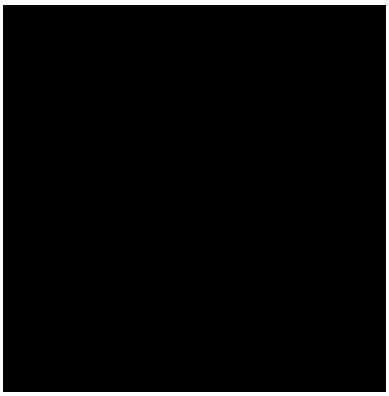

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Expression and characterization of pyranose oxidoreductases: attractive biocatalysts for food biotechnology

Ph.D. thesis

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Ai miei genitori, a Sonia e ad Andrea

Contents

Abstract

Enzymes belonging to the family of sugar-oxidoreductases, which catalyse the oxidation of free, unphosphorylated sugars, play a role in the carbohydrate metabolism of a wide range of higher fungi as well as plants. Although the function and biological role of most of these enzymes is yet not completely understood, these enzymes become increasingly important as biocatalysts for a variety of sugar transformations, in analytical biochemistry and for biosensors.

Among basidiomycetes, the flavoprotein pyranose 2-oxidase (EC 1.1.3.10, P2Ox) is widespread especially among lignin- or lignocellulose-degrading fungi, and has been implicated to play a role in the ligninolytic system. P2Ox catalyzes the C-2 oxidation of several aldopyranoses, including glucose and some other monosaccharides, to the corresponding 2-keto derivatives. During this oxidation, electrons are transferred to oxygen to yield hydrogen peroxide. These ketosugars are difficult to obtain by chemical means, which makes the enzyme P2Ox an attractive tool in carbohydrate chemistry. However, the enzymatic activity of wild-type P2Ox is restricted to a rather small number of sugars. One possible way to alter this limited substrate spectrum is the use of enzyme engineering, e.g., the use of directed evolution and gene shuffling of different but related genes encoding the same enzyme. To this end, heterologous expression has to be established for a number of genes from different sources.

The presented work reports the isolation and heterologous expression of the *p2ox* gene encoding P2Ox from the basidiomycete *Phanerochaete chrysosporium*, the identification of pyranose 2-oxidase genes in the genomes of the ascomycetes *Aspergillus nidulans* and *Aspergillus oryzae*, and their successful expression in *Escherichia coli.*

The catalytically related pyranose dehydrogenase (EC 1.1.99.29, PDH) was first isolated from *Agaricus bisporus* and was found to probably be limited to a rather narrow group of fungi, litter-decomposing *Agaricaceae* and *Lycoperdaceae*. PDH is an extracellular monomeric glycoprotein. It is unable to utilize oxygen as electron acceptor and relies on (substituted) quinones or complexed metal ions as alternatives. The enzyme displays a broad substrate specificity and intriguing variations in regioselectivity; oxidation at C-1,

C-2, C-3 or C-4, as well as double oxidation at C-1,2, C-2,3 or C-3,4 were observed, yielding several reaction products that are inaccessible by chemical means or other enzymatic transformations. Additionally, PDH shows a significantly higher activity towards galactose compared to P2Ox, and is able to oxidize a number of β-1,4-linked disaccharides and even oligosaccharides, making it a potentially very versatile biocatalyst for sugar transformations. PDH is a comparably novel enzyme, and published information is scarce.

A critical point in enzymatic processes is the availability of the biocatalyst and its stability under operational conditions. Most species that produce PDH grow slowly in the laboratory, enzyme titers and yields of active protein are generally low, as is the expression level of *pdh*-genes. This has long been a significant hindrance for investigations of these enzymes, especially of structural properties or technological applications, where larger enzyme amounts are required. Expression of the *pdh*-encoding genes in *E. coli* was, unlike for P2Ox, not successful (our unpublished information). Here we report the successful heterologous expression of a PDH-encoding gene in the filamentous fungi *Aspergillus nidulans* and *Aspergillus niger*.

Zusammenfassung

Enzyme der Klasse Oxidoreduktasen, welche die Oxidation von freien, nicht-phosphorylierten Zuckern katalysieren, spielen eine wichtige Rolle im Zuckerstoffwechsel von vielen höheren Pilzen und Pflanzen. Obwohl die Funktion und die biologische Rolle von den meisten dieser Enzyme noch nicht völlig verstanden wird, werden diese Enzyme immer wichtiger als Biokatalysatoren für eine Vielzahl von Zuckertransformationen, in analytischer Biochemie and für Biosensoren.

