost all MCOs contain four copper ions, organized in two distinct sites. One type 1 copper ion is located in the T1 center near the surface. This copper ion accepts the electron of the substrate. The electron is then transferred via a conserved motif (H-X-H) to the other copper ions in the trinuclear center. The trinuclear center contains one type 2 and two type 3 copper ions. The dioxygen molecule binds covalently to the copper ions of the trinuclear center and is reduced at this site (Figure 3)[11].

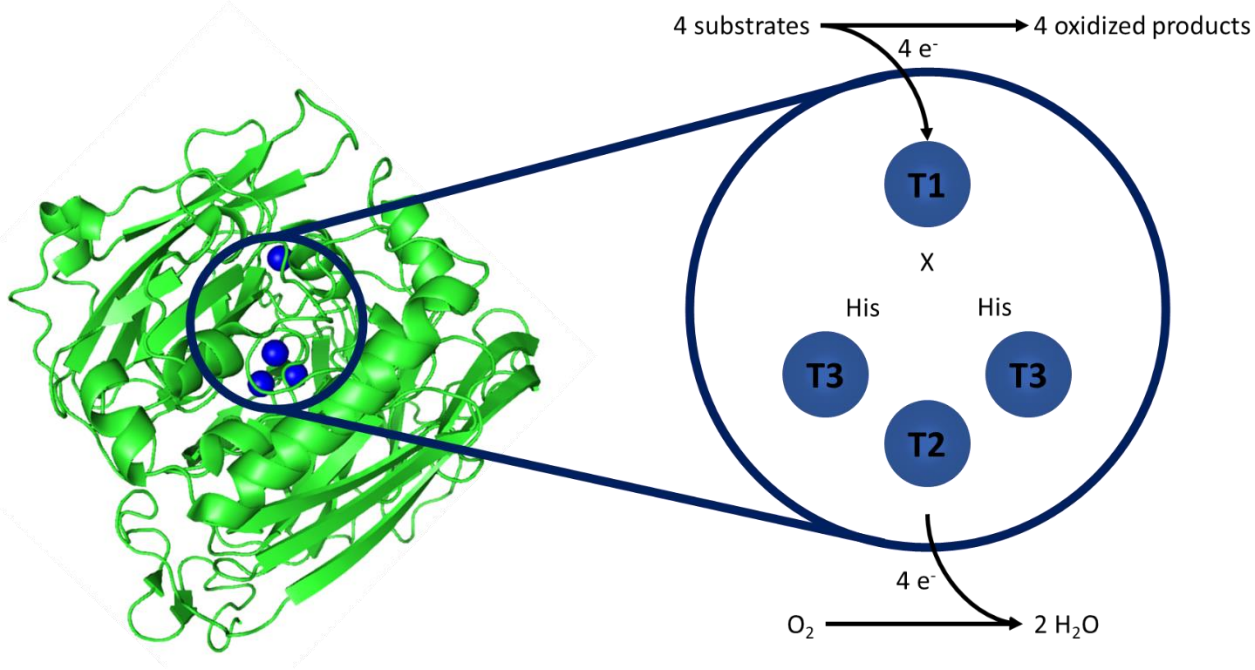

Figure 3: MCO reaction mechanism: Electron transfer takes place via the four bound copper ions. Substrates are oxidized at the T1 copper ion. Electrons are then transferred, one after the other, via the conserved His-X-His motif to the trinuclear center consisting of two T3 and one T2 copper ion. The trinuclear center is the site of oxygen reduction to water. (MCO structure on the left is modified from PDB 1GW0 [34])

The four copper ions are coordinated through conserved residues. T1 copper is fixed in a tight triagonal formation with one cysteine and two histidine residues. A fourth residue, most commonly a methionine, binds the T1 copper through a weaker and lengthier bond. Other residues, such as leucine or phenylalanine can substitute methionine. In the trinuclear center, the type two copper ion is bound by two histidine residues and, in most cases, a water molecule, whereas the type 3 copper pair is coordinated by three histidine residues per ion. The eight histidine residues of the trinuclear center come from different domains [33]. Most MCOs consist of one polypeptide chain, although some are oligomers, and form three domains (Figure 4). The exceptions are ceruloplasmin, which forms six domains and small laccases, which form two domains. Small laccases, furthermore, aggregate to trimers. These structural features of MCOs are highly conserved, although their protein sequences are very diverse [33]. Conserved protein sequences are those that form copper binding sites. It is possible to identify an enzyme as MCO by means of these sequences [35].

The substrate range of MCOs depends on the one hand on the conformation of its substrate binding pocket and on the other hand on the redox potential of the T1 copper site [36]. The substrate binding pocket restricts the access of substrates, for example a narrow binding pocket only allows access to small substrates [37]. A high redox potential of the T1 copper site facilitates the electron transfer from substrates to the enzyme [38]. The redox potential of the copper ion itself is increased by the inclusion into the protein structure. Binding stresses its geometry, which increases its redox potential [39]. Therefore, the immediate surrounding of

the T1 copper site has a large influence on the redox potential. Most significant is the impact of the axial ligand that coordinates the T1 copper ion. In most MCOs this ligand is a methionine, associated with a low redox potential. High redox potential MCOs have a phenylalanine and most medium redox potential MCOs a leucine instead of the methionine [33,36].

The MCO enzyme family includes different subgroups, namely ascorbate oxidases (EC 1.10.3.3), laccases (EC 1.10.3.2) and ferroxidases (EC 1.16.3.1) [8]. Sometimes bilirubin oxidases (EC 1.3.3.5) or ceruloplasmins are named as further subgroups. Ceruloplasmin is a ferroxidase and is the only MCO that contains six domains [8,40]. The subgroups differ mainly in their choice of substrate and biological function. The assignment of enzymes to these subgroups is, however, difficult, because some MCOs may react with substrates of different subgroups *in vitro* and their biological function is not known or not determinable [8,33].

1.2.1 Laccases

Laccases have been discovered more than one hundred years ago in the sap of the Japanese lacquer tree (*Rhus vernicifera*). The sap is released after wounding of the plant and is then hardened by the laccase to seal the opening [41]. The first application of laccases – even before their discovery – was the curing of lacquer. This is a technique traditionally applied to coat dishes and other items, known as lacquerware, in Asia [42]. Research interest and application possibilities have grown extensive since then. Laccases have not only been found in plants, but also in fungi, bacteria and insects [43–45]. Accordingly, they perform a wide variety of functions. Plant laccases are vital for the synthesis of lignin [46]. Fungal laccases are involved in the degradation of lignin for wood-decay, but also in detoxification, pathogenesis and fungal morphology [43]. Bacteria use their laccases for example for pigmentation, copper homeostasis, degradation of toxic compounds and degradation of lignin [44]. In insects laccase-like enzymes are involved in cuticle tanning [45]. The substrate range of laccases is subsequently large, covering phenols, aminophenols, diamines and some inorganic ions [43]. The substrates are oxidized at the T1 copper site to radicals, which can then react spontaneously. With the oxidation of four substrates, one dioxygen molecule is reduced to two water molecules (Figure 1d). The broad substrate spectrum of laccases is already being exploited for various biotechnological applications.

Laccases are mostly monomeric proteins that consist of three domains, although some oligomeric laccases have been described. The domains have similar structures, forming barrels out of ß-sheets that are oriented in a Greek key pattern. This structure is known as cupredoxin-like fold. The T1 copper site is located in the first domain (N-terminal). The copper ions of the trinuclear center are coordinated by residues from the first and the third (C-terminal) domains and are located at the interface of these two domains. The purpose of the second domain is probably to stabilize the structure (Figure 4a) [36]. The structure of the substrate

binding pocket varies strongly among different laccases and therefore their substrate spectra [36].

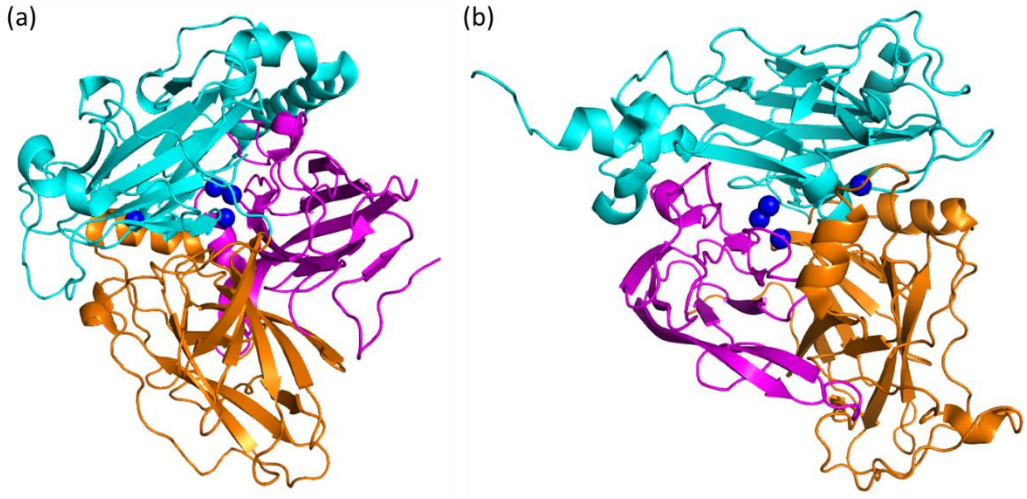

Figure 4: MCO structures: Laccase MtL (PDB 6F5K)[47] (a) and ascorbate oxidase Af_AO1 (PDB 1ASO)[48] (b): domain 1 (N-terminal) in magenta, domain 2 in orange and domain 3 (C-terminal) in turquois.

Several industries, such as textile or food, are employing laccases. In the textile industry, many commercial laccase-based applications have emerged in recent years [49]. Denim bleaching to achieve the so-called stone-washed effect is done with laccases. The bleaching ability of laccases has furthermore been exploited for the whitening of flax-pulp and as bleaching agents in cosmetics for hair and skin. In the food industry, laccases are employed to reduce oxygen content in beer to increase its shelf life and for the treatment of cork to avoid cork-taste in wine. Another interesting feature of laccases is its ability to induce polymerization of certain phenols. This has been employed for the bondage of medium-density fiber boards, in order to avoid the usage of toxic formaldehyde [50]. Technical lignins, containing mainly phenolic compounds, can be polymerized by laccases [51,52]. This is interesting, because technical lignins are an under-valued by-product of the pulp- and paper industry and are expected to accrue in even larger amount in the future, owing to the emerging third-generation biofuel production [53]. Today, most of the technical lignin is burned to produce energy. Through polymerization with laccases, it could be reused [54,55].

For biotechnological applications mostly fungal laccases are employed, because of their comparatively high redox potential [50]. One prominent example is the laccase from *Mycelophtora thermophila*, which is commercially available and described for several applications [54–57]. Plant laccases are more difficult to produce because heterologous expression of plant enzymes is difficult in the most common expression hosts, bacteria and yeast. Therefore, plant laccases are seldom employed. The advantage of bacterial laccases is their easy production and their potentially high tolerance to extreme conditions [58,59]. Especially activity at high pH ranges is of interest for many biotechnological applications.

Bacterial laccases have, however, not been studied in as much detail as fungal laccases and are generally considered to have a lower redox potential [60]. The need for other laccases, encompassing these characteristics for novel applications, drives the search for new laccases.

For that reason, we have heterologously expressed and characterized laccases in our research, specifically the bacterial laccase from *Streptomyces rochei* (*Sr*LA) and the fungal laccase from *Melanocarpus albomyces* (*Ma*L1) [61]. *Ma*L1 was previously described to have high tolerance to elevated temperatures and alkalinity [62]. *Sr*LA has a high similarity to a laccase from *Streptomyces ipomoea*, which is known to be resilient to high alkalinity and salinity [63]. Both enzymes were tried for polymerization of technical lignin and *Ma*L1 showed great potential for this application [61].

1.2.2 Ascorbate oxidases

Ascorbate oxidases are more substrate specific than laccases, typically oxidizing ascorbic acid to dehydroascorbate (DHA) (Figure 1d). In the substrate binding site, one histidine and two tryptophane residues stabilize the lactone ring of ascorbic acid. Various lactone ring-containing substrates may also be converted by ascorbate oxidases. Other substrates do not fit into the rather narrow substrate binding site [64] (Figure 4b). Furthermore, the redox potential of ascorbate oxidases is relatively low compared to laccases, limiting their possible substrates further [37]. Thus, research interest for ascorbate oxidases has been low, being overshadowed by the more versatile laccase family. The ascorbate oxidase of zucchini (*Cucurbita pepo* var. *melopepo* (1ASQ)) has, however, been the first MCO whose structure was elucidated [65]. This was a crucial step for understanding the reaction mechanism of MCOs in general. The industrial applications of ascorbate oxidases are still few, owing to their narrow substrate range and low research attention. They have been mostly applied as biosensors to detect ascorbic acid [66]. Ascorbate oxidases have been mainly described in plants [67] and only a few in fungi [8,68] and insects [69].

The biological function of ascorbate oxidases is still under consideration. There are a few theories for its role in plants. Some studies suggest that not the oxidation of ascorbic acid, but the reduction of oxygen is the purpose of the reaction [70]. Ascorbate oxidases remove oxygen without the formation of dangerous reactive oxygen species. Where photosynthesis takes place and oxygen accrues, close regulation of oxygen content is crucial. Both light and oxygen enhance the activity of ascorbate oxidases [71]. Plants normally release oxygen through stomata, but for certain plants, like those growing with low water availability, open stomata are a disadvantage and oxygen must be disposed of in other ways. Suitably, DHA, the oxidation product of ascorbate oxidases, was shown to induce stomata closure in tobacco (*Nicotiana tabacum* L., cv. Xanthi) [72]. Ascorbate oxidase expression is furthermore enhanced by auxin [73], a phytohormone critical for plant growth. The low oxygen content ascorbate oxidase

provides in growing tissue could be necessary for keeping stem cells in their pluripotent state. Further functions of ascorbate oxidase, through oxygen and ascorbic acid limitation and DHA signaling, have been suggested [67].

Only very few fungal ascorbate oxidases have been described so far and their functions have been even less investigated. Plant pathogenesis or the resistance to host defense mechanism might be possibilities, because putative ascorbate oxidase genes were found mainly in plant-pathogenic fungi [68]. One fungal ascorbate oxidase, the ascorbate oxidase from *Acremonium sp* (ASOM), has been characterized and described in more detail. [74–77]. ASOM was first isolated in 1992 by Murao et al. [75]. It is active at acidic and moderately warm conditions and does not oxidize any substrates other than ascorbic acid [75,77]. In our research, we characterized a putative MCO from *Aspergillus flavus* (*Af*_AO1). *Af*_AO1 turned out to be an ascorbate oxidase with activity on ascorbic acid, as well as, on a typical laccase substrate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [78].

1.3 Heterologous enzyme production

In the past, enzymes were mostly produced homologously in their native hosts [79]. Complex cloning steps and vector production, as well as problems with protein toxicity and folding, are thereby avoided. The yield is, however, generally low and the purification of the enzyme difficult. Many native hosts produce several isoforms of an enzyme, which are hard to separate. Cloning allows the addition of tags to the enzyme, which facilitate purification. Furthermore, native hosts might be pathogenic or difficult to cultivate. Heterologous expression allows for a choice of promoter that can regulate expression tightly and overexpress the enzyme. Protein engineering, is also much simpler in heterologous enzyme production, because changes in the gene sequence can be implemented effortlessly during the normal work-flow [80]. Production in a non-native expression host, that is heterologous expression, is therefore mostly preferable.

The workflow of heterologous expression includes selection of an expression system followed by gene design, cloning into a vector, transformation into a host organism, induction and purification. Each step presents a variable parameter whereby the process can be influenced. First, a host organism is chosen dependent on the enzyme of interest. Hosts can be bacteria, yeast, filamentous fungi, mammalian cells, insects, algae [81] or plants [80]. An important factor in the choice of host is post-translational modifications, which is strongly dependent on the host organism. Activity of enzymes can be impaired by faulty or lacking post-translational modification [82]. Bacterial enzymes are most commonly expressed in *Escherichia coli* [81], whereas fungal proteins are generally better expressed in yeast, such as *Pichia pastoris* [83]. Other parameters depend on the choice of host. The gene of interest is designed to reflect the codon-bias of the host organism. Vectors, media and growth conditions are specific for each

host organism. The purification method is not as strongly dependent on the host. Purification might be facilitated by secretion of the protein, which some hosts are capable of [84].

Several challenges and problems can arise with heterologous enzyme expression. Sometimes the enzyme cannot be expressed or only in low amounts, because the protein is toxic to the host organism or the folding is faulty, and the enzyme is not soluble. Unfolded proteins are generally found as inclusion bodies in the cells [85]. The growth of the host organism can be impaired by the insertion of the foreign gene. Furthermore, the produced protein can be instable in the environment of the host organism [81]. Several strategies have been developed to deal with these challenges. Many can be overcome by the right choice of host organism and expression conditions. However, heterologous expression of a novel protein requires intensive optimization of expression conditions.

Multi-copper oxidases pose specific challenges for heterologous enzyme expression, because they need to incorporate copper. Enzymes containing four copper ions per molecule of enzyme are considered fully copper-loaded. Only fully copper-loaded enzymes are folded correctly and can enfold their activity [86]. Copper has been shown to be incorporated sequentially into the enzyme structure in CueO, the MCO of *E. coli* and CotA, a laccase of *Bacillus subtilis*. The T1-copper is integrated first, followed by the T2 and T3 copper ions [86,87]. Therefore, copper must be present during enzyme expression. Expression media are supplemented with copper to ensure an abundance of copper. Even then, copper incorporation can be insufficient [86].

Copper has high oxidative power and is an essential nutrient, but it is also toxic. Copper content regulation is therefore necessary for all organism and elaborate copper-controlling and expelling mechanism have been established in nature [88,89]. *E. coli* has two copper homeostasis systems, necessitating different strategies for copper-containing enzyme expression. Under anaerobic conditions, one of the copper efflux systems is inactive. Therefore copper is incorporated to a higher intracellular concentration without oxygen [88]. This effect can be exploited when heterologously expressing copper-containing enzymes in *E. coli*. Growth of *E. coli* is reduced under anaerobic conditions and with high copper concentrations. For enzyme expression the culture is therefore first grown aerobe under optimal growth conditions. When a high cell density is reached, the *E. coli* culture is then kept under anaerobic conditions and copper is supplemented to the medium for the enzyme expression. This process ensures maximal intracellular copper concentrations present for enzyme folding [86].

MCO expression in yeast has advantages, but optimal expression conditions are difficult to predict. Copper supplementation to the media of yeast cultures is essential for the production of copper-loaded proteins. The ideal copper concentration is dependent on the yeast and on the enzyme. For laccase expression in *P. pastoris* copper concentrations, between 0.1 and

1 mM have been shown to be ideal. Yeasts seem to be capable of dealing with higher copper concentrations than *E. coli*. Nevertheless, growth is inhibited at high concentrations. A big advantage of yeast is their ability to bestow certain post-translational modifications, such as disulfide bonds and glycosylation. The post-translational modifications can be necessary for enzyme activity, but in some cases, they have been shown to even have diminishing effect on enzyme activity. Another advantage of yeast as expression host is the possibility to secrete the enzyme into the medium. Secretion signal sequences native to the enzyme or to the yeasts can be used and both have shown to be advantageous in some cases [80].

During the purification process of MCOs, copper ions can get dislodged from the enzyme structure. His-tags are commonly used in affinity chromatography for enzyme purification. Copper ions are, however, bound by His-tags and therefore extracted from the enzyme structure during the chromatography [90]. It is therefore unadvisable to use His-tags, but to switch to other tags or purification methods. In some cases, copper is lost when using other purification methods as well. If this happens, the enzyme can be reconstituted with copper by incubation with copper solutions [44].

1.4 References

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2 Polyphenol oxidases exhibit promiscuous proteolytic activity

A. Biundo[a,b,+], V. Braunschmid[a,c,+], M. Pretzler[d], I. Kampatsikas[d], B. Darnhofer[c,e,f], R. Birner-Gruenberger[e,f,g], A. Rompel[d], D. Ribitsch[a,c,*], G. M. Guebitz[a,c]

[a] *Institute of Environmental Biotechnology, University of Natural Resources and Life Sciences (BOKU), Konrad Lorenz Straße 22, 3430 Tulln, Austria*
[b] *Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari, Via Edoardo Orabona, 70125 Bari, Italy*
[c] *Austrian Centre for Industrial Biotechnology (ACIB), Konrad Lorenz Straße 22, 3430 Tulln, Austria and Petersgasse 14, 8010 Graz, Austria*
[d] *Universität Wien, Fakultät für Chemie, Institut für Biophysikalische Chemie, Althanstraße 14, 1090, Wien, Austria; http://www.bpc.univie.ac.at*
[e] *Medical University of Graz, Diagnostic and Research Institute of Pathology, Neue Stiftingtalstraße 6, 8010 Graz, Austria*
[f] *BioTechMed-Graz, Mozartgasse 12/II, 8010 Graz, Austria*
[g] *Vienna University of Technology, Institute for Chemical Technologies and Analytics, Getreidemarkt 9/164, 1060 Vienna , Austria*

[+] *These authors contributed equally: Antonino Biundo, Verena Braunschmid*

* *Corresponding author: Doris Ribitsch, e-mail: doris.ribitsch@boku.ac.at*

2.1 Abstract

Tyrosinases catalyse both the cresolase and catecholase reactions for the formation of reactive compounds which are very important for industrial applications. In this study, we describe a proteolytic activity of tyrosinases. Two different tyrosinases originating from mushroom and apple are able to cleave the carboxylesterase EstA. The cleavage reaction correlates with the integrity of the active site of tyrosinase and is independent of other possible influencing factors, which could be present in the reaction. Therefore, the cleavage of EstA represents a novel functionality of tyrosinases. EstA was previously reported to degrade synthetic polyesters, albeit slowly. However, the EstA truncated by tyrosinase shows higher degradation activity on the non-biodegradable polyester polyethylene terephthalate (PET), which is a well-established environmental threat.

2.2 Introduction

In nature, a limited number of enzymes catalyse hundred-thousands of reactions in a cell. One approach of nature to deal with the disproportion between enzymes and biochemical reactions, is the expression of “multifunctional enzymes” [1]. These enzymes are proteins that perform several distinct enzymatic reactions. Multifunctionality is very important for industrial applications and our understanding of enzymes. In this study, we describe a proteolytic functionality of polyphenol oxidases (PPOs).

Tyrosinases (EC 1.14.18.1 and EC 1.10.3.1), catechol oxidases (EC 1.10.3.1) and aurone synthases (EC 1.21.3.6) constitute the PPO family, which belongs to the type-III-copper protein class [2–4]. PPOs contain a dinuclear copper centre, which consists of two Cu ions (CuA and CuB) coordinated by the τ nitrogen atoms of three conserved histidine residues for each Cu ion [5,6]. PPOs are ubiquitous in the different domains of life [7]. They are important in biosynthetic pathways, such as the formation of brown colour in fruits after the loss of cell compartmentalization and the contact with atmospheric oxygen [8].

Most eukaryotic PPOs are expressed as latent pro-enzymes containing an active domain and a shielding C-terminal domain. Plant PPOs contain also an N-terminal signal peptide, which is thought to direct the enzyme into the lumen of thylakoids, whereas the C-terminal domain of PPOs covers the active site. The activation of the enzymes can be achieved via the cleavage of the C-terminal domain by proteases, at acidic pH or in the presence of detergents. In nature, the most probable activation mechanism is proteolytic maturation, although this is still under investigation [9,10]. *In vitro* activation of latent PPOs (lPPOs) is carried out by site-specific cleavage with proteases yielding active forms of PPOs (aPPOs) or chemically by sodium dodecyl sulphate (SDS), which partially unfolds the C-terminal domain, making the active site accessible. Members of the tyrosinase family possess cresolase and catecholase activity (Figure 5 A). The cresolase activity (monophenolase activity, EC 1.14.18.1), is responsible for the *ortho*-hydroxylation of monophenols and subsequent oxidation to *o*-quinones. On the other hand, the catecholase activity (diphenolase activity, EC 1.10.3.1), is specific for the oxidation of *o*-diphenols to *o*-quinones [11,12]. The highly reactive *o*-quinones can undergo further non-enzymatic rearrangements leading to complex polymers such as melanin [13]. The mechanistic division of PPOs into tyrosinases (cresolase and catecholase activity) and catechol oxidases (only catecholase activity) is still under consideration [14]. Moreover, aurone synthases can catalyse the formation of aurones from chalcones through hydroxylation and oxidative cyclization using a mechanism similar to tyrosinases [15]. However, certain aurone synthases do also possess hydroxylase activity, as previously reported [16].

Tyrosinases have been investigated for many different biotechnological applications [17]. They are widely used for cross-linking of proteins, because of their ability to convert surface-exposed

tyrosines of polypeptides into *o*-quinones, which in turn react spontaneously through 1,4-additions with the side chain of amino acids that contain a nucleophilic group [18]. This cross-linking activity has made tyrosinases very important in the food industry in order to modify the structure and texture of food proteins. In a similar attempt to immobilize a carboxylesterase from *Clostridium botulinum* (EstA, formerly published as Cbotu_EstA) to a suitable carrier, we have recently discovered an unusual activity of tyrosinases. Incubation of EstA with certain tyrosinases resulted in a complete cleavage of the EstA into a larger and a smaller subunit. The carboxylesterase EstA has been identified in the past to hydrolyse synthetic polyesters such as polybutylene adipate-*co*-terephthalate (PBAT) [19]. Processing of the carboxylesterase by tyrosinase resulted in a truncated version of EstA (ΔEstA) lacking 71 amino acids at the N-terminus. Interestingly, ΔEstA showed drastically improved activities on soluble and insoluble substrates, such as the recalcitrant polyester polyethylene terephthalate (PET), which strengthens their environmental and industrial relevance.

In this study, we test several PPOs in their active form towards EstA. Cleavage activity is present in three out of the five tested PPO-containing products. The cleavage is specific for a defined site between Ser71 and Ile72, which is not known as a typical protease cleavage site. To our knowledge this intermolecular cleavage activity was never reported before for members of the PPO family.

2.3 Results

2.3.1 Polyphenol oxidases exhibit proteolytic activity

In this study, the proteolysis activity was first discovered using a commercial tyrosinase preparation from *Agaricus bisporus* (*Ab*T) (Figure 5 B), initially intended for the purpose of enzyme immobilization on carriers. Although tyrosinase from *A. bisporus* is well studied and commercially available, enzyme formulations may consist of a mixture of several isoenzymes and may contain stabilizers and additional, undeclared enzymatic activities [20]. Hence, in order to confirm proteolytic activity of pure isoenzymes, the recombinantly produced fourth isoform of *A. bisporus* tyrosinase (*Ab*PPO4), was investigated in detail [21–24]. On L-tyrosine, the activated form of the recombinant tyrosinase (a*Ab*PPO4) displayed an activity of 2.8 U mg^{-1}. Upon cleavage with a*Ab*PPO4, analysis of the ΔEstA by liquid chromatography coupled to mass spectroscopy (LC-MS/MS) unveiled a specific non-tryptic cleavage site between Ser71 and Ile72, embedded in the motive NH_2-VNTR**SI**IGGN-COOH of EstA (Supplementary Figure 1 and Supplementary Tables 1 and 2). Interestingly, *in silico* studies of the cleavage site showed that the motif is not specific for any known protease [25] except for thermolysin. Thermolysin, however, was predicted to cut at 103 further sites throughout the sequence of EstA [26], which was not observed by SDS-PAGE for the cleavage by PPOs (Supplementary Table 3).

The 3D structure of ΔEstA was modelled for investigation of the cleaved part (Figure 5 C). The model was based on the crystal structure of EstA recently solved at 1.2 Å (PDB 5AH1) [19]. The structure revealed that the 71 amino acids cleaved off by the tyrosinase form a small *α*-helix and a long, solvent exposed loop that span over the active site of the enzyme.

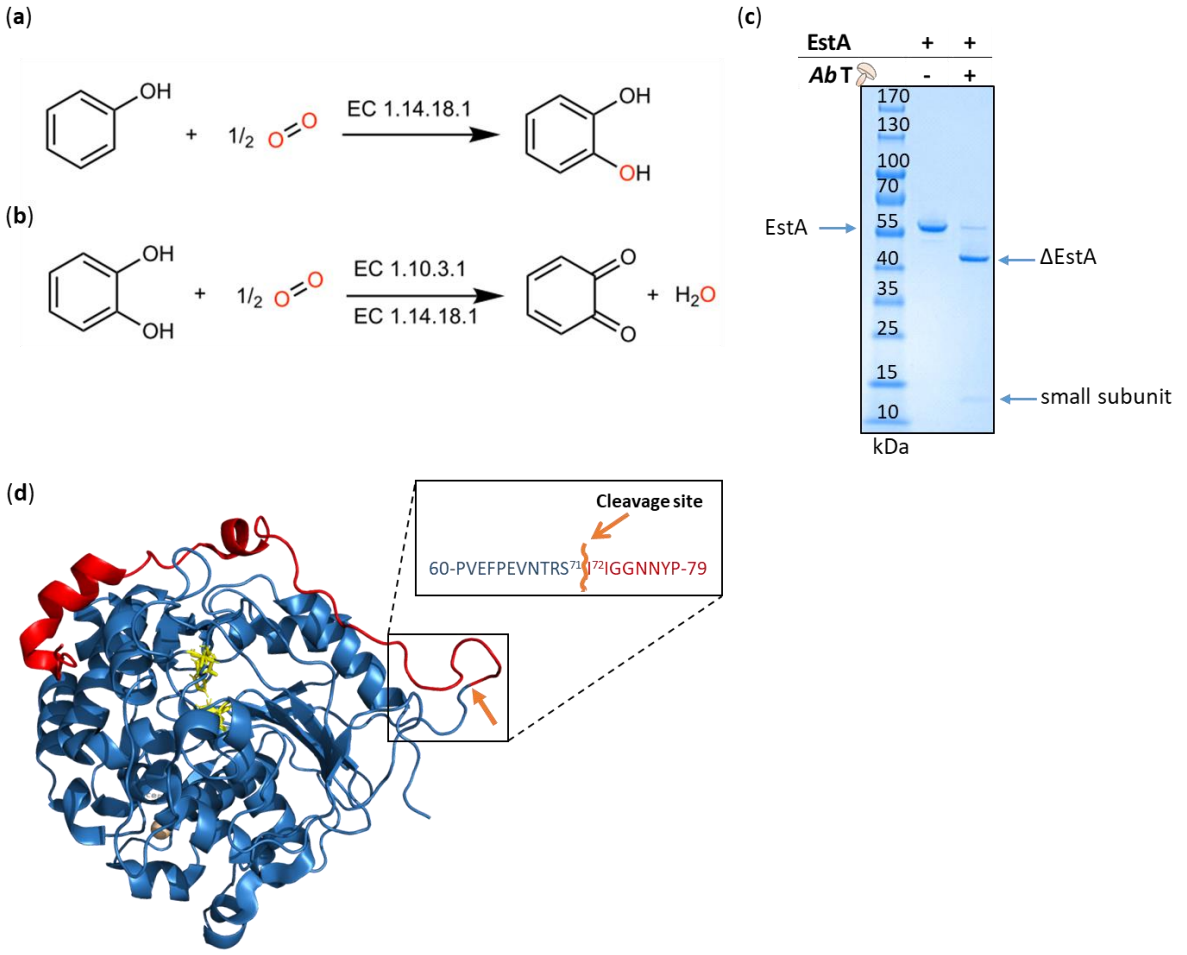

Figure 5: Activities of tyrosinases: Tyrosinases catalyse reactions from monophenols to *o*-quinones through monophenolase (**a**) and diphenolase (**b**) activity . Incubation of the commercial tyrosinase from *A. bisporus* (AbT) with the carboxylesterase EstA led to a cleavage of the latter into a big (ΔEstA) and a small subunit (**c**). EstA was cleaved at a specific site between Ser71 and Ile72. The 71 amino acids cleaved off form an α-helix and a loop on the surface of EstA (PDB 5AH1) (released N-terminal part in *red*; ΔEstA in *blue*, cleavage site in *orange*, catalytic triad of the active site in *yellow sticks*) (**d**).

The truncated enzyme exhibited a 1.9-fold increased activity, from 268 ± 5 U mg^{-1} to 523 ± 17 U mg^{-1}, on the model substrate *para*-nitrophenyl butyrate (*p*NPB) compared to the uncleaved enzyme (Supplementary Figure 2). Previous studies showed that this N-terminal *α*-helix might hinder the entrance of the substrate into the active site of EstA. This *α*-helix is positioned between the *α*-helices of the lid-domain, which are needed for interfacial activation (Figure 5 c)[27].

In order to identify a proteolytic activity conserved throughout the PPO family, experiments with other PPOs were conducted (Figure 6 a). Three members of the tyrosinase family were investigated, *i.e.* the recombinant a*Ab*PPO4 (from common mushroom)[21], the recombinant PPO2 from *Malus domestica* (a*Md*PPO2 from "Golden Delicious" apple)[28] and the tyrosinase isolated from *Juglans regia* leaves (a*Jr*TYR from walnut)[29,30]. In addition, another member of the PPO family, the aurone synthase purified from *Coreopsis grandiflora* petals (a*Cg*AUS1 from large-flowered tickseed)[16,31], was included in the study. The aurone synthase a*Cg*AUS1 is classified as a catechol oxidase as it is not reactive on classical tyrosinase substrates tyrosine or tyramine, however, it can hydroxylate its natural substrate isoliquiritigenin [16]. Recombinant a*Md*PPO2 exhibited the same proteolytic activity on EstA as a*Ab*PPO4 (Figure 6 a, lane 2 and 3, respectively). The two other members of the PPO family, a*Jr*TYR and a*Cg*AUS1, did not exhibit any cleavage of EstA (Figure 6 a, lane 4 and 5, respectively). The distinctive behaviour of the different PPOs is difficult to account for, due to high similarity of the structures of all tested enzymes and especially of their active sites. Hence, the recombinantly produced tyrosinase a*Ab*PPO4 [21] was used for further characterization of the cleavage (Figure 6 b).

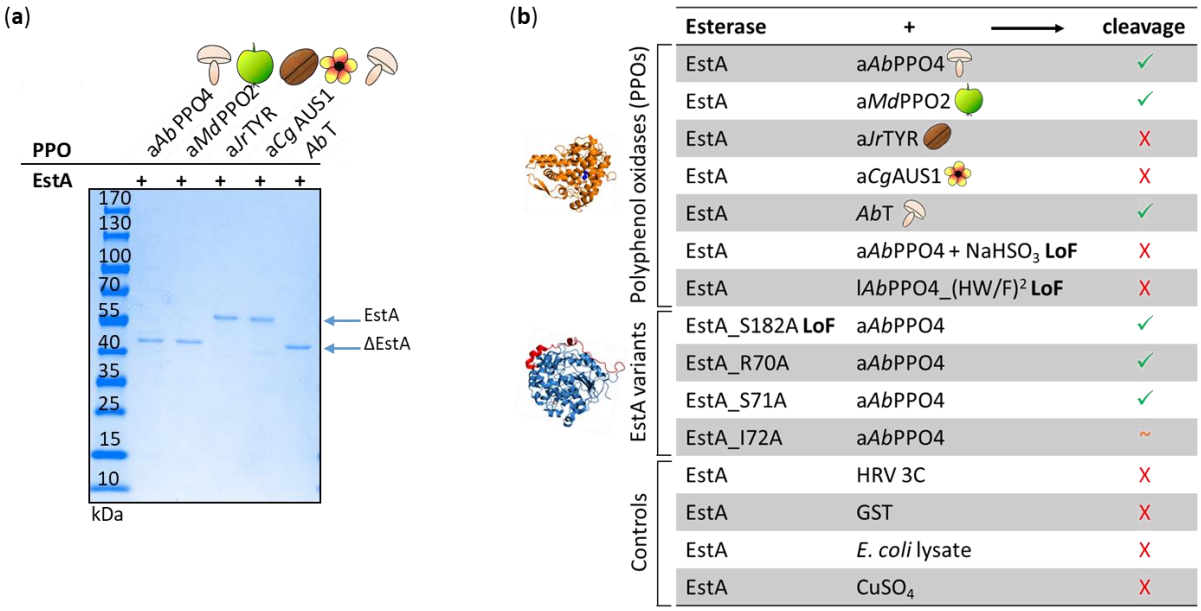

Figure 6: Cleavage with different PPOs: PPOs from *A. bisporus* (commercial: *Ab*T, recombinant: a*Ab*PPO4), *M. domestica* (a*Md*PPO2) *J. regia* (a*Jr*TYR) and *C. grandiflora* (a*Cg*AUS1) were tested for cleavage activity on EstA (**a**). Various experiments were carried out to characterize the reaction, summarized in (**b**).