In der Klasse der Basidiomyceten ist das Flavoprotein Pyranose 2-oxidase (EC 1.1.3.10, P2Ox) speziell in lignin- und lignocellulose-abbauenden Pilzen weit verbreitet und wird mit dem lignin-abbauenden System in Verbindung gebracht. P2Ox katalysiert die C-2 Oxidation von verschiedenen Aldopyranosen, einschliesslich Glukose und einiger anderer Monosaccharide, zu den entsprechenden 2-keto Derivativen. Während dieser Oxidation werden Elektronen zu Sauerstoff transportiert, was zur Synthese von Wasserstoffperoxid führt. Da die Keto-zucker nur schwierig auf chemischem Wege zu erzeugen sind, gewinnt das Enzym P2Ox an Attraktivität als Werkzeug in der Zuckerchemie. Die enzymatische Aktivität der Wildtyp P2Ox ist jedoch auf eine ziemlich kleine Anzahl von Zuckern beschränkt. Eine Möglichkeit dieses limitierte Substratspektrum zu erweitern, besteht im Enzyme-engineering, indem man Arbeitsmethoden wie “directed evolution” und “gene shuffling” von verschiedenen homologen Genen, die das gleiche Enzym kodieren, verwendet. Daher muss auch in Zukunft die heterologe Expression von zahlreichen Genen aus unterschiedlichen Quellen weiterentwickelt werden.

Die vorliegende Arbeit handelt von der Isolierung und heterologen Expression des *p2ox* Gens, das die P2Ox der Basidiomycete *Phanerochaete chrysosporium* kodiert, und von der Identifikation von Pyranose 2-oxidase Genen in den Genomen der Ascomyceten *Aspergillus nidulans* und *Aspergillus oryzae*, und deren erfolgreicher Expression in *Escherichia coli*.

Die katalytisch ähnliche Pyranose Dehydrogenase (EC 1.1.99.29, PDH) wurde zuerst aus *Agaricus bisporus* isoliert und wurde nur in einer kleinen Gruppe von Pilzen, nämlich den abfall-abbauenden *Agaricaceae* und *Lycoperdaceae*, vermutet. PDH ist ein

extrazelluläres, monomeres Glykoprotein. Es kann Sauerstoff nicht als Elektronenakzeptor verwenden und verwendet stattdessen (substituierte) Chinone und komplexierte Metallionen als Alternativen. Das Enzym zeigt eine breite Substratspezifität und eine verblüffende Variation in seiner Regioselektivität; Oxidation des C-1, C-2, C-3 oder C-4 als auch Doppeloxidation von C-1,2, C-2,3 oder C-3,4 wurden beobachtet, was zu verschiedenen Reaktionsprodukten, die mit chemischen Mitteln oder anderer enzymatischer Transformationen nicht erhältlich sind, führt. Zusätzlich zeigt die PDH signifikant höhere Aktivität mit Galaktose verglichen mit P2Ox und kann eine Vielzahl von β-1-4 verknüpften Disacchariden und sogar Oligosacchariden oxidieren, was sie zu einem vielseitigen Biokatalysator für Zuckertransformationen macht. Die PDH ist ein vergleichbar neues Enzym, und publizierte Informationen darüber sind rar.

Ein kritischer Schritt in enzymatischen Prozessen ist die Verfügbarkeit des Biokatalysators und seine Stabilität unter Prozessbedingungen. Die meisten Spezies, die PDH produzieren, wachsen nur sehr langsam im Labor und sowohl die Ausbeute an aktivem Enzym, als auch das Expressionslevel der *pdh*-Gene ist sehr gering. Dies war lange Zeit das grösste Hindernis in der Erforschung dieser Enzyme, speziell was deren Struktur und technologische Anwendungen, für welche man grosse Enzymmengen benötigt, betrifft. Die Expression der *pdh*-kodierenden Gene in *E.coli* war, im Gegensatz zur P2Ox, nicht möglich (unpublizierte Information). In dieser Arbeit berichten wir über die erfolgreiche, heterologe Expression eines *pdh*-kodierenden Gens in den filamentösen Pilzen *Aspergillus nidulans* und *Aspergillus niger*.

Introduction

Heterologous expression of recombinant proteins in filamentous fungi

Heterologous protein expression technologies are widely used in both basic research and industrial application. In discovery research, heterologous gene expression serves as an important tool to produce proteins and enzymes that are either available at limited levels or difficult to purify in their native context. Many enzymes and proteins have important commercial and industrial value; thus large-scale protein production is of great interest. Since different enzymes and proteins have various molecular and biochemical characteristics, different expression systems with different features are necessary to meet the growing needs of heterologous protein expression.

Because the diverse nature of enzymes and proteins requires that expression systems be customizable and scalable for each application, choosing the most suitable system for expression of the protein of interest becomes a major challenge.

When choosing a heterologous protein expression system, the following general aspects should be considered: (1) post translational modification and processing of proteins; (2) authenticity of the expressed proteins; (3) protein expression level and scale up requirements; (4) subcellular localization (e.g. intracellular, membrane bound, or secreted proteins); (5) cofactor requirement; and (6) costs, safety, and other regulatory issues. Some advantages and drawbacks for commonly used expression systems are listed in Table 1.

The attraction of filamentous fungi as production hosts is based on their natural ability to secrete large amounts of proteins (mainly hydrolytic enzymes) into the growth medium. Eukaryotic post-translational protein processing machinery is an added bonus, with a view of using fungi as expression hosts for proteins requiring elaborate post-translational modification – for example, in the form of protein glycosylation, proteolytic cleavage or formation of multiple disulfide bonds. As with other industrially exploited microbial hosts, filamentous fungi have gone through intricate strain improvement programmes. In the current setting, it is typical that a fungal cell factory used for the expression of

selected gene products is a high-protein-secreting mutant made by traditional random mutagenesis, for which the characteristics have further been modified by genetic engineering. In an overall comparison with other available expression systems for heterologous proteins, applied in liquid culture, filamentous fungi perform well and provide a potentially high-yielding and relatively cheap option.

Characteristics	***Escherichia coli***	**Filamentous fungi**	**Yeast**	**Insect cells**	**Mammalian cells**	**Plant cell culture**
Cell growth	Hours to days	Days to 1 week	Days to 1 week	Days to 1 week	Weeks	Months
Cost of growth medium	Low to medium	Low to medium	Low to medium	High	High	Medium to high
Expression level	Low to high	Low to high	Low to high	Low to high	Low to high	Low
Secretion capability	Secretion to periplasm	Secretion to medium	Secretion to medium	Secretion to medium	Secretion to medium	Secretion to medium
Post-translational modifications						
Protein folding	Refolding usually required	Refolding might be required	Refolding might be required	Proper folding	Proper folding	Proper folding
N-linked glycosylation	None	Mammalian-type core, no sialic acid, non-human sugars added	High mannose, no sialic acid, non-human sugars added	Complex, no sialic acid, non-human sugars added	Complex, non-human sugars added, e.g. by murine cells	Complex, no sialic acid, non-human sugars added
O-linked glycosylation, phosphorylation, acetylation, acylation	No	Yes	Yes	Yes	Yes	Yes

Table 1. Salient characteristics of liquid culture-based expression systems used for the production of heterologous proteins (Nevalainen et al.2005).

Filamentous fungi that dominate the markets as production hosts are the asexually reproducing *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei*. Consequently, most information on heterologous protein expression has come from studies using these particular fungi and the genetically well-characterized *Aspergillus nidulans*.

Aspergillus spp. as host strains

The *Aspergilli* are a large and diverse genus (~180 species) of filamentous fungi including several well known species with substantial commercial value (*A. oryzae* and *A. niger*) and medically significant molds; both as pathogens (*A. parasiticus* and *A. fumigatus*) and toxin-producing contaminants of food and feed (*A. flavus*), as well as lesser-known but valuable species such as *A. terreus*, source of the cholesterol lowering agent lovastatin. *A. nidulans* has been one of the most widely used filamentous fungi for basic genetic research, and continues to be used a model organism for the study of common eukaryotic cellular functions. *Aspergillus* will grow at a wide range of temperatures (10–50 °C), pH (2.0–11.0), and osmolarity (from nearly pure water up to 34% salt). It is also capable of continuous secretion of large amounts of metabolites into the culture medium, and its filamentous morphology allows separation of cells by simple filtration, simplifying recovery of valuable products. Filamentous fungi are widely used for solid-state fermentation of grain-based foods (miso, shoyu) and beverages (sake, shochu) and for industrial enzyme production, especially for their high titers of native hydrolytic enzymes, in particular amylases and proteases (e.g., 30 g/L of glucoamylase from *A.*
niger (Withers et al. 1998).

A complete physical map of the *A. nidulans* genome is completed and in 2001 the whole genome was sequenced. Genome sequences for *A. oryzae*, *A. fumigatus* and *A. niger* followed shortly and are at or nearing completion of annotation and public availability; genome sequences for *A. clavatus*, *A. flavus*, *A. parasiticus*, and *A. terreus* are also in various stages of development in public and private efforts. Over 300 genes have been cloned, mostly from *A.nidulans*, many by complementation of random mutants, which provides today's researcher with several hundred mutant strains to work with. The GRAS (generally recognized as safe) status of *A. niger* and *A.oryzae* makes them attractive as expression hosts, since this status should facilitate approval of new food or drug products. The development of transformation techniques for *Aspergillus* and other filamentous fungi lagged behind those for *E. coli* and *S. cerevisiae*, complicated by their multicellular morphology, thick chitinous cell walls, and lack of plasmids. The first reports of gene