2.3.2 Cleavage was independent of possible impurities

Although EstA was consistently stable during expression [19], the effect of other substances involved in the downstream processing of a*Ab*PPO4 that might cleave EstA, were investigated.

Both enzymes, a*Ab*PPO4 and EstA, were expressed in *Escherichia coli* BL21(DE3). First, the influence of *E. coli* BL21(DE3) cell-lysate on the purified EstA was examined to exclude any possible interference with protein stability. During 30 h of incubation, the enzyme EstA was stable in the presence of *E. coli* BL21(DE3) cell-lysate (Supplementary Figure 3 a).

Recombinant tyrosinases were purified via the N-terminal affinity tag glutathione-S-transferase from *Schistosoma japonicum* (GST tag), which was cleaved off by the cysteine protease 3C from human rhinovirus serotype 14 (HRV 3C) after purification. GST and HRV 3C are active on the tripeptide glutathione and on a specific recognition sequence (LEVLFQ/GP), respectively [32,33]. GST and HRV 3C could potentially contribute to the cleavage of EstA. To exclude this possibility, the two enzymes, GST and HRV 3C, were separately incubated with EstA at the assay conditions. Neither of these compounds showed an effect on EstA (Supplementary Figure 3 a). For the expression of the recombinant tyrosinases, copper sulphate was supplemented to the medium during expression, to permit the correct insertion into the tyrosinase active site. This salt was likewise tested and did not show any cleavage of EstA (Supplementary Figure 3 a).

2.3.3 Esterase activity does not contribute to cleavage

EstA showed the potential to hydrolyse synthetic polyesters [19]. Peptide (amide) bonds differ from ester bonds in the nitrogen atom bound to the carbonyl carbon. The presence of the nitrogen atom in amides confers higher resistance to cleavage since the so-called nitrogen inversion mechanism must take place for the bond to be hydrolysed [34]. However, the serine hydrolase enzyme family can cleave peptides [35] and EstA as a member might have this activity as well [19]. The stability of EstA was tested in each experiment, carrying the reactions out with EstA alone.

To further exclude any auto-proteolysis activity from the natural active site of the enzyme, an inactive EstA variant was constructed (EstA_S182A) and tested. Variant EstA_S182A contains a substitution of the nuclueophilic Ser182 of the catalytic triad to alanine that inactivates the enzyme. Inactivity was tested by esterase activity assays on *p*NPB (Supplementary Figure 4). This variant was incubated with a*Ab*PPO4 and showed identical cleavage pattern to the wild-type EstA (Supplementary Figure 3 b). Therefore, a contribution of EstA to its proteolytic cleavage could be excluded.

2.3.4 Inhibition of tyrosinase activity inhibits the cleavage

In order to characterise the proteolytic activity of PPOs and to potentially identify the presence of a second active site, inhibition of their natural activity, *i.e.* polyphenol oxidase activity, was examined. Sulphites are well-known irreversible inhibitors of PPO activity. The inactivation of PPOs is achieved by binding of the sulphite ion to a histidine residue that coordinates one of the copper ions of the active site, which inhibits the activity irreversibly [36]. In this study, the

loss of PPO activity of a*Ab*PPO4 was verified by an activity assay with dopamine (Supplementary Figure 5). Indeed, inactivation of a*Ab*PPO4 through sodium bisulphite effectively inhibited the proteolysis of EstA (Supplementary Figure 3 c) confirming the involvement of the PPO's active site in proteolytic cleavage.

Furthermore, to confirm these results, the gene encoding for the latent *Ab*PPO4 (l*Ab*PPO4) was mutated, to generate loss-of-function variants. The loss-of-function l*Ab*PPO4 variants, l*Ab*PPO4_(HF)^2 and l*Ab*PPO4_(HW)^2, were constructed to disrupt the coordination of the copper ions in the active site. Accordingly, histidines at positions 91 and 251 were exchanged for phenylalanine in l*Ab*PPO4_(HF)^2 and histidines at positions 57 and 282 to tryptophan in l*Ab*PPO4_(HW)^2. None of the inactive *Ab*PPO4 variants cleaved EstA (Supplementary Figure 3 d). Hence, these results confirmed the multifunctionality of a*Ab*PPO4.

2.3.5 Substitutions in the cleavage site affect the cleavage

Proteases are highly specific for certain amino acid sequences. The physicochemical properties of the amino acids surrounding the cleavage site can influence the specificity and activity of the protease. The peptide motif of the identified cleavage site in EstA was Arg70-Ser71-Ile72 at positions P2-P1-P1' (Figure 7 a), according to the Schechter and Berger nomenclature [37]. These three amino acids are diverse in their physicochemical properties. Arginine is positively charged with a long side chain, whereas serine is relatively small and polar, but uncharged. Isoleucine, moreover, is uncharged, but bulkier and non-polar. In order to characterize the influence of these amino acids on the proteolysis by PPOs, we exchanged these residues in single substitutions for alanine. Alanine is a small, uncharged and non-polar residue and differs substantially from all of the exchanged residues. The variants created were specifically: EstA_R70A, EstA_S71A and EstA_I72A. Cleavage of these variants with a*Ab*PPO4 conveyed diverse results (Figure 7 b). The exchange of Arg70 by alanine led to the biggest change in physicochemical properties, decreasing size and charge. This variant was completely cleaved by a*Ab*PPO4, as shown for wild type EstA (Figure 7 b, lane 7). Proteolysis of EstA_S71A resulted in a similar pattern (Figure 7 b, lane 8). Interestingly, cleavage of the variant EstA_I72A took place, but it was not complete after 30 h (Figure 7 b, lane 9). Proteolysis of this variant yielded less cleavage product in comparison to that of the wild-type enzyme, after 30 h. For this variant, a band at a molecular weight slightly lower than EstA was observed. This band could be an intermediate between EstA and the truncated EstA. Analyses of this product could help elucidate the underlying mechanism. From these results, we can deduce that the amino acid in position P1' is important for the cleavage reaction and the comparatively long side chain of Ile72 is involved in the mechanism.

2.3.6 Cleavage increases hydrolytic activity of the esterase

EstA cleaved by PPOs was truncated by 71 amino acids. The truncation opened the entrance to the active site and exposed a hydrophobic patch on the surface of the enzyme [27]. Hydrophobic regions on the surface can increase the sorption to hydrophobic substrates, such as polyesters [38]. Previously, we have reported the increased activity of the recombinantly expressed truncated version of EstA on the soluble substrate *p*NPB and on the insoluble polyester PET [27]. This truncated EstA missed the same 71 amino acids as did the ΔEstA cleaved by PPOs. The carboxylesterase EstA, truncated by proteolytic cleavage with tyrosinases, exhibited drastically higher activities than both the un-cleaved and the recombinant truncated enzyme. Analysis of PET hydrolysis showed that the total amount of release products, terephthalic acid (Ta) and mono-2-hydroxyethyl terephthalate (MHET), was 32-fold higher than the products released by the full-length EstA (Figure 7 c and d). On the other hand, the EstA recombinantly produced as the truncated carboxylesterase released only 8-fold more hydrolysis products than the wild-type [27]. This could be due to differences in folding caused by the high hydrophobicity present on the surface during the expression of the truncated version. The shielding of hydrophobic patches during expression is important for the correct folding of proteins. Protein folding is driven by the minimalization of energy and the maximization of Van-der-Waals forces. Therefore, hydrophobic residues are usually located on the inside of proteins or in cavities [39,40].

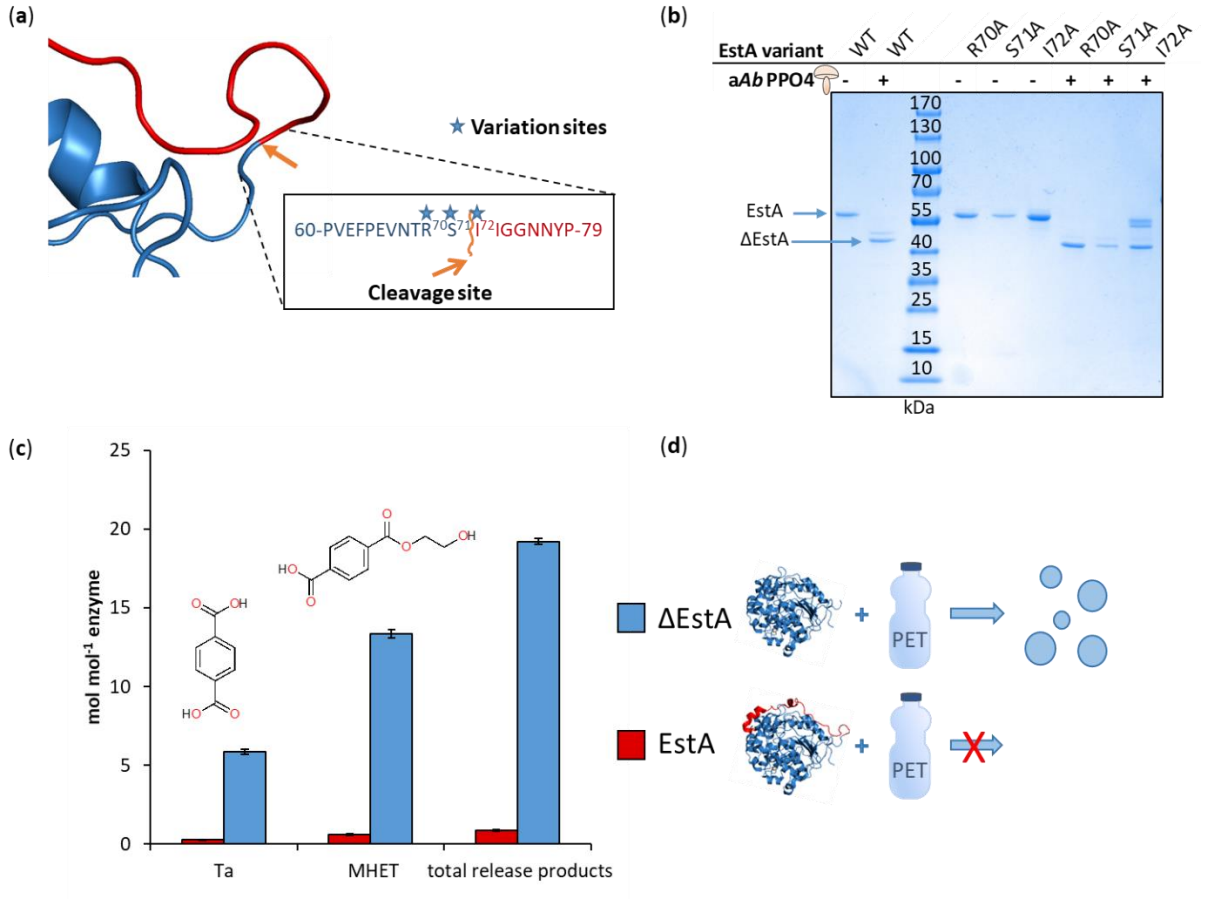

Figure 7: Characterization of EstA variants: EstA variants with substitutions in the cleavage site (R70A, S71A and I72A) were created (**a**) to elucidate the contribution of these residues. The cleavage of these variants with a*Ab*PPO4 was monitored and compared to the cleavage of wild type (WT) EstA (**b**). HPLC analyses of the hydrolysis products showed a higher increase in MHET and Ta after hydrolysis with the truncated (ΔEstA) compared to the full-length EstA. Measurements were performed in triplicates and the standard deviation is given in the error bars (**c**). EstA hydrolysed PET more efficiently when cleaved by tyrosinases (**d**).

2.4 Discussion

In this work, five PPOs from plant and fungal domains of life were tested for their proteolysis activity on the carboxylesterase EstA. Out of the five PPOs, three enzyme products (commercial *Ab*T, a*Ab*PPO4 and a*Md*PPO2) were found to proteolytically cleave EstA. *Ab*PPO4 and *Ab*T are tyrosinases from common mushroom (*A. bisporus*), while *Md*PPO2 is a tyrosinase from apple (*M. domestica*) and therefore they are from different domains of life. Other active PPOs from plants, a*Jr*TYR from walnut (*J. regia*) and a*Cg*AUS from large-flowered tick-seed (*C. grandiflora*) did not show any cleavage of EstA at the tested conditions, despite their high similarity to a*Ab*PPO4 and a*Md*PPO2. A comparison of 3D-models did not reveal crucial differences (Supplementary Figure 6). Thorough analyses of the cleavage mechanism were performed with different forms of *Ab*PPO4.

*Ab*PPO4 cleaved EstA at a specific site between Ser71 and Ile72. The cleavage at this site resulted in the truncation of EstA, removing 71 amino acids at the N-terminus. As a result, the truncated EstA showed a two-fold increased activity towards the soluble substrate *p*NPB and a 32-fold increased hydrolysis of the polymer PET, compared to the full-length enzyme.

Although the activity of ΔEstA was drastically improved, its activity is still lower than the activity of PETase from *Ideonella sakaiensis* [41]. EstA is, however, an anaerobic enzyme, expressed under aerobic conditions. Anaerobic conditions might increase its activity on PET. Therefore the comparison of the two enzymes is difficult. Analysis of auto-proteolysis and activity of impurities, potentially present in the enzyme preparations, showed that the cleavage of EstA was dependent on the presence of tyrosinases.

In our study, we described for the first time the cleavage of a full-length protein by tyrosinases. Therefore, it is probable that this cleavage followed a different mechanism compared to previously reported studies [42]. Recently, it was reported that an isoform of the *Md*PPO2 (namely *Md*PPO1) from *M. domestica* showed self-cleavage due to a peptide present in the linker between the C-terminal domain and the active site. In the mentioned study, the self-cleavage was deactivated only upon deletion of the entire peptide, while mutagenesis experiments did not influence the self-cleavage of *Md*PPO1 [9]. The (auto)proteolytic process of PPOs does not resemble the mechanism of any of the known proteases like serine proteases, cysteine proteases or metalloproteases. PPO-induced proteolysis is quite slow (hours to days), while proteases usually cleave their substrate in seconds or minutes. Proteases often contain catalytic triads (serine [43] or cysteine proteases [44]) or metals like zinc (metalloproteases [45]) at their active sites, which function as activators for water, thereby allowing for hydrolytic peptide bond cleavage. In PPOs, however, similar catalytic motifs are not present and therefore their proteolytic activity cannot be explained with the known protease mechanisms.

Models of *Ab*PPO4 show a single active site. Furthermore, the presented results that focused on the inactivity of *Ab*PPO4, showed that the cleavage was correlated with tyrosinase activity. Therefore, we can conclude that the active site of *Ab*PPO4 plays a crucial role in the presented reaction. The described results point to a catalytic promiscuity of *Ab*PPO4 for proteolytic activity.

Substitutions of the residues Arg70, Ser71 and Ile72 of EstA to alanine suggested the importance of the amino acid Ile72, which is directly adjacent to the cleavage site. Further substitutions would be needed to determine the exact influence of residues, which are proximal and distal to the cleavage site. Furthermore, substitutions to amino acids, other than alanine, could identify side chains that are important for the reaction.

This is the first time an intermolecular proteolysis of folded proteins by tyrosinases has been reported, to our knowledge. Multifunctionality of enzymes is very attractive for industrial applications. Further investigations of this newly found activity of PPOs might not only lead to new applications, but could help understand the promiscuity of this very important and useful class of enzymes.

2.5 Methods

2.5.1 Chemicals and reagents

Pre-stained protein marker IV was obtained from Peqlab (Germany) and the protein determination kit was from Biorad (USA). All other chemicals and reagents were purchased from Sigma Aldrich (Germany) or Carl Roth (Germany) and were at least of analytical grade. The commercially available *Ab*T, sodium bisulphite as well as the substrates *p*NPB and dopamine hydrochloride were procured from Sigma Aldrich. Q5® High-Fidelity DNA Polymerase, *Sap*I and T4 DNA ligase were purchased from New England Biolabs (NEB, Germany) and *Esp*3I from Fisher Scientific (Austria). The pGEX-6P-1 (GE Healthcare, Austria) and pENTRY-IBA51 (IBA, Germany) vectors were used as templates and nucleotide primers were synthesised by Sigma-Aldrich.

2.5.2 Construction and cultivation of bacterial strains

The strain *E. coli* BL21(DE3) was used for the expression of all esterases in lysogeny broth (LB) medium supplemented with the appropriate antibiotic. The esterase EstA and its variants were cloned into the expression vector pET26b(+) and expressed in LB medium containing 40 µg mL^{-1} kanamycin sulphate. Media, bacterial strains and plasmids for the production of *Ab*PPO4 and *Md*PPO2 were as previously described [21,28].

For the generation of enzymatically inactive variants of *Ab*PPO4 the gene encoding the latent form of the tyrosinase [21] was cloned into the pENTRY vector. Therefore the required *Sap*I recognition sequences was added to both ends using the two primers pENTRY-*lAbPPO4*_fwd and pENTRY-*lAbPPO4*_rev. The amplicon was cut-ligated [46] into pENTRY-IBA51 using *Sap*I and T4 DNA ligase. Mutagenesis was carried out in two steps introducing one point mutation each, applying the Q5® Site-Directed Mutagenesis Kit (NEB, Germany). The four pairs of mutagenic primers (Supplementary Table 4) targeted one histidine at the active centre each (H91F and H251F for l*Ab*PPO4_$(HF)^2$, H57W and H282W for l*Ab*PPO4_$(HW)^2$). Sequences were verified by Sanger sequencing (Microsynth, Austria). The genes with the newly introduced bulky amino acids at the copper-coordination site were subcloned into the expression vector pGEX-6P-SG (*vide infra*) by cut-ligation with the type IIS restriction endonuclease *Esp*3I and T4 DNA ligase.

The adaptation of the pGEX-6P-1 vector to the StarGate® cloning system (IBA, Germany) was carried out by Q5® Site-Directed Mutagenesis in two steps. In the first step the two recognition sequences for *Esp*3I in pGEX-6P-1 were removed. One of them was removed by introducing a silent point mutation in the recognition sequence located inside the *lac*I gene (primers pGEX-6P-1_C4295A_fwd & rev) and the other by deletion of a 121 bp segment containing the multiple cloning site (primers pGEX-6P-1 -> pGEX-6P-SG_fwd & rev). The primers for the deletion step were also used to introduce 28 bp containing the two *Esp*3I recognition (6 bp) and cutting (5

bp) sequences required for the StarGate® cloning system as well as a single *Sma*I recognition site (6 bp) located between the two nonpalindromic *Esp*3I recognition sequences.