cloning (Kinghorn and Hawkins, 1982) and transformation in *Aspergillus* (Ballance et al.1983; Tilburn et al. 1983) were in 1982 and 1983, respectively. Several laboratory methods for *Aspergillus* transformation are currently in use. The most popular technique, adapted from that originally developed for yeast, is the chemical treatment of nucleated protoplasts produced by enzymatic digestion of the fungal cell wall. Refinements of this method have increased transformation efficiencies by several orders of magnitude since its conception, from <10 transformants/μg DNA to several hundred. Other methods include electroporation and a biolistic technique. The plant transformation workhorse *Agrobacterium tumefaciens* has been used to transform *Aspergillus* and other fungi. Methods for the genetic manipulation of *Saccharomyces* and *E. coli* were greatly facilitated in each case by the discovery of native plasmids; unfortunately, like most fungi, the Aspergilli lack natural extrachromosomally replicating DNA elements. Accordingly, researchers have attempted to construct artificial plasmids for *Aspergillus* (Aleksenko et al. 1996). Heterologous gene expression from these vectors was of much interest, because transcription from a plasmid would be free of any possible positional effects on genes integrated into different chromosomal loci. However, the transformants show a high phenotypic instability on selective medium.

For developing production strains, chromosomal integration is preferred for genetic stability. This also allows for recombination of desired traits by subsequent breeding, and eliminates the need for addition of selective agents to the medium, a not-inconsiderable expense in an industrial setting.

Shortly after the first transformation of *Aspergillus*, work began on vectors combining a selectable marker with fungal promoter and terminator sequences for gene expression. Vectors bearing the *E. coli lacZ* and *uidA* gene sequences, encoding β-galactosidase and β- glucuronidase (GUS), respectively, were successfully demonstrated as reporter constructs in *A. nidulans* and *A. niger* (van Gorcom et al. 1986; van Gorcom & van den Hondel, 1988; Roberts et al. 1989). By1990 several workable expression systems had been developed to a commercially viable level (reviewed in Davies 1991a; van den Hondel et al. 1991; Kinghorn and Unkles 1994); however, transformation and controlled expression in *Aspergillus* remains considerably more problematic than in yeast or *E. coli*.

Aspergillus promoters have been explored for use in heterologous expression, with an aim of gaining further control over expression levels or timing, eliminating catabolite repression or other "crosstalk" between host metabolism and the introduced expression construct; or more economical means of induction such as pH or simple salts rather than a gratuitous inducer, which can add considerable expense to production scale fermentation. The *A. oryzae* α-amylase (Christensen et al. 1988), *A. niger* pacA (acid phosphatase; pH inducible (Macrae et al. 1988)), *A. nidulans* gpdA (glyceraldehyde- 3-phosphate dehydrogenase; strong, constitutive (Punt et al., 1990)) promoters have been tried.

Most work on expression constructs in *Aspergillus* has focused on promoters and associated repressors and activators, rather than other components of the transcription and translation machinery, e.g., sequence elements such as transcription initiation sites and terminators, poly-adenylation sequences, and translational initiation sites. A number of different 3′ untranslated regions from native genes, ranging from less than 100 bp up to 600–800 bp in length, have been used as terminators for heterologous gene expression, with more or less equal success anecdotally.

The production of fermentation-derived enzymes has since grown to an industry with a global market estimated to be worth nearly US$5 billion by 2009, of which filamentous fungi account for roughly half of the production (FreedoniaGroup 2006); *Aspergillus* is the leading genus for enzymes, with new industrially useful activities still being discovered (Guimaraes et al. 2006). A 2004 review lists over 70 biotechnologically important native fungal enzymes in current use, comprising 32 classes of activity; 22 of these are produced in eight *Aspergillus* species, representing 14 enzyme families (Schauer and Borriss 2004). A 2005 review lists 80 recombinant proteins of fungal origin, all enzymes that have been expressed to date in 12 filamentous fungi; of these 55 were produced in four *Aspergillus* species; *A. awamori*, *A. nidulans*, *A. niger* and *A. oryzae* (Yoder and Lehmbeck 2004). Additionally the authors cite another 40 heterologous proteins produced in eight filamentous fungi, 30 of these in the four *Aspergillus* species. Most of these are of animal or fungal origin, largely enzymes.

The enzymes are used in large industrial applications such as corn starch conversion for ethanol fermentation (amylases), detergent additives (proteases, lipases), and processing

textiles (cellobiohydrolase, cellulases), paper (cellulases, hemi-cellulases) or leather (proteases, lipases, cellulases). Over two-thirds of the native enzymes are used in food, feed and beverage applications, e.g., production of high-fructose corn syrup (amylase, invertase), fruit juice and wine (pectinases, cellulases, glucosidases), baking (glucose oxidase, amylase, metalloproteinase), brewing and distilling (amylases, glucanases), and dairy (lactase, proteases). Sixteen recombinant fungal enzymes were approved for use in food as of 1998, of which 13 are produced in *Aspergillus* (Archer 2000). The first recombinant fungal product approved for food use in the U.S. was the aforementioned bovine chymosin (Ward 1990); fungal aspartic proteases are also used as a chymosin substitute in cheese making. The replacement of animal products such as calf chymosin with recombinant fungal products may serve to generate goodwill in the growing vegetarian population, and hopefully offset negative perceptions of GMO products.

The growing knowledge of fungal metabolism and unique capabilities of *Aspergillus* assure it a continued place among the most biotechnologically useful organism known to humanity.

Sugar oxidoreductases

Oxidoreductases of free, non-phosphorylated sugars are typical of a large number of higher fungi. Glucose 1-oxidase (EC 1.1.3.4) catalyzes the oxidation of glucose at C-1 and is typical for a wide range of Ascomycetes and Fungi imperfecti (Volc et al. 1997). The hemoflavoprotein cellobiose dehydrogenase (CDH, EC 1.1.99.18; formerly cellobiose oxidase) has been described in cellulolytic cultures of *Phanerochaete chrysosporium* and *Trametes versicolor* (Westermark and Eriksson 1974). It oxidizes various sugars, typically β-1,4-linked di- or oligosaccharides, at the position C-1 to the corresponding lactones; cellobiose (glc-β-1,4-glc) is the preferred substrate (Bao et al. 1992; Henriksson et al. 1998).

Among basidiomycetes, the flavoprotein pyranose 2-oxidase (EC 1.1.3.10, P2O) is widespread (Danneel et al. 1992; Volc et al. 1995; Leitner et al. 1996), especially among lignin- or lignocellulose-degrading fungi, and has been implicated to play a role in the

ligninolytic system (Daniel et al. 1994). It has been purified and characterized from several white-rot fungi including *P. chrysosporium* (Volc and Eriksson 1988; Artolozaga et al. 1997), *Pleurotus ostreatus* (Shin et al. 1993), *Trametes multicolor* (Leitner et al. 2001b) and *Trametes (Coriolus) versicolor* (Machida and Nakanishi 1984). Typically, P2O is a large, homotetrameric protein containing one covalently bound flavin adenine dinucleotide per subunit. The crystal structure of P2O from *T. multicolor* has been elucidated recently (Hallberg et al. 2004). Preferred in vivo substrates are most probably D-glucose, D-galactose, and D-xylose, which are abundant in lignocellulose and are oxidized to 2-keto-D-glucose (D-*arabino*-hexos-2-ulose, D-glucosone), 2-keto-D-galactose (D-*lyxo*-hexos-2-ulose, D-galactosone), and 2-keto-D-xylose (D-*threo*-pentos-2-ulose, D-xylosone), respectively. The first industrial application that was studied on a larger scale was the conversion of glucose to pure fructose via the intermediate 2-keto-D-glucose by the American company Cetus in the early 1980s (Neidleman *et al.* 1981). In subsequent studies several other mono- and disaccharides were converted to the corresponding 2-ketosugars on a preparative scale and the reaction products were identified (Freimund *et al.* 1998; Huwig *et al.* 1994; Leitner *et al.* 2001a). These chemically well defined 2-ketosugars have lately attracted increased interest as rare sugar intermediates for subsequent transformations or synthesis (Giffhorn 2000; Giffhorn *et al.* 2000).

A somewhat different but catalitically related enzyme, pyranose 2-dehydrogenase (henceforth abbreviated PDH, EC 1.1.99.29), was first isolated from the edible mushroom *Agaricus bisporus* (Volc et al. 1997). It was since found to probably be limited to a rather narrow group of fungi, litter-decomposing *Agaricaceae* and *Lycoperdaceae* (Volc et al. 2001). PDH is an extracellular monomeric protein of about 75 kDa, is heavily glycosylated and is unable to utilize oxygen as electron acceptor. Suitable electron acceptors are 1,4-benzoquinone, 3,5-di-tert-butyl-1,2-benzoquinone, 2,6-dichloroindophenol and ferricyanide, amongst others. PDH is capable of oxidizing a broader variety of monosaccharides, oligosaccharides and glycosides compared to P2O; almost all major sugar components of wood polysaccharides, such as D-glucose, D-galactose, D-xylose, D-arabinose, cellobiose, D-glucono-1,5-lactone, yield comparable

activities, with none of them appearing to be a prominently preferred substrate (Volc et al. 1997).

While P2O shows high C-2 regioselectivity, the enzyme from *A. bisporus* oxidizes glucose to both 2-keto-glucose and 3-keto-glucose, and then further to 2,3-diketo-glucose. The preference for C-2, C-3 or C2 + C-3 is, however, substrate dependent and changes with the source of the enzyme. Galactose, for instance, is exclusively oxidized at C-2 (*A. bisporus*), glucose and the D-glucosyl-moiety of non-reducing oligosaccharides at C-3 (*Macrolepiota rhacodes* and *A. meleagris*, resp.), whereas xylose is oxidized at C-2 and C-3 (*A. meleagris*) (Volc et al. 2004). Additionally, P2O is unable to oxidize β-1,4-linked disaccharides (including lactose), whereas PDH from a number of *Agaricus* sp. shows significant activity towards lactose. Some of these enzymes display almost exclusive selectivity for oxidation at C-1, whereas others produce mixtures of C-2/C-1 oxidation products in variable ratios, the highest being 1.7 (*A. xanthoderma*) and 1.1 (*A. meleagris*) (Volc et al. 2004).