2.5.3 Expression and purification of enzymes

The enzymes were expressed and purified. EstA wild-type and variants were expressed and purified as described by Perz *et al* [19]. Genes encoding the EstA variants were codon optimized for the *E. coli* codon usage and synthesized in a pET26b(+) vector by GenScript (USA). The activated forms of a*Ab*PPO4 and a*Md*PPO2 as well as the enzymatically inactive variants l*Ab*PPO4_(HF)^2 and l*Ab*PPO4_(HW)^2 were prepared as previously described [21,28]. *Jr*TYR was isolated from walnut leaves and purified applying cation exchange chromatography [29]. *Cg*AUS1 was isolated from the petals of *C. grandiflora* and purified using a series of anion and cation exchange steps [16].

2.5.4 Determination of proteolytic activity of polyphenol oxidases

For the proteolysis of EstA and its variants, 4 mM of each esterase variant were mixed with 0.6 µM of PPOs in 0.1 M Tris-HCl buffer pH 6. The final reaction volume was 300 µL. The reactions were carried out at 23 °C and shaking at 300 rpm for 30 hours, if not stated otherwise. Each reaction was done at least twice to replicate the results. To characterise the cleavage reactions, various substances were added to the reaction mixture in place of the PPOs. Specifically, 0.5 mM copper sulphate, 0.6 µM GST, 1.3 µM HRV 3C and 30 µL of the *E. coli* BL21(DE3) cell-lysate were individually added. The *E. coli* lysate was obtained through the lysis of *E. coli* BL21(DE3) by ultrasonication three times for 45 sec with an amplitude of 60% and 2 min breaks in between sonication steps. The *E. coli* lysate was prepared by centrifugation at 20 817 x *g* for 5 min.

Latent *Ab*PPO4 variants, l*Ab*PPO4_(HF)^2 and l*Ab*PPO4_(HW)^2, were activated by the addition of 2 mM sodium dodecylsulphate (SDS) to the reaction mixture. To inhibit tyrosinase activity, a*Ab*PPO4 was incubated with 10 mM sodium bisulphite for 1 h at 23 °C. The tyrosinase was subsequently separated from the sodium bisulphite by ultrafiltration with Vivacon 2 ultrafiltration devices with 30 kDa cut-off and a Hydrosart® membrane (Sartorius, Germany) and washing with 400 µL 0.1 M Tris-HCl buffer pH 6. The washing and centrifugation was repeated three times. Inhibition was verified by measurement of the activity on 15 mM dopamine. The activity was measured photometrically at 475 nm (ε_{475} = 3.1) in 0.1 M Tris-HCl buffer pH 6 (Supplementary Figure 7).

2.5.5 Characterization of enzymes

2.5.5.1 Protein analyses

Protein concentration was determined by Bradford Assay [47] with a Bio-Rad Protein determination kit (Bio-Rad, USA) using bovine serum albumin as a protein standard. Photometric measurements were performed with a Tecan plate reader Infinite M200 PRO

(Tecan, Switzerland). Proteolytic cleavage of EstA and its variants as well as the purity of freshly produced enzymes was determined with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.[48] Precast gels were purchased from Bio-Rad (USA), with 4% stacking and 15% separating gel and were run at 150 V. As molecular mass marker pre-stained protein marker IV (Peqlab, Germany) was used. Gels were stained with 1.25 g L-1 Coomassie Brilliant Blue solution containing 30% ethanol and 10% acetic acid. Destaining was performed with the same solution excluding Coomassie Brilliant Blue G 250.

2.5.5.2 Biochemical characterization

The activity of the carboxylesterases was determined spectrophotometrically with the model substrate *p*NPB (100 µM final concentration) in 0.1 M potassium phosphate buffer pH 7 (ε_{405} = 8.31 M^{-1} cm^{-1}).[49,50] Enzymatic activity of polyphenol oxidase preparation was tested in 50 mM sodium citrate buffer pH 6.8 at 25 °C with 1 mM L-tyrosine as the substrate and 2 mM SDS for activation of latent enzymes [21]. The resulting formation of dopachrome (ε_{475} = 3600 M^{-1} cm^{-1}) was monitored photometrically and the enzymatic activity was determined from the linear part of the absorption-time curve. One unit (U) of enzymatic activity was defined as the amount of enzyme catalysing the formation of 1 µmol reaction product per minute under the conditions specified above.

2.5.5.3 Mass spectroscopy

For LC-MS/MS analysis protein bands were excised from gels, reduced, alkylated and tryptically cleaved according to the manufacturer´s instructions (Promega, USA). Peptide extracts were dissolved in 0.3% formic acid, 5% acetonitrile and separated by nano-HPLC (Dionex Ultimate 3000) equipped with a µ-precolumn (C18, 5 µm, 100 Å, 5 x 0.3 mm) and a Acclaim PepMap RSLC nanocolumn (C18, 2 µm, 100 Å, 150 x 0.075 mm) (all Thermo Fisher Scientific, Austria). Samples of 20 µL were injected and concentrated on the enrichment column for 2 min using 0.1% formic acid as isocratic solvent at a flow rate of 5 µL min^{-1}. The column was then switched into the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 250 nl min^{-1} at 60 °C and separated using the following gradient: solvent A: water, 0.1% formic acid; solvent B: acetonitrile, 0.1% formic acid; 0-2 min: 4% B; 2-90 min: 4-25% B; 90-95 min: 25-95% B, 96-110 min: 95% B; 110-110.1 min: 4% B; 110.1-125 min: 4% B. The sample was ionized in the nanospray source equipped with stainless steel emitters (Thermo Fisher Scientific, Austria) and analyzed in a Thermo Orbitrap Velos Pro mass spectrometer in positive ion mode by alternating full scan MS (m/z 380 to 2000) and MS/MS by CID of the 20 most intense peaks. The LC-MS/MS data were analyzed by searching a homemade database containing the protein sequence and common contaminations with Mascot 2.3 (MatrixScience, UK) and Proteome Discoverer 1.3. Carbamidomethylation on cysteine was entered as fixed and oxidation on methionine as variable modification. Detailed search criteria were used as follows: semi trypsin; max. missed cleavage sites: 2; MS/MS ion

search with decoy database search included; precursor mass tolerance +/- 0.05 Da; product mass tolerance +/- 0.7 Da; acceptance parameters: $p < 0.05$; minimum 2 peptides; ion score cut off: 20 and FDR 1%.

2.5.6 Degradation of Polyester

Amorphous PET films (0.5 x 1 cm) were thoroughly washed [27]. Washed PET films were incubated with 5 µM EstA in 0.1 M potassium phosphate buffer pH 7 at 50 °C and 100 rpm for 120 h. Supernatants were collected and analysed by high performance liquid chromatography (HPLC) (Agilent Technologies, USA)[27].

2.5.7 Modelling

Structural data for the enzymes was derived from the PDB using the following entries: 5AH1 (EstA), 5M6B (a*Ab*PPO4), 5CE9 (a*Jr*TYR1), 4Z0Y (a*Cg*AUS1). For a*Md*PPO2 a homology model was prepared using the SWISS-MODEL Server [51] with the coordinates of *Md*PPO1 (59.6% sequence identity, PDB entry 6ELS) as the template.

2.6 Data Availability

The datasets generated and/or analysed during the current study are included in the publication and supplymentary information or available from the corresponding author on reasonable request or publicly available on Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB; a member of wwPDB; https://www.rcsb.org/) (PDB IDs for individual structures are given in the manuscript).

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2.8 Author Contributions

A.B. and V.B. performed the main experiments and analysed the data. M.P., I.K. and A.B. produced the enzymes. B.D. and R.B. performed MS analyses. D.R., A.B. and V.B. planned the experiments. A.B. and V.B. wrote the manuscript. D.R. and G.M.G. corrected the manuscript. A.R., D.R. and G.M.G. supervised the work. All authors discussed the presented data and commented on the manuscript.

2.9 Competing interests

The authors declare no competing interests.

2.10 Figure legend

2.11 References

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3 Comparison of a fungal and a bacterial laccase for lignosulfonate polymerization

Verena Braunschmid [a,b], Karin Binder [b], Sarah Fuerst [b], Raditya Subagia [a], Caroline Danner [a], Hedda Weber [c], Nikolaus Schwaiger [c], Gibson S. Nyanhongo [a,b], Doris Ribitsch [a,b,*] and Georg M. Guebitz [a,b]

[a] *Institute of Environmental Biotechnology, Department of Agrobiotechnology, University of Natural Resources and Life Sciences (BOKU), 3430 Tulln an der Donau, Austria; verena.braunschmid@boku.ac.at (V.B.); raditya.subagia@boku.ac.at (R.S.); carolinedanner0@gmail.com (C.D.); g.nyanhongo@boku.ac.at (G.S.N.); guebitz@boku.ac.at (G.M.G.)*

[b] *Austrian Centre for Industrial Biotechnology (ACIB), 3430 Tulln an der Donau, Austria; karin.binder@boku.ac.at (K.B.); sarah.fuerst@boku.ac.at (S.F.)*

[c] *Sappi Papier Holding GmbH, 8101 Gratkorn, Austria*

***** *Corresponding author. Tel.: +43 1 47654-97487; fax: +43 1 47654-97409. E-mail address: doris.ribitsch@boku.ac.at.*

Abstract: Lignin is the second most abundant biopolymer on earth and accrues in large amounts in the pulp- and paper industry. While currently lignins are mainly used for low-value applications such as energy production, recently the potential of laccases for upgrading lignins has been demonstrated. In this study, two laccases of fungal and bacterial origin were characterized regarding their potential to polymerize lignosulfonate. The laccases *Ma*L1 from *Melanocarpus albomyces*, and *Sr*LA from *Streptomyces rochei* were heterologously expressed and showed typical characteristics of laccases, like acidic pH optima for ABTS at pH 4 and 5, respectively, and temperature optima at 50 °C and 80 °C. Polymerization of lignosulfonate with *Ma*L1 led to an almost two-fold increase of the molecular weight, according to size exclusion (SEC) multiangle laser light scattering (MALLS) analysis. In contrast, *Sr*LA showed considerably less activity on lignosulfonates, as measured based on oxygen consumption and SEC-MALLS.

Keywords: laccase, lignin, lignosulfonate polymerization

3.1 Introduction

Laccases are a well explored group of enzymes belonging to the multi-copper oxidase (MCO) family. MCOs are oxidoreductases that oxidize various substrates while reducing oxygen to water. As their name suggests, MCOs contain four copper ions, organized in two sites. The type 1 copper obtains one electron from a substrate, transferring it via an His-Cys-His motive to the trinuclear center. The trinuclear center contains one type 2 and two type 3 copper ions and is the site of the oxygen reduction to water. In this reaction, four electrons from substrate molecules are transferred to one molecule of O_2, reducing it to two molecules of water [1]. Laccases can oxidize various substrates, including phenols, aminophenols, diamines and some inorganic ions [2]. Their broad substrate range is necessary for the wide variety of functions laccases perform in nature. Plant laccases are vital for the synthesis of lignin [3], whereas fungal laccases are involved in the degradation of lignin for wood-decay, detoxification, pathogenesis and fungal morphology [2]. Bacteria use their laccases for example for pigmentation, copper homeostasis, degradation of toxic compounds and degradation of lignin [4]. The multitude of biological functions is mirrored in the wide variety of industrial applications of laccases. In food industry laccases are, for instance, employed for reducing oxygen content in beer to increase its shelf life or for the treatment of cork to avoid cork-taste in wine. Additionally, many commercial laccase-based products have been developed for the textile industry, for example to bleach denim fabric. The bleaching ability of laccases has furthermore been exploited for the whitening of flax-pulp, as well as in cosmetics for hair and skin. Another interesting ability of laccases is its ability to induce polymerization of certain phenols. This has been employed for the binding of medium-density fiber boards to avoid the usage of toxic formaldehyde [5].

Lignin is the second most abundant natural polymer on earth. It provides structural integrity to plants, forming a complex matrix to strengthen the cell walls [6]. The biosynthesis of lignin is catalyzed by laccases and peroxidases via a radicalization of its three main precursors, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These radicals cross-link with each other to form an irregular polymer [7]. Lignin comprises a major part of lignocellulosic biomass [6] and accrues in great quantities in cellulose extracting industries. This industrially produced lignin, called technical lignin, is a main by-product of the pulp and paper industry and will accumulate in large amounts from the emerging second-generation biofuel production. Depending on the cellulose extraction mechanism used, different technical lignins, such as lignosulfonate, KRAFT lignin or organosolv lignin are produced [8]. The majority of the 100 million tons of technical lignin produced each year are used for energy production [9]. To a small extent, technical lignin has been employed in value-added applications, for example as low-cost binders in pigment composites or plywood. Furthermore, lignin has the potential to be a valuable precursor for high-value chemicals and materials. Vanillin, aldehydes, DMSO and

other platform chemicals could be produced from lignin, due to its richness in aromatic compounds [9]. In addition, there is a large amount of research for the production of lignin-based materials, such as carbon fibers [10] and materials to replace petroleum-derived precursors in polyesters [11]. Further studies have shown the potential of technical lignin as coating agents for paper [12] or pesticide delivery-systems [13]. The two last mentioned applications require the polymerization of technical lignin with laccases.

The polymerization of technical lignin proceeds analogously to the biosynthesis of lignin. Laccase-mediated radicalization leads to cross-linking of the fractionated lignin present in technical lignin. Several studies have been aimed at optimizing and upscaling this process. The ensuing material is water-insoluble, but brittle. The addition of plasticizers has been shown to improve its characteristics, creating opportunities for wider application of this lignin-material [14]. The polymerization of lignin is strongly influenced by reaction conditions like oxygen-supply [15], the type of technical lignin and lignin concentration [16]. Traditionally (potentially toxic) mediators were employed for lignin polymerization, while more recently it has been shown that laccases alone can efficiently polymerize lignosulfonates, provided that sufficient oxygen is supplied [17]. Subsequently, mediator-free modification of lignosulfonate with laccases has been applied for paper-coating binders [12], pesticide delivery systems [13] and to study the mechanism and material in more depth [14,15]. When laccases are applied without mediators, the properties of the enzyme play a crucial role, since, in contrast to mediator-supported reactions, it interacts directly with the substrate. Presently, a fungal laccase from *Myceliophthora thermophila* (*Mt*L) is mainly applied for the polymerization of lignosulfonate [12–14,16]. However, only few other laccases, mainly fungal, like *Trametes villosa* laccase [15], have been assessed for this purpose. Hence, there is a need to investigate the potential of novel laccases, including bacterial sources, for lignosulfonate polymerization. This study focuses on the characterization of two laccases for the polymerization of lignosulfonate.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

If not indicated otherwise, chemicals were obtained from Sigma–Aldrich (Munich, Germany). Lignosulfonate was kindly provided by Sappi Papier Holding GmbH, Gratkorn, Austria. The commercial laccase MtL was obtained from Novozymes (51003; Bagsværd, Denmark).

3.2.2 Expression and Purification

The fungal laccase MaL1 (CAE00180.1) was expressed in *Pichia pastoris*. The gene was codon optimized for *P. pastoris*, a C-terminal Strep-tag added and cloned into a pPICZαB vector by GenScript (GenScript Biotech, Leiden, The Netherlands). Glycosylation sites were determined by in silico with NetNGlyc 1.0 Server (DTU Health Tech, Kongens Lyngby, Denmark). The bacterial laccase, *Sr*LA (WP_086876778.1), was expressed in *Escherichia coli*. The *Sr*LA gene sequence was codon optimized for *E. coli* and equipped with a C-terminal

Strep-tag. The gene was synthesized in a pET-26b(+) plasmid by GenScript. For amplification, all plasmids were transformed into *E. coli* XL10 (Agilent Technologies, Santa Clara, CA, USA). Cells were grown over night in a Lennox Broth (LB; Carl Roth, Karlsruhe, Germany) at 37 °C. The plasmids were purified with a Promega Midiprep kit (Madison, WI, USA).

The pPICZαB vector containing the gene coding *Ma*L1 was linearized with SacI (New England Biolabs, Ipswich, MA, USA). The transformation into *P. pastoris* KM71H (ThermoFisher Scientific, Waltham, MA, USA) was achieved by electroporation according to manufacturer's protocol (MikroPulserTM, Bio-Rad, Hercules, CA, USA). Transformed cells were grown on yeast extract peptone dextrose agar containing 100 µg/mL zeocin (Alfa Aesar, Ward Hill, MA, USA). Clones containing inserts were determined by colony PCR. For expression, clones containing the insert were cultivated in 100 mL buffered glycerol complex medium with 0.1 M phosphate buffer (BMGY) medium supplemented with 100 µg/mL zeocin, at 28 °C and 150 rpm overnight (OD_{600} = 2-6). The cells of this culture were harvested and resuspended in 10 mL BMGY + zeocin medium and cultivated for 72 h at 28°C and 150 rpm in 100 mL baffled flasks. Gene expression was induced twice a day by the addition of 0.5% methanol. At the first induction, 0.1 mM $CuSO_4$ was added. Samples were taken after 0, 5, 24, 48 and 72 h and deglycosylated by EndoH (New England Biolabs, Ipswich, MA, USA,). Deglycosylated as well as glycosylated samples were analyzed by SDS-PAGE. After 72 h, the culture was centrifuged for 20 minutes at 3428 rcf and the supernatant, containing the enzyme stored at -20 °C. *Ma*L1 was purified from the supernatant and concentrated with Vivaspin20 (Sartorius AG, Göttingen, Germany), followed by buffer exchange to 0.1 M TRIS/HCl buffer pH 7 (Trizma®base) with PD10 desalting columns (GE Healthcare, Chicago, IL, USA).

The *Sr*LA gene was expressed in *E. coli* BL21-Gold(DE3) (Agilent Technologies, Santa Clara, CA, USA). It was transformed by heat-shock and selected on nutrient agar (Merck, Darmstadt, Germany) containing kanamycin sulfate (Carl Roth, Karlsruhe, Germany) at a final concentration of 40 mg/mL. Freshly transformed *E. coli* was cultivated overnight in LB + kanamycin medium at 37 °C and 150 rpm. This culture was used to inoculate 200 mL LB + kanamycin medium to an OD600 of 0.1. The cells were grown at 30 °C and 120 rpm. At an OD_{600} of 0.6 the gene expression was induced with 0.05 mM isopropyl-ß-D-thiogalactopyranoside (IPTG; Santa Cruz Biotechnology, Heidelberg, Germany). Copper was added in form of 0.25 mM $CuSO_4$ and the temperature was decreased to 25 °C. The culture was incubated at 120 rpm for four more hours. Then the shaking was stopped, and the cells cultivated for 20 h at 25 °C, without shaking. The cells were harvested by centrifugation at 3428 rpm for 30 minutes and the pellet stored at -20 °C. Cells were lysed using sonication (Digital sonifier, Branson, Danbury, CT, USA) with 5 pulses of 10 seconds each, interspersed by 2 minutes breaks, while cooled on ice. Cell debris was cleared away by centrifugation at 20800 rcf for 15 minutes and filtering through 0.22 m polyethersulfone filters. SrLA was purified

via strep-avidin affinity chromatography according to manufacturer's protocol (IBA GmbH, Goettingen, Germany) using an ÄKTApure system (GE Healthcare, Chicago, IL, USA).