Lactose and lactose derivatives as raw materials for biotechnology

Cheese whey is the remaining liquid following the precipitation and removal of milk casein during cheese-making. Lactose represents about 4.5 – 5 % w/v of cheese whey, accumulating to approximately 6 mio tons annually world wide from 145 mio tons of liquid whey (Siso 1996). Because of the low concentration of milk constituents (approx. 6 – 7 % dry matter), whey has commonly been considered a waste product, and about half of the world cheese whey production is not treated but disposed of as effluent, causing considerable environmental problems due to the high organic content and large volumes. These issues are likely to gain importance in the near future due to the observed and projected increase in worldwide cheese production (Timmermans 1999) as well as centralization of cheese production plants, and stricter legislative regulations regarding effluent quality (Siso 1996).

Direct utilization of lactose in food and pharmaceuticals is necessarily limited, due to the high percentage of lactose intolerant adults (approx. 70%), and alternative ways to exploit

the renewable resource lactose are of considerable commercial interest. Enzymatic sugar transformations present a great potential for the regioselective synthesis of various keto sugars, and the synthesis of industrially important compounds from keto sugars has already been demonstrated. By combining enzymatic and chemical reactions, a vast number of sugar-derived compounds can be constructed (Giffhorn 2000; Giffhorn et al. 2000). Enzymatic synthesis can be an interesting alternative that would aid in turning the low-cost by-product lactose into a marketable commodity.

Lactulose – medical, nutritional and technological properties

Lactulose (4-O-ß-D-galactopyranosyl-D-fructose) is an isomerization product of lactose consisting of a galactose and a fructose moiety. Lactulose does not occur in nature, but does occur in very small quantities in heated milk as a product of noncatalysed isomerisation. Lactulose can not be split at the ß-1-4-glycosidic bond by human intestinal enzymes, and is metabolized by saccharolytic intestinal (colonic) bacteria (Huchzermeyer and Schumann 1997; Schumann 2002). It constitutes a favorable food for lactic acid bacteria over a number of proteolytic pathogenic microorganisms. Recently, the term „prebiotic“ was introduced in food science for a „non-digestible food ingredient that beneficially affects the host by stimulating growth and/or activity of one or a limited number of bacterial species in the colon“ (Gibson and Roberfroid 1995). In contrast to other prebiotics, however, lactulose has been mainly used as a medicinal drug thus far, and is registered as such in over 100 countries. For chronic constipation, lactulose applications of 10 – 40 g/day have proven reliably efficient and safe in long-term use (Blei and Cordoba 2001). For hepatic encephalopathy and chronic portal systemic encephalopathy, lactulose has been used for more than 30 years in prophylaxis and treatment of all degrees of severity in dosages of up to 90 g/day (Conn 1994). A rather old indication of the prebiotic efficacy of lactulose is its potential for treatment of salmonella carrier state. Lactulose is used in this indication in dosages of up to 60 g/day (Schumann 2002).

Most beneficial effects can be attributed to the prebiotic properties of lactulose, which are thoroughly researched since 1957, and, in contrast to most other prebiotics, established not only through animal testing or in vitro trials, but clinical research. Among these physiological effects of lactulose are, briefly summarized, an increase in osmotic pressure along with a drop in colonic pH, depletion of colonic ammonia, changes in bacterial metabolism such as decreased urea degradation and protein degradation, increased carbohydrate metabolism, increased utilization of nitrogen containing compounds for bacterial growth, changes in the composition of the colonic microflora, increased peristalsis, inhibition of toxin-producing enzymes, shortening of toxin residence time, prevention of gallstones and decrease of serum lipids (Schumann 2002).

These properties make lactulose a very interesting compound for food technology, and it can be predicted that lactulose is a highly attractive ingredient for functional food, with its application in food technology mainly hampered by its relatively high price. Another potentially interesting application of lactulose is as an additive to animal feed. As more and more countries ban antibiotics in animal nutrition, lactulose may be an alternative for the prevention and treatment of infections or carrier-states as well as for growth enhancement. The future perspectives for the prebiotics market are very promising: in 1997 the market value of prebiotic compounds sold was 2 million $, in 2004 this value reached 85 million $, and for the year 2010 it is estimated that sales will value 180 million $ world-wide; these figures only include food applications, so when considering the proposed use in animal feed it is obvious that the market potential is much wider.

Lactulose is currently synthesized by alkaline isomerization from lactose. This method offers only low yields and moderate selectivity, and various degradation reactions occur; complexation by equimolar addition of boric acid increases yield, but requires removal of the acid (van Zundert and Hoffmann 1999; Mayer et al. 2004), resulting in cost intensive waste management and product purification. Alternative enzymatic processes should offer significant advantages over this method to be economically viable. There is, however, no enzyme known thus far catalyzing the direct isomerization of lactose to lactulose.