The BioRad Bradford assay (Bio-Rad, Hercules, CA, USA) was used according to manufacturer's instructions for protein concentration determination with bovine serum albumin (BSA, Fermentas, Waltham, MA, USA) as protein standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to Laemmli [18] was applied to determine protein size and purity, as well as expression success. Proteins were separated in precast 4-15% stain-free gels (Mini PROTEAN TGX; BioRad, Hercules, CA, USA). Pre-stained protein marker IV (Peqlab, Erlangen, Germany) was run alongside enzymes as protein standard. Proteins were stained with Coomassie Brilliant Blue.

3.2.3 Copper determination

The QuantiChromTM Copper Assay Kit (DICU-250; BioAssay Systems, Hayward, CA, USA) was used according to manufacturer's protocol for 96-well plates for spectrophotometric (Infinite 200 Pro; Tecan, Männedorf, Switzerland) copper determination. Samples were diluted 1:4 in buffer from original protein concentrations of 0.68 mg/mL for *Ma*L1, and 1.02 mg/mL for *Sr*LA.

3.2.4 Laccase activity assay with ABTS

The activity of the enzymes on ABTS (Alfa Aesar, Ward Hill, MA, USA) was determined spectrophotometrically by the time-dependent change of absorbance at 420 nm. Enzymes were diluted in buffer and mixed with 10 mM ABTS dissolved in water. The absorbance was measured immediately in 20 cycles in 10 s intervals. For the determination of the pH optima, the pH of the dilution buffer was varied from pH 3 to pH 8 at 25 °C. For pH 3, citrate-phosphate ($\varepsilon_{420\ nm,\ pH\ 3}$ = 34.683 L/mmol/cm) was used. Sodium acetate buffer ($\varepsilon_{420\ nm,\ pH\ 4}$ = 33.115 L/mmol/cm, $\varepsilon_{420\ nm,\ pH\ 5}$ = 31.332 L/mmol/cm) was used for pH values 4 and 5. For the pH values from 6 to 8 sodium phosphate buffer ($\varepsilon_{420\ nm,\ pH\ 6}$ = 21.103 L/mmol/cm; $\varepsilon_{420\ nm,\ pH\ 7}$ = 12.070 L/mmol/cm; $\varepsilon_{420\ nm,\ pH\ 8}$ = 5.251 L/mmol/cm) was applied. The temperature optimum was determined at the optimal pH of 4 for MaL1 and 5 for *Sr*LA, in a temperature range from 20 °C to 70 °C. For the measurements at different temperatures a photometer with connected water bath (Hitachi U-2900; Metrohm Inula GmbH, Vienna, Austria) was used. One unit of enzyme activity was defined as one µmol of product generated per minute.

For determination of kinetic parameters, V_{max} and K_m, activity was determined with ABTS concentrations in a range from 0.5 to 30 mM at 25 °C and optimal pH values. Lineweaver-Burk diagrams were applied to calculate the kinetic parameters [19].

Storage stability of enzymes was assessed by regular activity measurements during the incubation of the enzymes. Enzymes were incubated at 23 °C for 72 h, at pH 7 and at their optimal pH value.

3.2.5 Substrate screening

The substrates 2,6-dimethoxyphenol (DMP; f.c. 0.1 mM), syringaldezine (f.c. 0.05 mM), catechol (f.c. 2 mM), guaiacol (f.c. 20 mM), sinapic acid (f.c. 0.1 mM), ferulic acid (f.c. 0.1 mM), vanillic acid (f.c. 0.1 mM), gallic acid (f.c. 0.1 mM), and tannic acid (f.c. 0.1 mM) were dissolved according to their solubility in buffer, methanol or water and subsequently diluted to their final concentration in buffer. Sodium-acetate buffer pH 4 (100 mM) was used, except for catechol, which was diluted in sodium-acetate buffer pH 5 (50 mM), and syringaldezine, which was diluted in sodium-phosphate buffer pH 6 (100 mM). Enzymes were incubated for 30 minutes with the substrates and monitored by a wavelength scan from 200 nm to 800 nm in 1 nm steps. As a control, buffer was incubated with the substrates and measured likewise. Activity of enzymes on substrates was appraised by differences in the spectra of substrates incubated with enzymes to controls.

3.2.6 Lignin polymerization

Dried lignosulfonate was dissolved in distilled water to a concentration of 10 or 20% (w/V). The dissolved lignosulfonate had a pH of 3.4. Other pH values were set with 5 M NaOH. For the polymerization reaction, enzymes were added at final concentrations of 0.05 U/mL for the pH optimum of *Ma*L1, 0.025 U//mL for the comparison of *Ma*L1 to *Mt*L, and 0.25 U/mL for *Sr*LA to start the reaction. The final lignosulfonate concentration was 10% (w/V) in a volume of 1.5 mL. Samples were stirred at approximately 800 rpm at 23 °C for 20 h.

3.2.7 Oxygen measurement

The oxygen content in the reaction mixture was measured optically with a sensor dye immobilized inside the reaction vessel and an optical probe (FirestingO2; PyroScience GmbH, Aachen, Germany). A 2-point calibration was performed before each measurement, setting 100% oxygen content at oxygen concentration of ambient air and 0% oxygen content in the presence of pure nitrogen.

3.2.8 Size exclusion chromatography

The molecular weight of lignosulfonate was determined via size exclusion chromatography (SEC) as previously described [13]. Lignosulfonate samples were diluted in mobile phase, 50 mM $NaNO_3$/ 3 mM NaN_3, to a final concentration of 1 mg/mL. As a standard, 1 mg/mL BSA was used for normalization, band broadening and alignment of the MALLS detector. Of the samples 100 µL were injected into the chromatography system, containing a PL aquagel-OH Mixed Guard column (PL1149-1840, 8 µm, 7.5 × 50 mm^2, Agilent, Santa Clara, CA, USA) and PL aquagel-OH MIXED H separation column with a mass range of 6-10000 kDa (PL1549-5800, 4.6 × 250 mm^2, 8 µm, Agilent, Santa Clara, CA, USA). The system was further equipped with a quaternary/binary pump, an autosampler 1260 series from Agilent Technologies (Santa Clara, CA, USA). As detectors a diode array detector (DAD), a refractive index (RI) detector (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) and a MALLS HELEOS DAWN II detector (Wyatt Technologies, Dernbach, Germany) were used. The run time was 90 minutes.

For data acquisition and analysis, the software programs Openlab Chemstation CDS (Agilent Technologies, Santa Clara, CA, USA) and ASTRA (Wyatt Technologies, Dernbach, Germany) were applied.

3.3 Results and Discussion

3.3.1 Laccases

In this study, a fungal and a bacterial laccase, namely *Ma*L1 from *Melanocarpus albomyces* (*Ma*L1; CAE00180.1) and *Sr*LA from *Streptomyces rochei* (*Sr*LA; WP_086876778.1) were investigated in terms of their ability to polymerize lignosulfonate. The laccases were selected to represent a variety of characteristics which could have an influence on polymerization of lignosulfonates in the absence of redox mediators. The laccase *Ma*L1 originates from a thermophilic ascomycete. Fungal laccases are known for their comparatively high redox potential [5]. *Ma*L1 has been previously described to have activity at an alkaline pH range and pronounced thermal stability [20]. These characteristics make *Ma*L1 a promising candidate for various applications, including lignin polymerization. The laccase *Sr*LA exhibits high similarity to a laccase from a different *Streptomyces* species, namely *S. ipomoea* (*Si*LA), with a homology of 99.7% (Figure S1). *Si*LA is highly stable at an alkaline pH and at high salt concentrations [21]. *Ma*L1 was previously shown to polymerize KRAFT and organosolv lignin under alkaline conditions [22]. Therefore, these enzymes were expected to be capable of lignosulfonate polymerization.

*Ma*L1 and *Sr*LA were heterologously expressed in *Pichia pastoris* (*Ma*L1) and *Escherichia coli* (*Sr*LA). The amino acid sequence of the fungal laccase *Ma*L1 exhibits nine potential N-glycosylation sites (Figure S2). Glycosylation is essential for the functionality of most glycoproteins. Therefore, *Ma*L1 was expressed in the eukaryotic expression system *P. pastoris*, which can perform N-glycosylation. N-Glycosylation by *P. pastoris* consists of two N-Acetylglucosamine residues and a branched oligomannosyl chain of nine to sixteen residues [23]. Expression of the codon-optimized *Ma*L1, including a C-terminal Strep-tag, was successful, as shown by activity on 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) of the supernatant. SDS-PAGE of samples taken during the expression confirmed expression of *Ma*L1, indicated by a protein band appearing at the molecular mass of the enzyme at 70 kDa after deglycosylation (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This size fits well to the theoretical size, based on the amino acid sequence of 67.9 kDa and the reported size of *Ma*L1 of 71.3 kDa [24]. Deglycosylation of *Ma*L1 led to a shift in the SDS-PAGE by approximately 30 kDa. The calculated molecular weight for N-glycosylations by *P. pastoris* lies between 2.1 and 3.3 kDa [23]. The average size of each glycosylation of *Ma*L1 was 3.3 kDa, according to the 30 kDa shift in the SDS-PAGE and the nine potential sites. Therefore, we can assume that each potential N-glycosylation site was glycosylated. The purification via affinity chromatography was, however, not possible, probably

due to the tag being hidden inside the enzyme structure or it being cleaved off. The secretion of the enzyme into the medium allowed the harvest of pure enzyme by removal of the media components through buffer exchange. The bacterial laccase *Sr*LA was codon optimized for and expressed in *E. coli* under micro-aerobic conditions and purified via Strep-Tactin® affinity chromatography. *E. coli* is a convenient expression host for bacterial proteins when glycosylation is not necessary. *E. coli* exhibit an increased level of intracellular copper when oxygen supply is low. The elevated copper content in the cells boosts the incorporation of copper into enzymes [25]. *Sr*LA exhibited a slightly higher molecular weight of 38 kDa, according to SDS-PAGE, than the 34 kDa expected based on the amino acid sequence (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This slight increase could be due to the presence of acidic amino acids [26]. For the correct folding and activity of the expressed laccases, copper was added to the media. Copper content determination showed a copper content of 5.17 mol/mol enzyme for *Ma*L1 and of 3.96 mol/mol enzyme for *Sr*LA. These values are in accordance with the four copper ions expected for a fully copper loaded laccase [1].

3.3.2 Biochemical characterization

The optimal operation conditions of *Ma*L1 and *Sr*LA were determined on the standard substrate ABTS. *Ma*L1 and *Sr*LA exhibited their highest activity at pH 4 and pH 5 respectively (**Fehler! Verweisquelle konnte nicht gefunden werden.**a). The pH optimum of *Ma*L1 was broad and there was only a minor difference in activity between pH values of 3 and 5. *Sr*LA showed a pronounced pH optimum at pH 5. Laccases generally prefer an acidic environment for the conversion of ABTS, with optimal pH values usually between 3 and 5. The optimal pH of laccases, however, depends strongly on the substrate [2]. The laccase from *Myceliophthora thermophila*, (*Mt*L) for example, was reported to catalyze ABTS most efficiently at a pH below 3 [27,28], while the optimum for polymerization of lignosulfonates was reported to be at pH 7 [12].

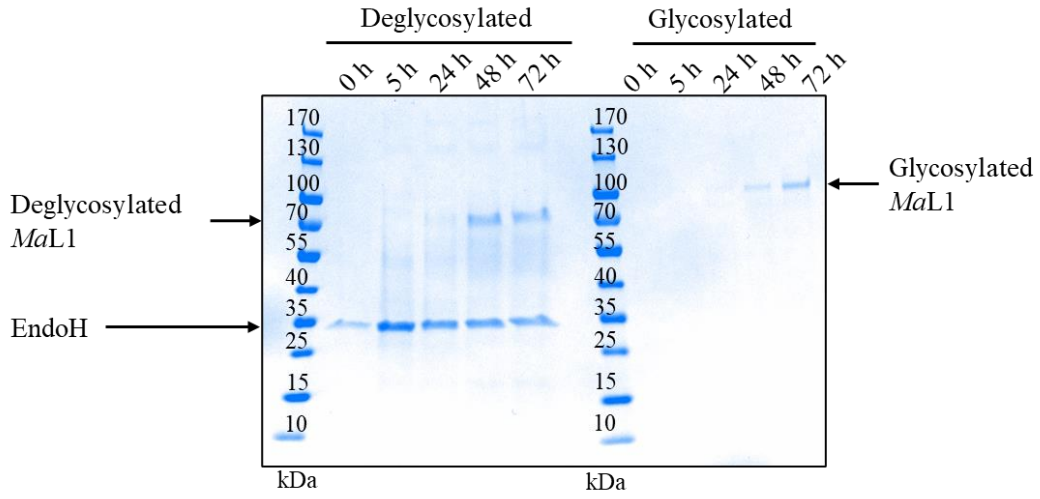

Fig. 1: Surveillance of enzyme expression of *Ma*L1: SDS PAGE of samples of supernatant of the expression culture taken after 0, 5, 24, 48 and 72 h.

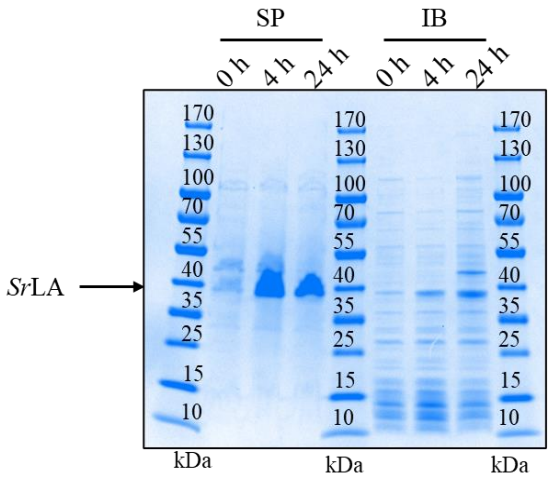

Fig. 2: Surveillance of *Sr*LA production: SDS PAGE of soluble proteins (SP) and inclusion bodies (IB) of E. coli culture at 0, 4 and 24 h of expression time.

On ABTS as a substrate and at 23 °C, the bacterial laccase *Sr*LA revealed a turnover number of k_{cat} = 2.12 ± 0.11 s^{-1} at its pH optimum of 5. In comparison, the fungal laccase *Ma*L1 showed maximal activity at pH 4, with a k_{cat} = 1.84 ± 0.04 s^{-1}. At their pH optima, *Ma*L1 displayed a lower K_m of 0.32 ± 0.01 mM, than *Sr*LA with a K_m of 0.57 ± 0.04 mM, hinting at a higher affinity to ABTS of *Ma*L1, than of *Sr*LA (Figure S 3). Most laccases show low K_m values for ABTS, although values differs widely for other substrates [2]. The K_m values of *Sr*LA and *Ma*L1 to ABTS are in a typical range for laccases [29–32].

The optimal temperature of ABTS conversion with *Ma*L1 was 50 °C (**Fehler! Verweisquelle konnte nicht gefunden werden.**b). *Sr*LA was most active at a temperature of 80 °C (**Fehler! Verweisquelle konnte nicht gefunden werden.**b), which is rather high. Laccases usually exhibit temperature optima between 50 °C and 70 °C [2]. The laccase from *Myceliophthora thermophila* (*Mt*L), for example, has been reported to have a comparatively low temperature optimum of 30 °C at its optimal pH on ABTS [28]. The temperature optima of *Ma*L1 and *Sr*LA were therefore typical for laccases.

*Ma*L1 and *Sr*LA both showed high storage stability, retaining 68% and 75% activity after 72 h at 23 °C and pH 7. At their optimal pH, their storage stability was lower, but still high for *Ma*L1 with a residual activity of 42% after 72 h. The activity of *Sr*LA decreased faster at pH 5, with only 13% of the initial activity remaining after 72 h (Figure S4).

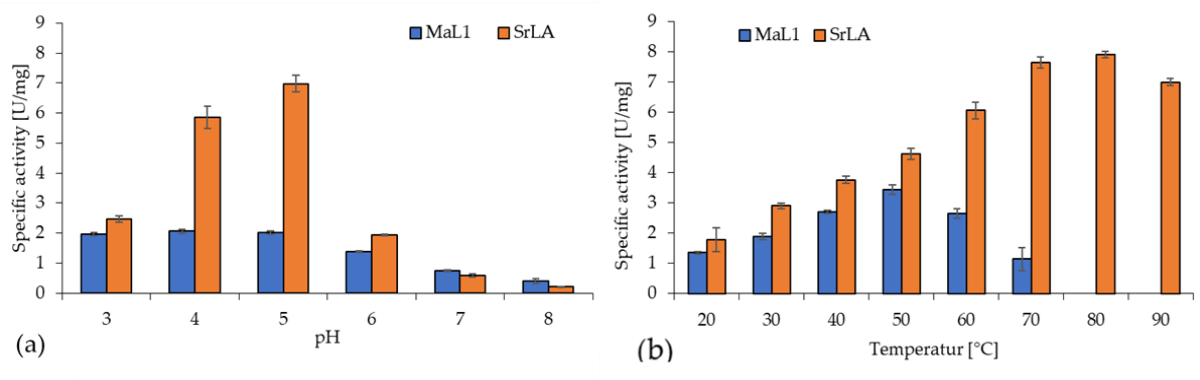

*Fig. 3:*Characteristics of the laccases *Ma*L1 and *Sr*LA on ABTS: activity of *Ma*L1 and *Sr*LA on ABTS at (a) different pH values and 23 °C and (b) different temperatures at their respective pH optima of pH 4 and 5, respectively. Error bars represent standard deviation of triplicates.

The spectrum of convertible substrates varies strongly between different laccases and is dependent on the redox potential, as well as the accessibility of the active site [33]. In this study, activity on eight typical laccase substrates, at concentrations from 0.05 to 20 mM, was examined (Table S1). Both laccases were able to oxidize 2,6-dimethoxyphenol (DMP), syringaldezine, guaiacol, sinapic acid and ferulic acid to a different extent. Noticeably, *Sr*LA oxidized many of these substrates to a lesser extent than *Ma*L1. Both exhibited no activity on catechol, gallic acid and vanillic acid. Laccases are known for their broad substrate range, which enables their use in a vast variety of applications. A broader substrate range of a laccase indicates its versatility for industrial applications [5].

3.3.3 Polymerization of lignosulfonate

The activity of laccases can be on the one hand determined by the chemical change in the substrate and on the other hand by examining the oxygen consumption during the reaction. This is possible, because laccases reduce oxygen to water stoichiometrically to the oxidation of their substrates [1]. The pH value has both an effect on the substrate (e.g. redox potential) and on the enzyme. Hence, it is important to study the pH dependency for laccase catalyzed reactions. Therefore, the optimal pH of *Ma*L1 and *Sr*LA for lignosulfonate polymerization was determined. Oxygen consumption was monitored, and molecular weights of the products were determined after 20 h. Oxygen consumption could be detected for the reaction with *Ma*L1 at all pH values (Figure S5). The fastest and most pronounced drop in oxygen was at a higher pH range of 6 to 8. Molecular weight determinations, through SEC-MALLS analyses, confirmed these observations (**Fehler! Verweisquelle konnte nicht gefunden werden.**) and the highest increase of MW from 126 kDa to 220 kDa was seen at pH 7. Despite a clear pH optimum of *Ma*L1 for ABTS as a substrate at pH 4, the increase of molecular mass was most pronounced at higher pH values. This would indicate increasing activity on lignosulfonates with increased pH levels.

*Sr*LA consumed only marginal to no oxygen during the reaction with lignosulfonate at all pH values (Figure S6). Most oxygen was consumed at the low pH values. Molecular mass

determination, through SEC-MALLS analyses, confirmed only marginal polymerization activity of *Sr*LA when dosed at the concentration where it exhibits the same activity as *Ma*L1. However, an increase in molecular mass of 42.4 kDa from, 171.0 kDa to 213.4 kDa, was observed at pH 8, when this enzyme was dosed at the concentration where it exhibits 5-fold higher activity than *Ma*L1 (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

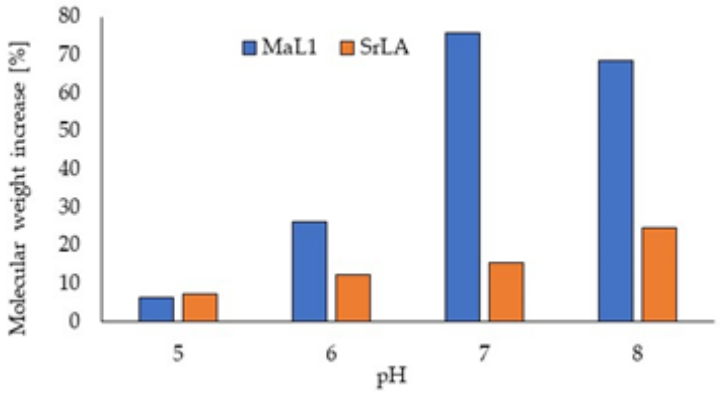

Fig. 4: Lignosulfonate polymerization with the laccases *Ma*L1 (0.05 U/mL) and for *Sr*LA (0.25 U/mL): relative molecular weight increase at different pH values.

*Si*LA, the laccase from *S. ipomoea*, exhibiting a high similarity to *Sr*LA, was previously shown to polymerize different lignins, however lignosulfonate polymerization was not examined. In the study by Moya *et al.,* best polymerization results with *Si*LA were obtained at pH 9 for most of the lignins [22], showing a similar correlation of pH and molecular weight increase to our results.

We can only speculate about the reasons for the higher activity of *Ma*L1 on lignosulfonate, than *Sr*LA. The glycosylation of *Ma*L1 is one possible explanation. A recent study has shown that attachments to the laccase can increase its polymerization activity [34]. The same study has shown that the polymerization product varies with reaction conditions and attachments to the laccase. Although this study was only concerned with catechol polymerization, the same could be true for lignosulphonate polymerization. It is a distinct possibility that the product varies for different reaction conditions and laccases. Nevertheless, size exclusion chromatography, especially when coupled to MALLS analysis, is well suited for describing the polymerization reaction [12,35].

in recent studies, Laccases from *Myceliophthora thermophila* (*Mt*L) have been most widely used on lignosulfonate polymerization [12–14,16] in the presence of oxygen, but without mediators. Therefore, the lignosulfonate polymerization activity of MaL1 was compared to that of *Mt*L. At the same enzyme concentration, the oxygen consumption, as indicated by a more pronounced decrease of the oxygen saturation, of *Ma*L1 was higher than that of *Mt*L (**Fehler! Verweisquelle konnte nicht gefunden werden.**). This indicates higher activity of *Ma*L1. The oxygen content decreases twice during the 20 h reaction with *Ma*L1. Once after 100 min and

a second time after 600 min. This could be explained by the heterogeneity of the substrate. At the first decrease of oxygen, easily oxidizable substrates are consumed. More difficult substrates are oxidized at a later stage, causing the second drop in oxygen.

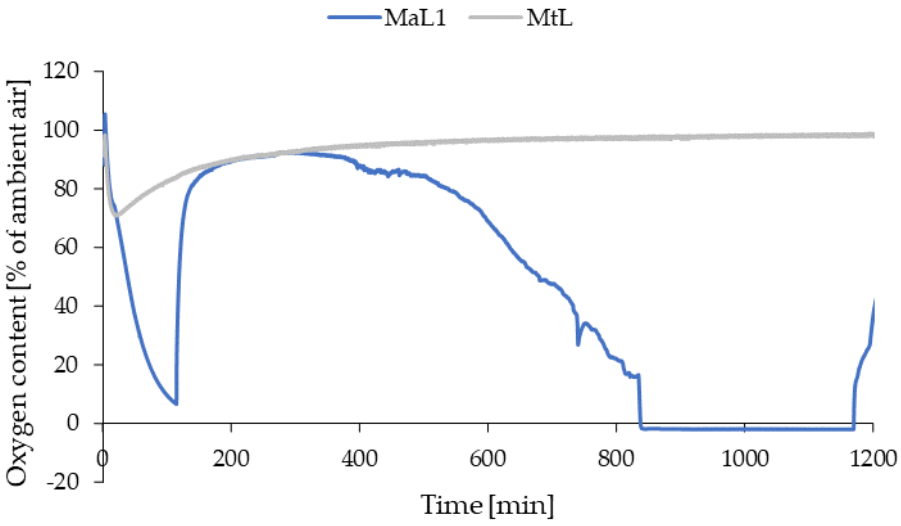

Fig. 5: Comparison of activity on lignosulfonate of *Mt*L to *Ma*L1 (both 0.025 U/L) at pH 7 according to oxygen consumption.

3.4 Conclusion

The laccases *Ma*L1 and *Sr*LA were successfully expressed, although purification of *Ma*L1 proved to be difficult, because of insufficient binding to the strep-avidin column. Due to secretion, purity of MaL1 was sufficient after desalting and buffer exchange. Both enzymes incorporated copper at the expected amount of approximately 4 mol/mol enzyme. Characteristics of *Ma*L1 and *Sr*LA on ABTS, like pH and temperature optima and affinity to ABTS, were typical for laccases. Both *Ma*L1 and *Sr*LA proved to be comparatively stable.

*Ma*L1 preferred high pH values for the polymerization of lignosulfonate. This property makes it interesting for the application on other technical lignins. *Sr*LA showed lower polymerization activity on lignosulfonate, which is surprising considering its over 99% similarity to a laccase from a different *Streptomyces* species. The laccase from *Streptomyces ipomoea* was previously shown to be active on lignin, although lignosulfonate was not among the tested lignin species [22].

The laccase *Ma*L1 is a promising candidate for lignin polymerization and to valorize this under-used resource. Further studies will show the full potential of both laccases, and especially, if the purification could be optimized. For future studies, upscaling and a more detailed molecular characterization of the produced lignosulfonate polymers is of high interest, especially regarding their applicability.

3.5 Acknowledgements

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4 A fungal ascorbate oxidase with unexpected laccase activity

Verena Braunschmid [1,2], Sarah Fuerst [2], Veronika Perz [1], Sabine Zitzenbacher [1], Javier Hoyo [3], Cesar Fernandez-Sanchez [4], Tzanko Tzanov [3], Georg Steinkellner [2,5], Karl Gruber [2,6], Gibson S. Nyanhongo [1,2], Doris Ribitsch [1,2,*] and Georg M. Guebitz [1,2]

[1] *Institute of Environmental Biotechnology, Department of Agrobiotechnology, University of Natural Resources and Life Sciences (BOKU), 3430 Tulln an der Donau, Austria; verena.braunschmid@boku.ac.at (V.B.); veronika.perz@meduni.graz.at (V.P.); sabine.zitzenbacher@perrigo.com (S.Z.); g.nyanhongo@boku.ac.at (G.S.N.); guebitz@boku.ac.at (G.M.G.)*

[2] *Austrian Centre for Industrial Biotechnology (ACIB), 3430 Tulln an der Donau, Austria; sarah.fuerst@boku.ac.at (S.F.); georg.steinkellner@acib.at (G.S.); karl.gruber@uni-graz.at (K.G.)*

[3] *Grup de Biotecnologia Molecular i Industrial, Department d'Enginyeria Química, Universitat Politècnica de Catalunya, Rambla Sant Nebridi, 08222 Terrassa, Spain; javier.hoyo@upc.edu (J.H.); tzanko.tzanov@upc.edu (T.T.)*

[4] *Instituto de microelectronica de Barcelona (IMB-CNM), CSIC, Campus UAB, 08193 Bellatera, Spain; cesar.fernandez@imb-cnm.csic.es (C.F.-S.)*

[5] *Innophore GmbH, 8010 Graz, Austria*

[6] *Structural biology, Institute of Molecular Bioscience, University of Graz, 8010 Graz, Austria*

***** *Correspondence: doris.ribitsch@boku.ac.at*

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Abstract: Ascorbate oxidases are an enzyme group that has not been explored to a large extent. So far, mainly ascorbate oxidases from plants and only a few from fungi have been described. Although ascorbate oxidases belong to the well-studied enzyme family of multi-copper oxidases, their function is still unclear. In this study, *Af*_AO1, an enzyme from the fungus *Aspergillus flavus*, was characterized. Sequence analyses and copper content determination demonstrated *Af*_AO1 to belong to the multi-copper oxidase family. Biochemical characterization and 3D-modeling revealed a similarity to ascorbate oxidases, but also to laccases. *Af*_AO1 had a 10-fold higher affinity to ascorbic acid (K_M = 0.16 ± 0.03 mM) than to ABTS (K_M = 1.89 ± 0.12 mM). Furthermore, the best fitting 3D-model was based on the ascorbate oxidase from *Cucurbita pepo* var. *melopepo*. The laccase-like activity of *Af*_AO1 on ABTS (V_{max} = 11.56 ± 0.15 µM/min/mg) was, however, not negligible. On the other hand, other typical laccase substrates, such as syringaldezine and guaiacol, were not oxidized by *Af*_AO1. According to the biochemical and structural characterization, *Af*_AO1 was classified as ascorbate oxidase with unusual, laccase-like activity.

Keywords: ascorbate oxidase; laccase; multi-copper oxidase; ABTS

4.1 Introduction

Ascorbate oxidases catalyze the oxidation of ascorbic acid to dehydroascorbate (DHA), however, their specific function has not yet been fully elucidated [1,2]. There are indications, that ascorbate oxidases are involved in signaling pathways in plants since DHA induces stomata closure in tobacco [3]. Mostly, the functions of ascorbate oxidases are, however, not expected in the oxidation of ascorbic acid, but rather in the reduction of oxygen levels [2]. Maintenance of a stable redox state requires the control of oxygen levels, especially where oxygen is produced by photosynthesis. Furthermore, in some tissues hypoxia is essential to keep stem cells in their pluripotent state [2]. In fungi, the functions of ascorbate oxidase are even less explored. There are indications that they might be involved in plant pathogenesis [4] or fungal development [5,6]. The biotechnological applications of ascorbate oxidases are consequently limited. The most common application is for biosensors to detect ascorbic acid, an important dietary and physiological antioxidant [7].

Ascorbate oxidases belong to the enzyme family of multi-copper oxidases (MCO). The MCO family is one of the earliest and best-studied enzyme groups [8,9]. As their name suggests, MCOs utilize copper to oxidize various substrates, concomitantly reducing oxygen to water [10]. The MCO family is highly diverse and includes, besides ascorbate oxidases (EC 1.10.3.3), laccases (EC 1.10.3.2), ceruloplasmin, and ferroxidases (EC 1.16.3.1) [1]. The enzyme groups differ mainly in their substrate specificity. The structure of MCOs is largely conserved across the various groups. Most MCOs, like ascorbate oxidases and laccases, consist of three domains, except ceruloplasmin, which is comprised of six domains [10]. Common and mechanistically important features in all MCOs, are the four copper ions organized in two sites. The mononuclear site contains one T1 or blue copper-ion and is the site of substrate oxidation. The redox potential of the T1 copper is crucial for the catalytic efficiency of the enzyme [11]. From the T1 copper, the electrons are transferred via a conserved His-X-His motive to the second copper-containing site, the trinuclear site. The trinuclear site contains one T2 copper and a pair of T3 copper ions. There, a four-electron reduction of dioxygen to water takes place [12,13]. Conserved domains, typical for MCOs, coordinate the copper ions, and facilitate the identification of MCO family members [14].

So far, most ascorbate oxidases were found and studied in plants [2], only a handful were found in fungi [1,4,15] and insects [16]. Consequently, literature on fungal ascorbate oxidases is rare. The best described fungal ascorbate oxidase, ASOM from *Acremonium* sp.–HI-25, was described in 1992 [17]. Ascorbate oxidases were also found in the fungi *Myrothecium verrucaria* [18], *Physarum polycephalum,*[19] and *Pleurotus ostreatus* [20], but those are atypical, since they probably do not contain copper ions. Xie et al. describe an ascorbate oxidase-like enzyme in the filamentous fungus *Podospora anserine*, whose gene deletion

decreased ascorbate oxidase activity [5], but this enzyme was not isolated and therefore not characterized in depth.

Ascorbate oxidases, in contrast to laccases, have narrow substrate specificity, almost exclusively oxidizing ascorbic acid to DHA, but can also accept other lactone-ring containing substrates [21]. The high substrate specificity is probably due to their rather small substrate cavity. One histidine and two tryptophan residues stabilize the lactone ring of ascorbic acid in the cavity [22,23].

In contrast to ascorbate oxidases, laccases have attracted a lot of attention [1]. Since their discovery in the sap of the Japanese lacquer tree (*Rhus vernicifera*) in 1883 [8], laccases were found not only in plants, but also in fungi, and more recently in bacteria and insects [12,24]. The wide range of potential laccase-substrates in nature is reflected in the high diversity of industrial applications, ranging from textile and food processing to chemical synthesis [9].

Ramos et al. identified *Aspergilli* fungi as a good source for the identification of novel MCOs after thorough genomic analysis [25]. Takeda et al. expressed an ascorbate oxidase (ASOM) in *Aspergillus nidulans* [26]. Furthermore, two protein sequences of *A. nidulans* (or *Emericella nidulans*) have been identified as ascorbate oxidase [1], but those have not been characterized in depth. Furthermore, *Aspergilli* have been used to express laccases from other fungi [27]. A few laccases native to *Aspergilli* have been described as well [28–30]. For *A. flavus*, for example, five putative laccases have been identified by genome sequencing [31]. Some laccases from *A. flavus* have already been characterized and applied, but lack of protein sequences impedes the classification of these enzymes [32–35].

This paper focuses on the characterization of an oxidoreductase from *Aspergillus flavus* to elucidate its classification within the MCO family. We found that *Af*_AO1 was highly active on ascorbic acid, but also oxidized 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), one of the most commonly used laccase substrates. A structural modeling of *Af*_AO1 suggested a high similarity to ascorbate oxidase of plants. Therefore, we concluded that *Af*_AO1 is a putative ascorbate oxidase with unprecedented laccase-like activity.

4.2 Results

4.2.1 Sequence Analyses and Copper Content Determination

In the genome sequence of the saprotrophic and pathogenic fungus *Aspergillus flavus*, five putative laccases were found (Ref. seq.: XP_002384796.1, XP_002382290.1, XP_002382290.1, XP_002381510.1, XP_002378028.1) [31]. Of the five putative laccases found in *A. flavus* four contain the signal for extracellular expression. Out of these laccases *Af*_AO1 was best expressed in *Pichia pastoris*. *Af*_AO1 has the locus tag AFLA_123160 and was annotated as putative laccase (Ref. Seq.: XP_002381510.1).

*Af*_AO1 contained 3.3 mol copper per mol of enzyme, which is characteristic for MCOs [1,11,36]. Furthermore, the protein sequence of *Af*_AO1 comprised the typical MCO consensus sequences essential for the binding of the four copper ions (Figure 1) [14] and an absorbance spectrum typical for laccases (Figure S1). Therefore, *Af*_AO1 was identified as a member of the MCO enzyme family. Additionally, *Af*_AO1 contained a conserved region typical for the ascorbate oxidase fungal superfamily [37].

```
         10          20          30          40          50          60
HLVLHDDSFQ PDHILRVTAQ DVNQACMDRY SVLINGSLPG PQLNIQEGKV NWIRVYNDME

         70          80          90         100         110         120
DLNVTMHWHG LSAFTAPFSD GTPMASQWPI PPGHFFDYEV RPEVGYAGTY FYHSHVGFQA

        130         140         150         160         170         180
LTAWGALIVE SAQPSPYQYD EERIIALSDF FTKTDEEIEN GLTSTNFTWS GETSAVLVNG

        190         200         210         220         230         240
QGRLATNATG SCKLAAISVE PGKTYRLRFI GATALSFVSI SLESHDVLEI IEADGHYTKP

        250         260         270         280         290         300
VNTSYLQISS GQRYSVLLKA KTEAELQQAK SRQFYFQLTT MGRPTVLTTF AVLEYPSPTT

        310         320         330         340         350         360
TDLITVPVTP PLPVANITYG WLDYTLEPYY PDLDFPTVEE VTRRIIINVH QNISDRTVWL

        370         380         390         400         410         420
QNGYDWVETF PKSPYLVDIY AGTLDLDASY KRAIASGYAF DNQTRLFPAK MGEVLEIVWQ

        430         440         450         460         470         480
NQGAVSNGGV ENHPFHAHGR HFYDIGGGDG LYNLTENEAR LKGTHPVIRD TTMLYAYRKT

        490         500         510         520         530         540
TTALEPSGWR AWRIRVTAAG VWMVHCHVLQ HMLMGMQTAF AFGDQTAIKA QSGTPAEGYL

        550         560
TYGGSAYGNV THFPPVKHFF N
```

Figure 1: Sequence of *Af*_AO1: conserved regions are underlined, and copper-coordinating residues marked in grey. The sequence is displayed, as it was expressed without signal peptide.