Recently, an enzymatic process based on the transglycosylation from lactose to fructose leading to lactulose was suggested. The reaction was performed using β-galactosidase

from *Aspergillus oryzae* or β-glucosidase from *Pyrococcus furiosus* (Mayer et al. 2004), as well as permeabilized whole cells of *Kluyveromyces lactis* (Lee et al. 2004). Both methods resulted in good yields of lactulose (16 g/l and 44% relative to lactose for CelB from *P. furiosus*, (Mayer et al. 2004); and 6.8 g/l/h for whole cells of *K. lactis*, (Lee et al. 2004)). However, both methods are limited in yield as well as attainable purity of reaction products due to the nature of the transglycosylation reaction – even at fructose concentrations close to saturation alternative products arise, especially in later stages of the process, and β-glucosidase activity surpasses transglycosylation activity, resulting in a decrease of lactulose concentration.

Tagatose

D-Tagatose is an isomer of D-galactose and rarely found in nature, except in strongly heated milk, in certain cheese such as Roquefort and some cultures of mycobacteria when grown on galactose. It has many properties resembling those of sucrose, including a high sweetness (92 % as compared to sucrose in 10 % solution) and a similar bulk value. The caloric value of tagatose for humans is only 1.5 kcal/g (Kim 2004) and it has prebiotic properties (Bertelsen et al. 1999). It has been approved as a "generally recognized as safe (GRAS)" material (Levin 2002), and can be used in confectionary, beverages, health food and dietary products as low-calorie, full-bulk sweetener. The market is expected to partly compete with the market for sugar-substituting polyalcohols such as sorbitol, xylitol, mannitol or isomalt, but, due to certain unique properties such as the lack of a cooling effect, its prebiotic properties and involvement in browning reactions during heat treatment, tagatose can be expected to create new market opportunities (Kim 2004).

Tagatose can be synthesized by isomerization of galactose via a number of chemical or enzymatic processes, as was recently reviewed (Kim 2004). Among those are isomerization by a calcium catalyst (requiring a neutralization step before purification) and enzymatic isomerization using L-arabinose isomerase, which can be obtained from several microbial sources, including thermotolerant microorganisms. Using an immobilized L-arabinose isomerase from *Geobacillus stearothermophilus*, Kim and

coworkers were able to produce 230 g/l tagatose with a productivity of 9.6 g/(l·h) and a conversion rate of 46 % (Kim et al. 2003). A disadvantage of these isomerization methods is that only the equilibrium between galactose and tagatose can be reached, and the remaining galactose has to be removed in subsequent steps. An alternative process is via the oxidative synthesis of galactose to 2-keto-galactose, analogous to the Cetus process for the production of pure fructose (Neidleman et al. 1981)

Biofuel cells

Bioelectronics is a rapidly advancing interdisciplinary field that aims at integrating biomolecules and electronic elements into functional systems. The properties of biocatalysts that are unmatched by conventional redox catalysts – activity at ambient temperature and neutral pH, and more importantly, selective catalytic activity – together with our ability to modify or tailor the functions or properties of these biocatalysts, make these biomolecules attractive building blocks for functional devices such as biofuel cells (Willner 2002; Barton et al. 2004). Biofuel cells can be classified into microbial and enzymatic fuel cells. The latter allow for substantial concentration of the biocatalyst and removal of mass-transfer barriers that are encountered in microbial fuel cells, therefore enzymatic biofuel cells produce higher current and power densities, approaching the range of applicability to micro- and miniscale electronics applications (Barton et al. 2004). Possible applications of biofuel cells are for implantable power, such as microscale cells implanted in human or animal tissue, or power derived from ambient fuels, such as plant saps (Heller 2004). Biocatalysts have evolved to function in complex physiological environments, efficiently and selectively catalyzing reactions at physiological temperature and pH, and involving fuels and oxidants present in such environments. They can use substrates that are typically found *in vivo* even though only in low concentrations (e.g., glucose 5 mM; oxygen 0.1 mM). This makes enzymatic biofuel cells a promising future application as an implantable power source, and a number of implantable medical devices might benefit from these power supplies, such as the cardiac pacemaker, or prosthetic applications including artificial hearing or vision

(Barton et al. 2004). The second possible application, the exploitation of ambient fuels, is attractive in situations where power needs for small electronic devices are distributed, disconnected, and long term, e.g., for electronic sensors monitoring air quality, weather, presence of biohazards, etc. For this application the fuel can be derived from carbohydrates contained in plants, such as sucrose-, glucose- or fructose-rich plant saps, or lignocellulose hydrolysates containing various pentoses and hexoses.