4.2.2 Redox Potential And Substrate Spectrum

The redox potential of an MCO is a characteristic parameter for these enzymes and an important factor for its substrate range [11]. Cyclic voltammetry (CV) was performed to elucidate the redox potential of *Af*_AO1 (Figure 2). The CV of *Af*_AO1 showed an oxidation peak at 500 and a reduction peak at 410 mV indicating a redox potential (E_0) of 455 ± 10 mV against normal hydrogen electrode (NHE) for the T1 copper ion of *Af*_AO1.

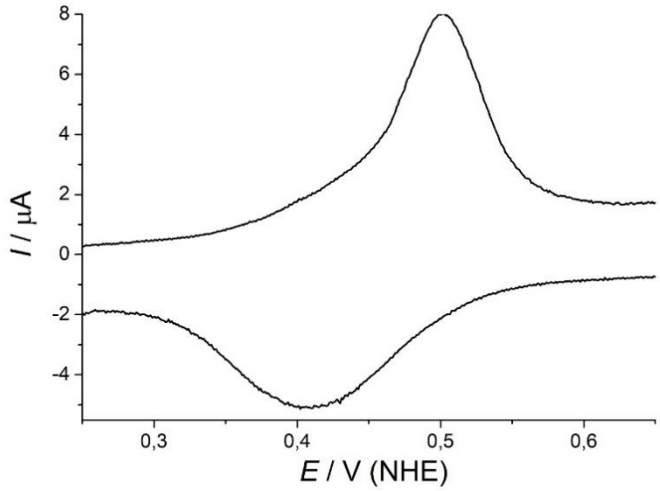

Figure 2: Redox potential of *Af*_AO1: Cyclic voltammogram of *Af*_AO1 at 10 mV·s^{-1}, exhibiting an oxidation peak at 500 mV and a reduction peak at 410 mV, indicating a redox potential at 445 ± 10 mV against normal hydrogen electrode (NHE).

A screening of various typical MCO-substrates showed a high specificity of *Af*_AO1 for ascorbic acid and the typical laccase substrate, ABTS. Interestingly, *Af*_AO1 did not oxidize any other tested laccase substrates (Table 1 and Figure S2). Accordingly, the activity of *Af*_AO1 was further characterized on ABTS and ascorbic acid (Figure 3).

Table 1: Substrate screening: *Af*_AO1 converted, of all tested substrates, only ascorbic acid and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Activity was measured at conditions preferred by each substrate. Activity was determined at 25 °C, except for ascorbic acid, which was measured at 30 °C.

Substrate	Activity	Buffer
DMP	-	sodium acetate pH 4
Syringaldezine	-	sodium-phosphate pH 6
Catechol	-	sodium acetate pH 5
Guaiacol	-	sodium acetate pH 4
Sinapic acid	-	sodium acetate pH 4
Ferulic acid	-	sodium acetate pH 4
Vanillic acid	-	sodium acetate pH 4
Gallic acid	-	sodium acetate pH 4
Tannic acid	-	sodium acetate pH 4
Ascorbic acid	+	sodium acetate pH 4
ABTS	+	sodium acetate pH 5

4.2.3 Enzyme Optima and Kinetics

On ABTS, *Af*_AO1 showed the highest activity at a pH-value of 3.4, while it preferred a pH of 5.0 for the conversion of ascorbic acid. The temperature optimum at 40 °C, on the other hand, was identical for both substrates (Figure 3). Results for enzyme kinetics differed strongly for the conversion of ABTS and ascorbic acid. Kinetic analyses were performed at the optimal pH value for each substrate. The maximum velocity V_{max} of *Af*_AO1 on ABTS was 11.56 ± 0.15 µM/min/mg and the affinity of the enzyme to ABTS was moderate, with a K_M of 1.89 ± 0.12 mM. On ascorbic acid, *Af*_AO1 exhibited a significantly higher V_{max} of 424.89 ± 23.19 µM/min/mg. The affinity of *Af*_AO1 to ascorbic acid was approximately ten times

stronger than to ABTS, with a K_M of 0.16 ± 0.03 mM (Figure S3). *Af*_AO1 retained 70% of its ascorbate oxidase activity after 24 h at 40 °C (Figure S4).

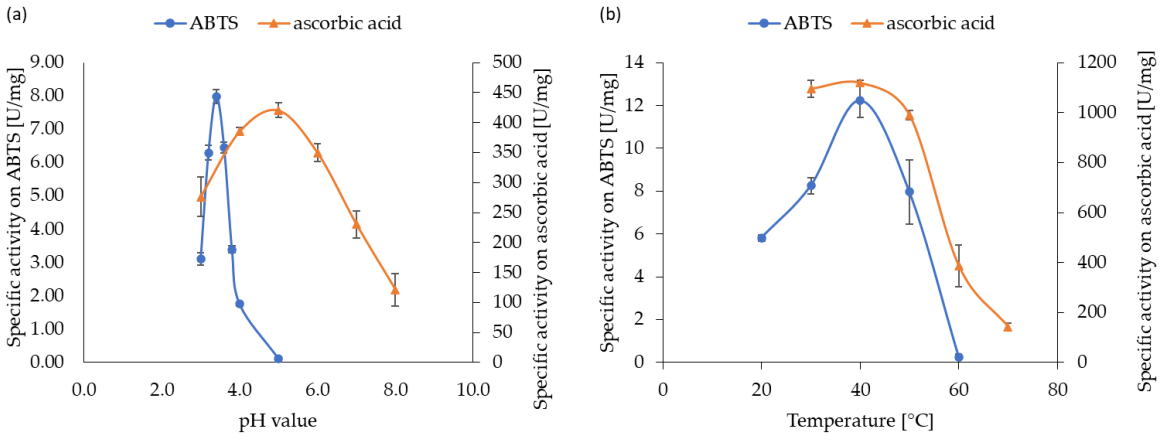

Figure 3: Optimum reaction conditions of *Af*_AO1: (**a**) pH optimum of *Af*_AO1 on ABTS was determined at 25 °C and that on ascorbic acid at 30 °C. (**b**) Temperature optimum of *Af*_AO1 on ABTS and ascorbic acid was measured at the respective optimal pH values of pH 3.4 and pH 5, respectively. Experiments were conducted in triplicates. The error bars depict standard deviation.

4.2.4 Modeling of *Af*_AO1

For structural investigation of *Af*_AO1, we modeled its 3D-structure based on known homologous structures derived from different alignments. Despite the overall moderate sequence identity (~32%) and similarity (~50%) to available template structures, we were able to generate a model of *Af*_AO1. The five models built on two laccases from *Steccherinum murashkinskyi* (5E9N [38] and 5MEW [39]), a laccase from *Lentinus tigrinus* (2QT6 [40]), a laccase from *Cerrena maxima* (3DIV [41]) and an ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (1ASQ [42]) ranked similarly high according to their Z-score for dihedrals and overall packing. Manual inspection of the quality of the models regarding overall alignment and the position of the histidines and copper ions, revealed that the best model was built on the structure of the ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (PDB Code: 1ASQ [42]) (Figure 4). This model was used as a representative structure and for model analysis. Of 561 target residues 453 (80.7%) were aligned to the template residues. Among these aligned residues, the sequence identity was 32% and the sequence similarity was 47% (Figure S5). In this model, the copper ions were in a similar position compared to the ones in the template, which was not always the case at different modeling attempts using alternate alignments and selected template structures. Furthermore, the overall amino acid placements, regarding the copper-binding sites, were similar to the template structure in the model. The most differences compared to the template were near the proposed active site (Figure S6).

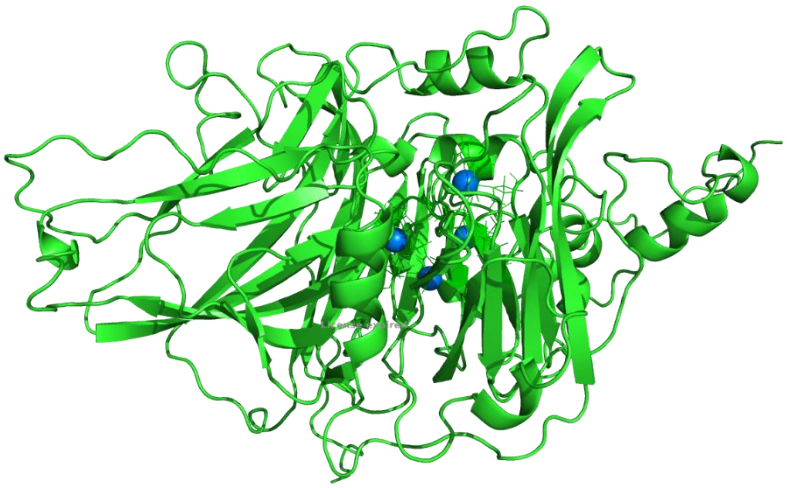

Figure 4: Model of *Af*_AO1: Proposed 3D-structure of *Af*_AO1 based on the structure of the ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (PDB Code: 1ASQ [42]). Copper atoms are displayed in blue and coordinating residues are represented as sticks.

4.3 Discussion

In this study, we investigated a novel ascorbate oxidase *Af*_AO1 from *A. flavus*. The gene sequence was published as part of a genome sequencing study by Nierman et al. [31]. MCO members contain four copper ions that are coordinated by conserved amino acid residues. These conserved regions make it relatively easy to identify an enzyme as belonging to the MCO family [14]. The *Af*_AO1 sequence contained all MCO-characteristic copper-coordinating sequences. Furthermore, analysis of copper content showed that the expressed enzyme contained 3.3 mol copper per mol enzyme. This is consistent with the copper content expected for MCOs [1]. Therefore, *Af*_AO1 was identified as a member of the MCO family.

The determination of function from sequence is considerably more difficult for MCO members than the grouping into this family [14]. *Af*_AO1 contained a region typical for the fungal ascorbate oxidase family. The fungal ascorbate oxidase superfamily comprises, besides fungal ascorbate oxidases, also plant ascorbate oxidases and laccases, as well as, laccase-like enzymes [37].

At the T1 copper site, MCOs oxidize their substrates, then transfer the electrons to the trinuclear-copper center, where oxygen is reduced to water. The redox potential of the T1 copper site is characteristic for each enzyme and has an impact on its substrate range [11]. The T1 copper site of *Af*_AO1 exhibited a redox potential of 445 ± 10 mV against NHE. Laccases are grouped according to their redox potential. High redox potential laccases have a redox potential above 710 mV. Laccases with a redox potential between 460 and 710 mV are called medium redox potential laccases and all below that are called low redox potential laccases [12]. Fungal laccases are generally found in the medium to high redox potential range, while low redox laccases are mainly bacterial [43]. The differences in the redox potential of laccases can be explained by the big variety of functions, and thereby the wide range of substrates, of laccases. Ascorbate oxidases generally exhibit a considerably lower redox

potential. They mainly oxidize one substrate, ascorbic acid, but do so under different conditions. Therefore, the range of redox potential could be smaller than for laccases. For comparison, the fungal ascorbate oxidase ASOM (from *Acremonium* sp.) has a redox potential of 197 mV [44] and the ascorbate oxidase from zucchini of 139 mV [45]. Cucumber ascorbate oxidase has a relatively high redox potential of 350 mV [46]. According to its redox potential *Af*_AO1 could be either a low redox potential laccase or a high redox potential ascorbate oxidase.

The redox potential usually correlates with the substrate range of an enzyme. Generally, a substance can be oxidized by a substance with higher redox potential. Therefore, the higher the redox potential of an MCO the higher the redox potential of their substrates can be and therefore the range of oxidizable substrates increases. Oxidoreductases are, however, not only restricted by their redox potential, but also by the accessibility of their substrate-binding site [11]. Laccases normally have a very wide substrate spectrum. Ascorbate oxidases, in contrast, are very specific for ascorbic acid and lactone ring containing substrates. This narrow substrate range can be explained by the comparatively low redox potential and by amino acids restricting the access to the active site. The active site of ascorbate oxidases is generally very narrow, and the lactone ring of the substrate is stabilized by a histidine and two tryptophan residues [23]. *Af*_AO1 showed a very narrow substrate range for ascorbic acid and for ABTS. The narrow substrate range is in accordance with its rather low redox potential, compared to that of laccases. On the one hand, the narrow substrate range is unusual for laccases and typical for ascorbate oxidases, suggesting that *Af*_AO1 is an ascorbate oxidase. On the other hand, ABTS is a characteristic laccase substrate and typically not oxidized by ascorbate oxidases, as it does not contain a lactone ring. Xie et al. found that the deletion of the gene of a putative ascorbate oxidase in *P. anserine* reduced its ability to oxidize ABTS and ascorbic acid. This enzyme was accordingly classified as ascorbate oxidase, but with laccase-like activity [5]. To our knowledge, no other ascorbate oxidases with ABTS oxidizing ability have been reported so far.

The pH optimum of laccases depends strongly on the substrate [47]. For the oxidation of ABTS low pH optima of around 3 to 5 are usually reported [48–50]. For ascorbate oxidases in plant optimal pH of 5 to 6.5 were reported [51–53]. ASOM, the only fungal copper-containing ascorbate oxidase described until now, has a pH optimum of pH 4 or 4.5 on ascorbic acid [15,17]. The pH optimum of *Af*_AO1 on ABTS of pH 3.4 fits very well to reported data for laccases. *Af*_AO1 showed the maximal activity on ascorbic acid at pH 5. This pH value lies within the range expected for ascorbate oxidases, although it is higher than that of ASOM [15,17].

The temperature optimum for *Af*_AO1 on both substrates was 40 °C. For most enzymes, the temperature optimum is not dependent on the substrate. The temperature optimum of *Af*_AO1

was lower than the average temperature optimum of laccases, which is between 50 °C and 70 °C [47]. On the other hand, some laccases with considerably lower temperature optima have been reported as well. A laccase isolated from *Polyporus* sp. for example, exhibited the highest activity at a temperature of 25 °C [54]. For ascorbate oxidases temperature optima range from 37 °C in orange peel [53] to 50 °C in wheat [55]. The fungal ascorbate oxidase ASOM exhibits optimal activity at a temperature of 45 °C [17]. Laccases, because of their high variety in substrates and functions, have a wide variety of environments and conditions that they need to be able to function in. This is reflected in the wide range of optimal temperatures and pH-values. The optimal temperature and pH range of ascorbate oxidases is narrower. Most of the described ascorbate oxidases act inside plants and the environment for their functionality is thereby restricted and only vary slightly from plant to plant. The temperature optimum of *Af*_AO1 was therefore in the range of both laccases and ascorbate oxidases.

Kinetic analyses on both substrates revealed a significantly higher maximum conversion rate for ascorbic acid than for ABTS. The V_{max} on ABTS was 11.6 µM/min/mg, while it was 431.19 µM/min/mg on ascorbic acid. Furthermore, the K_M for ascorbic acid (0.16 mM) was approximately 10-fold lower compared to ABTS (1.9 mM) indicating a higher affinity of *Af*_AO1 for ascorbic acid than for ABTS. Although some laccases have even lower affinity to ABTS than *Af*_AO1, such as laccase from *Klebsiella pneumoniae* with a K_M of 5.33 mM [56], most laccases have a significantly higher specificity for ABTS [57,58]. The affinity of *Af*_AO1 to ascorbic acid is similar to those reported for ascorbate oxidases [51,59]. ASOM, for example, exhibits a K_M of 0.29 mM [15]. The higher specificity and conversion rate for ascorbic acid suggested that *Af*_AO1 is an ascorbate oxidase, but even low activity on ABTS is uncommon for ascorbate oxidases.

Lastly, the three-dimensional structure of *Af*_AO1 was modeled applying a comparative modeling approach using the YASARA modeling suite [60]. The most appropriate templates were identified and include mostly laccase structures (like PDB Codes: 5E9N [38], 5MEW [39], 2QT6 [40], 3DIV [41]), but also an ascorbate oxidase structure was among the best templates (PDB Code: 1ASQ [42]), indicating some sequence similarities of *Af*_AO1 to an ascorbate oxidase. The best template was automatically assigned as the laccase structure from *Steccherinum murashkinskyi* with the PDB-Code 5E9N [38]. Despite the better resolution of this template structure, the second best model using the ascorbate oxidase from *Cucurbita pepo* var*. melopepo* as a template [42], was used as a representative model for *Af*_AO1, because of a better coverage of the alignment (80.7% versus 66.3%) and having a similar overall sequence identity of about 32% (versus 33% to the laccase).

Taken together these results suggest that *Af*_AO1 is an ascorbate oxidase with laccase-like activity. Only very few ascorbate oxidases from fungi have been reported so far, while some untypical ascorbate oxidases from fungi containing heme in the active site are known [18–20].

*Af*_AO1 is a copper-containing MCO, exhibiting the conserved copper-coordinating ligands, as well as, 3.3 mol/mol copper. A single copper-containing fungal ascorbate oxidase, ASOM from *Acremonium* sp.-HI-25, is described in literature to date [17]. Furthermore, *Af*_AO1 exhibited an untypical, laccase-like activity, oxidizing ABTS. Similar behavior was only reported for a putative ascorbate oxidase or laccase in *P. anserine* that, if knocked out, decreases the activity of *P. anserine* on ABTS, as well as, on ascorbic acid.

*Af*_AO1 is therefore a two-fold unusual ascorbate oxidase, stemming from fungi and showing activity on ABTS.

4.4 Materials and Methods

4.4.1 Chemicals and Reagents

If not indicated otherwise, chemicals were obtained from Sigma–Aldrich (Munich, Germany).

4.4.2 Expression and Purification of *Af*_AO1

The gene of *Af*_AO1 was codon optimized for the expression in *Pichia pastoris* and synthesized directly in a pPicZαB vector without its natural signal peptide and without any tag by GenScript (GenScript Biotech, Leiden, The Netherlands). *Af*_AO1 was recombinantly expressed in *P. pastoris* KM71H cells (ThermoFisher Scientific, Waltham, MA, USA). The vector was amplified in *Escherichia coli* BL21-Gold(DE3) (Agilent Technologies, Santa Clara, CA, USA) and isolated with a Promega Midiprep kit (Madison, WI, USA). The vector was linearized with *SacI* (New England Biolabs, Ipswich, MA, USA) and transformed into *P. pastoris* via electroporation (MikroPulserTM, Bio-Rad, Hercules, CA, USA) according to manufacturer's protocol. Colonies including the insert were determined by colony PCR. Freshly transformed *P. pastoris* cells were cultivated in yeast extract peptone dextrose medium supplemented with 100 µg mL^{-1} zeocin, at 28 °C and 150 rpm, overnight. This culture was used to inoculate 100 mL buffered minimal dextrose medium in 1 L baffled flasks to an OD_{600} of 0.1. The expression was induced after 60 h of incubation at 28 °C with 0.5% methanol twice a day over a period of five days. Additionally, 0.1 mM $CuSO_4$ were added with the first induction for the proper folding of the enzyme. The supernatant containing the protein was collected through centrifugation and the protein purified by desalting using an ÄKTApure (GE Healthcare, Little Chalfont, UK) system with a HiPrep™ 26/10 desalting column. A 10 mM sodium acetate buffer pH 5 was applied as buffer. The enzyme concentration was determined photometrically using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as protein standard. The purity of the enzyme was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [61] using precast 4–15% gradient gels (Bio-Rad, Hercules, CA USA) (Figure S7). As a molecular mass standard pre-stained protein marker IV (Peqlab, Erlangen, Germany) was used. Gels were stained with Coomassie Brilliant Blue.

4.4.3 Characterization of *Af*_AO1

4.4.3.1 Sequence Analyses

Protein sequence was on the one hand analyzed manually, by looking for conserved residues, and on the other hand by the conserved domain search of NCBI [37].

4.4.3.2 Copper Determination

Copper concentration was determined spectrophotometrically (Infinite 200 Pro; Tecan, Männedorf, Switzerland) with a QuantiChrom™ Copper Assay Kit (DICU-250; BioAssay Systems, Hayward, CA, USA) according to manufacturer's protocol in 96-well plates. Samples had a concentration of 1.1 mg/mL.

4.4.3.3 Redox Potential

Gold foil surface was activated using 0.1 M KNO_3 performing 20 cycles from 0.8 to - 2.2 V at 0.1 $V \cdot s^{-1}$. A pre-polymerization solution of pyrrole in phosphate-buffered saline (PBS) (5 mL) was prepared by mixing 600 µL enzyme, 194 µL pyrrole and KCl at a final concentration of 0.1 M. The resulting solution was vortexed for 3 min and deoxygenated with nitrogen for at least 30 min, prior to the electrochemical polymerization of pyrrole. The charge accumulated during the electrosynthesis of polypyrrole (PPy) films at 0.85 V was fixed to 100 mC cm^{-2}. The gold electrode coated with PPy was cleaned extensively using milliQ water to remove the non-immobilized enzyme and unreacted pyrrole.

PBS was deoxygenated with N_2, for at least 30 min, prior to starting the cycling voltammetry experiments in a three-electrode cell using a µAutolab Potentiostat (Ecochemie, Utrecht, The Netherlands). The counter electrode was a platinum wire in spiral geometry and the reference electrode was Ag/AgCl/3 M KCl (Metrohm 6.0726.100). Cyclic voltammograms (CVs) were performed scanning towards positive potentials in a home-made glass cell with a reaction area of 33 mm^2.

4.4.3.4 Substrate Screening and Absorption Spectrum

The activity of *Af*_AO1 was determined on 2,6-dimethoxyphenol (DMP; f.c. 0.1 mM), syringaldezine (f.c. 0.05 mM), catechol (f.c. 2 mM), guaiacol (f.c. 20 mM), sinapic acid (f.c. 0.1 mM), ferulic acid (f.c. 0.1 mM), vanillic acid (f.c. 0.1 mM), gallic acid (f.c. 0.1 mM), and tannic acid (f.c. 0.1 mM). Therefore, substrates were solved in ethanol, methanol, water, or buffer, according to their solubility and diluted to their final concentration in buffer. For syringaldezine 100 mM sodium-phosphate buffer pH 6, for catechol 50 mM sodium acetate buffer pH 5 and for all other substrates 100 mM sodium acetate buffer pH 4 was used. Substrates were incubated with 0.1 mg/mL *Af*_AO1. Absorption was determined through a spectrophotometric scan with wavelengths between 200–800 nm in 1 nm steps. Interaction of enzyme and substrate was ascertained by differences in spectra of reactions with enzyme compared to blanks.

For the absorption spectrum *Af*_AO1 was diluted in citrate-phosphate buffer pH 3.4 and measured, as for the substrate screening.

4.4.3.5 Enzyme Characterization on ABTS

Enzyme activity on ABTS was determined spectrophotometrically, as described previously [62]. The enzyme was mixed with 10 mM ABTS solved in water and the absorbance at 420 nm determined immediately with a plate reader. To determine optima, the enzyme was incubated at temperatures ranging from 20 °C to 60 °C and at a pH range from pH 3 to pH 6. For pH values from pH 3 to 4 citrate-phosphate buffer was used ($\varepsilon_{460nm,\ pH3–3.5}$ = 34.683 L/mmol/cm; $\varepsilon_{460nm,\ pH3.6–4.5}$ = 33.115 L/mmol/cm). Sodium acetate buffer was used for pH values from 4.8 to 5.5 ($\varepsilon_{460nm,pH4.6–5.5}$ = 31.332 L/mmol/cm) and sodium phosphate buffer for pH 6 ($\varepsilon_{460nm,pH6}$ = 21.103 L/mmol/cm). For the determination of the temperature optimum the pH was set to the optimal pH of 3.4. The absorption was measured in 20 cycles with an interval time of 10 s. One unit (U) of laccase activity was defined as the amount of enzyme producing 1 µmol of reaction product per minute under the conditions specified.

To determine kinetic values enzyme activity was determined at a range of ABTS concentrations from 0.5 mM to 20 mM. The activity measurement was conducted, as described above, at 25 °C. The enzyme was diluted in 0.1 M citrate buffer at pH 3.4. Kinetic values, V_{max} and K_M, were determined with a Lineweaver-Burk diagram [63].

4.4.3.6 Enzyme Characterization on Ascorbic Acid

Activity on ascorbic acid was determined spectrophotometrically (U-2900; Hitachi, Austria) by the decrease of the substrate absorbance, as previously described [64]. Ascorbic acid was diluted to 0.5 M in buffer and incubated for 2 min at 30 °C and 350 rpm and for another 5 min, after the addition of 25 µL *Af*_AO1. The reaction was stopped by the addition of 750 µL HCl at a concentration of 0.2 M and the absorbance was measured at 245 nm (ε_{245} = 10 mL/µmol*cm). For the blank, the enzyme was added together with the HCl after the second incubation. To determine the temperature optimum the temperature of the water bath and photometer were set to 30 °C to 70 °C and the reaction was carried out at the optimal pH of 5. For the different pH values for the pH optimum determination appropriate buffers were chosen. For pH values between 6 and 8 phosphate buffer and for pH values between 3 and 6 sodium acetate buffer was used. To determine enzymatic stability, *Af*_AO1 was incubated at 40 °C and activity measured after 0.5, 1, 2, 4, 5 and 24 h. One unit (U) of ascorbate oxidase activity was defined, identical to one unit of laccase activity.

Kinetic parameters were determined by activity measurements as described, at optimal pH and 30 °C, with ascorbic acid concentrations of 0.05 to 1 mM. With Lineweaver-Burk approximation V_{max} and K_M were determined [63].

4.4.4 Modeling of *Af*_AO1

Models of *Af*_AO1 were built based on templates, identified through sequence comparisons. YASARA v.18.2.7.W.64 was used for the building of the models. In total 42 models were built, and the best scoring models were compared manually. The best model was based on the structure of the ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (PDB Code: 1ASQ [42]).

This model was chosen because of the overall alignment and the good fit regarding the copper-binding site and the most similar copper and histidine positions in the model compared to the template.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1: Wavelength scan of *Af*_AO1 compared to known laccases, Figure S2: Substrate screening, Figure S3: Kinetic parameters of *Af*_AO1, Figure S4: Stability of *Af*_AO1, Figure S5: Alignment *Af*_AO1 with ascorbate oxidase from *Cucurbita pepo* var. *melopepo* (1ASQ), Figure S6: Homology model in cartoon representation compared to the template (PDB-Code 1ASQ), Figure S7: SDS-PAGE of purified and deglycosylated *Af*_AO1.

Author Contributions: V.B., S.F., V.P. and S.Z. expressed the enzyme, performed the majority of the experiments and analyzed the data. J.H. and C.F.-S. under the supervision of T.T. performed all experiments concerning redox potential. G.S. modeled the enzyme under the supervision of K.G. D.R., S.Z., V.P. and V.B. planned the experiments. V.B. wrote the manuscript and G.M.G. and D.R. corrected it. G.M.G., D.R. and G.S.N. supervised the work. Funding was acquired by G.M.G., D.R. and G.S.N. Authors discussed and commented on the presented data and the manuscript.

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4.5 Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
*Af*_AO1	MCO from *aspergillus flavus* (NRRL 3357)
ASOM	ascorbate oxidase from *Acremonium* sp. – HI-25
CV	cyclic voltammogram
DHA	dehydroascorbate
DMP	2,6-dimethoxyphenol
K_M	Michaelis constant
MCO	multicopper oxidase
NHE	normal hydrogen electrode
PBS	phosphate-buffered saline
PPy	polypyrrole
V_{max}	maximum velocity

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5 Conclusion

The aim of this thesis was the characterization of novel oxidoreductases for potential application in biotechnology. Oxidoreductases are widespread in nature and comprise of a large group of enzymes catalyzing the transfer of electrons from an electron donor to an electron acceptor molecule mainly by the use of co-factors, such as oxygen. Since many chemical and biochemical transformations involve oxidation/reduction processes, oxidoreductases have become interesting catalysts in biotechnology.

Polyphenoloxidase (PPOs) are a group of oxidoreductases which hydroxylation and oxidation activities on phenols are already well known. However, proteolytic cleavage of other proteins by PPOs has not been described before and could be due to a multifunctionality of the enzyme. In our study, the tyrosinase of common mushroom, *Ab*PPO4, and of apple, *Md*PPO2, cleaved a carboxylesterase from *Clostridium botulinum*, EstA. The cleavage was dependent on tyrosinase activity, as inhibition studies showed. Other influencing factors were ruled out as activity source. Neither *E. coli* lysate, $CuSO_4$, nor enzymes used for purification and activation of the tyrosinases had an impact on the stability of EstA under assay conditions. The proteolytic activity was, therefore, an activity of the tyrosinases themselves. This proteolytic activity differed from the activities of other proteases. It was slower and not executed by traditional proteolytic motives. PPOs lack catalytical triads, as found in serine or cysteine proteases or the motives found in metalloproteases, such as Zn-proteases. Upon cleavage the activity of EstA was increased significantly, especially on PET, making it attractive for PET degradation or functionalization. This novel proteolytic activity of tyrosinases might have other, so far unforeseen, application possibilities as well.

Another group of frequently employed oxidoreductases are laccases, especially from fungi. We investigated laccases from the fungus *Melanocarpus albomyces*, *Ma*L1, and the bacterium *Streptomyces rochei*, *Sr*LA, in our studies. The fungal laccase *Ma*L1 was a typical laccase, with a characteristic substrate profile and typical pH- and temperature optima, of pH 4 and 50 °C, on the standard substrate ABTS. It was active on lignosulfonate at a surprisingly high pH range of pH 6-8. The polymerization of technical lignins with laccases is a promising avenue for making use of this under-used industrial side stream. Especially the activity at high pH values is interesting for technical lignins, other than lignosulfonate, that have inherently high pH values.

Bacterial laccases are not as well studied and as often employed as fungal laccases but have high potential. The bacterial laccase *Sr*LA from *Streptomyces rochei*, exhibited typical laccase characteristics. Its substrate profile and pH optimum of pH 5 were standard for laccases. *Sr*LA was, however, most active at an unusually high temperature of 80 °C and relatively stable which are very useful features for various industrial applications.

Ascorbate oxidases are oxidoreductases closely related to laccases, but less studied and employed. In our studies, the ascorbate oxidase from *Aspergillus flavus*, *Af*_AO1, showed a new activity, as it was a fungal ascorbate oxidase that exhibited laccase activity. Very few fungal ascorbate oxidases are described, and most are not studied in great detail. Ascorbate oxidases that are active on the typical laccase substrate ABTS are still less known. *Af*_AO1 exhibited characteristics typical for ascorbate oxidases with a temperature optimum at 40 °C and an optimal pH of 5 for ascorbic acid. The activity of *Af*_AO1 on ABTS (V_{max} = 11.6 µM/min/mg) was less pronounced than on ascorbic acid (V_{max} = 431.19 µM/min/mg) and specificity was higher on ascorbic acid, with a K_m 10-fold lower than on ABTS. Even a low activity on ABTS is unusual for ascorbate oxidases.

All of the enzymes used in this study were heterologously expressed to different levels in soluble form. Heterologous expression is useful for enzyme characterization and production. Although it poses its own difficulties and some enzymes fall through the grid that might be useful. Investigating and characterizing novel enzymes and activities is the first step to expanding our toolbox of biocatalysts and has been successfully performed in this study. Hence, this thesis broadens our understanding of specific enzymes and lead to a better knowledge about enzymes in general. Promiscuity and multifunctionality for example are relatively newly discovered attributes of enzymes and has been investigated in more detail.

I. Appendix

I.1 Publications

Antonino Biundo, **Verena Braunschmid**, Matthias Pretzler, Ioannis Kampatsikas, Barbara Darnhofer, Ruth Birner-Gruenberger, Annette Rompel, Doris Ribitsch, and Georg M. Guebitz. 2020. "Polyphenol Oxidases Exhibit Promiscuous Proteolytic Activity." Communications Chemistry 3 (1): 1–8. https://doi.org/10.1038/s42004-020-0305-2. (shared 1st author)

Verena Braunschmid, Karin Binder, Sarah Fuerst, Raditya Subagia, Caroline Danner, Hedda Weber, Nikolaus Schwaiger, Gibson S. Nyanhongo, Doris Ribitsch, and Georg M. Guebitz. 2021. "Comparison of a Fungal and a Bacterial Laccase for Lignosulfonate Polymerization." Process Biochemistry 109 (October): 207–13. https://doi.org/10.1016/J.PROCBIO.2021.07.001.

Verena Braunschmid, Sarah Fuerst, Veronika Perz, Sabine Zitzenbacher, Javier Hoyo, Cesar Fernandez-Sanchez, Tzanko Tzanov, et al. 2020. "A Fungal Ascorbate Oxidase with Unexpected Laccase Activity." International Journal of Molecular Sciences 21 (16): 5754. https://doi.org/10.3390/ijms21165754.

Silvio Curia, Antonino Biundo, Isabel Fischer, **Verena Braunschmid**, Georg M. G.M. Gübitz, and J.F. Joseph F. Stanzione. 2018. "Towards Sustainable High-Performance Thermoplastics: Synthesis, Characterization, and Enzymatic Hydrolysis of Bisguaiacol-Based Polyesters." ChemSusChem 11 (15): 2529–39. https://doi.org/10.1002/cssc.201801059.

I.2 Contributions to scientific conferences

Presentations:

- Presentation at ECB 2018 "Laccase: Old enzyme with new applications" **Verena Braunschmid**, Karina Stadler, Antonino Biundo, Sarah Fürst, Andreas Ortner, Sabrina Bischof, Gibson S. Nyanhongo, Doris Ribitsch, Georg M. Gübitz
- Presentation at RRB 2019 "Designing multi-copper oxidases for bio-transformation and upgrading of lignin" **Verena Braunschmid**, Sarah Fürst, Karin Binder, Gibson S. Nyanhongo, Doris Ribitsch, Georg M. Guebitz
- Presentation at 2nd VIP 2019 "Bio-transformation and upgrading of lignosulfonate with engineered enzymes" **Verena Braunschmid**
- Presentation at DBio 2019 "Engineering of laccases for the upgrading of lignosulfonate" **Verena Braunschmid**, Sarah Fürst, Karin Binder, Gibson S. Nyanhongo, Doris Ribitsch, Georg M. Guebitz
- Presentation at MECP 2020+1 "Characterization of a novel ascorbate oxidase with laccase activity" **Verena Braunschmid**, Sarah Fürst, Doris Ribitsch, Georg M. Gübitz

Posters:

- Poster at DocDay Tulln 2017 "Aromatic amino acids at the surface influence the hydrolytic activity of Thc-cutinase 2 on aromatic substrates" **Verena Braunschmid**, Antonino Buindo, Doris Ribitsch, Georg M. Gübitz
- Poster at ESIB 2017 "Fusion of a Substrate-binding domain to the laccase AfLacc1 increases its ability to polymerize lignin" **Verena Braunschmid**, Antonino Buindo, Sarah Fürst, Gibson S. Nyanhongo, Doris Ribitsch, Georg M. Gübitz
- Poster at ISBP 2018 "Applying and optimizing Laccases for the production of lignin-based bio-polymers" **Verena Braunschmid**, Karina Stadler, Antonino Biundo, Sarah Fürst, Andreas Ortner, Sabrina Bischof, Wolfgang Bauer, Karin Hofer, Gibson Nyanhongo, Doris Ribitsch, Georg Gübitz
- Poster at DepoTech 2020 " KASKADE - Enzymatischer Abbau persistenter Mineralölkohlenwasserstoffe" **Verena Braunschmid**, Marion Sumetzberger-Hasinger, Karin Müllern, Maximilian Lackner, Karl Putz, Markus Plank, Norbert Rüttinger, Doris

Ribitsch and Andreas P. Loibner

- Poster at DBio 2021 “PlL – an enzyme designed by nature to degrade petroleum contaminations” **Verena Braunschmid**, Thore Diefenbach, Julia Otte, Karin Müllern, Marion Sumetzberger-Hasinger, Doris Ribitsch, Andreas P. Loibner, Georg M. Guebitz

I.3 Other presentations

- Poster presentation at best of Science Call 2016 “Synthesising lignin- based wood protective coatings with engineered enzymes” **Verena Braunschmid**, Karina Stadler, Andreas Ortner, Doris Ribitsch, Gibson S. Nyanhongo, Georg M. Gübitz (Prize: 3rd best presentation)
- Presentation at Kyoto University (Japan) within the scope of a doctoral school excursion “Engineering enzymes for the valorisation of lignosulfonate” **Verena Braunschmid**