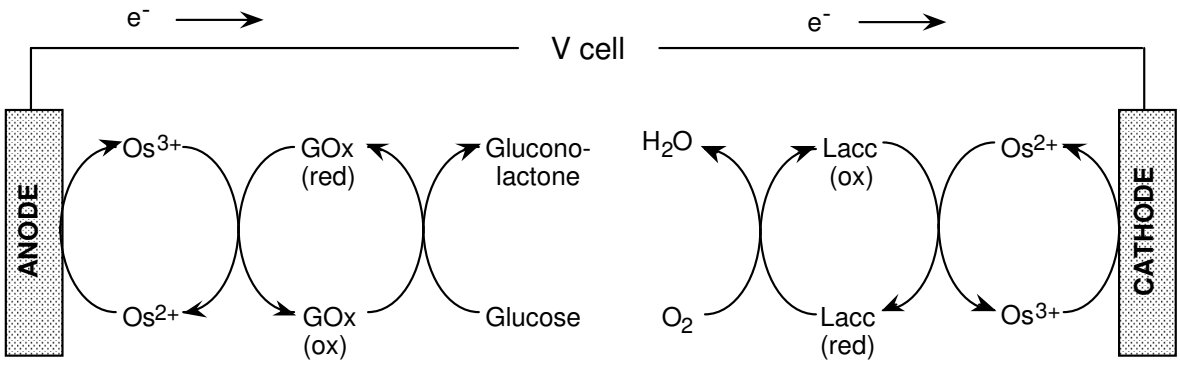

Figure 1. Biofuel cell. Bioelectronic elements generate electrical energy by the bioelectro-catalysed oxidation of glucose. GOx, glucose oxidase; Lacc, laccase

In enzymatic biofuel cells, suitable redox enzymes are connected to electrodes by redox active substances that shuttle electrons between the enzyme and the electrode and hence replace the natural cosubstrate of the enzyme. The construction of an electrically contacted enzyme electrode (e.g., with glucose oxidase as biocatalyst) and an oxygen-reducing biocatalytic electrode (e.g., consisting of laccase) creates an anode and a cathode that generate electrical power through the bioelectrocatalytic oxidation of the appropriate substrate, in this example glucose (Willner 2002). Glucose oxidase (GOx) is the most commonly used glucose-oxidising enzyme employed in conjunction with biofuel cells. GOx is relatively selective for glucose, with only few other sugars being oxidised by this enzyme at significantly lower rates. Another major disadvantage is that GOx is anomer selective, using only β-glucose as its substrate. In addition, the K_m value

for GOx is relatively high, e.g. for the commonly used *Aspergillus niger* enzyme it is in the range of 30 mM. Redox active compounds (mediators) that shuttle electrons between the enzyme and the electrode and that have been used for bioelectrocatalysis are often based on redox polymers, with polymeric backbones containing dispersed redox centres. Widely used are osmium redox polymers (Heller 1992), but others such as ferrocene-modified polysiloxane or polypyrrole were also successfully applied (Foulds & Lowe 1988, Hale et al. 1991). These Os hydrogels conduct electrons through electron-transferring collisions with other mobile redox centres which are tethered to the polymer backbone (Heller 2004).

Since most of the possible or proposed applications aim at long-term use of the biofuel cell with lifetimes ranging from months to years, stability is a key aspect (Barton et al. 2004). In addition, a wide substrate specificity for the biocatalyst, both pertaining to the electron donor as well as the electron acceptor, is desirable.

P2Ox has several advantages over the commonly used glucose oxidase; these advantages include (i) its high affinity for glucose (K_m of 0.74 mM for glucose for TmP2Ox vs. approx. 30 mM for *A. niger* GOx), (ii) the lack of any anomer preference so that both α- and β-glucose are oxidised essentially equally well, and (iii) the much wider sugar substrate specificity of P2Ox, including fructose, xylose, galactose, arabinose amongst others as substrates. Because of this latter aspect, many other sugars in addition to glucose can be oxidised from e.g., a sucrose or a lignocellulose hydrolysate and thus these can be used for small scale energy production. Recently, it was shown that different osmium-functionalised polymers can efficiently 'wire' (electrically connect) P2Ox to the electrode and thus mediate the electron transfer between enzyme and electrode. Because of this it should be possible to use P2Ox with these or other redox polymers in combination with a suitable cathode for biofuel cells. In these first preliminary experiments stability of the sensors was investigated as well. Using one type of Os polymer, about 6% of the original response of the sensor was lost after 18 h of operation (Timur et al. 2005). Even though this is encouraging for further work, it also shows the necessity of further improvement of biocatalyst stability for the intended long-term applications in biofuel cells.

Objectives/Aim of the thesis

Lactulose, tagatose and lactobionic acid are, as outlined in the Introduction, commercially interesting substances that can be synthesized from the abundantly available raw material lactose or, in case of tagatose, its hydrolysis product galactose. Isomerization of lactose to lactulose is, however, not catalyzed by glucose isomerases that are used for conversion of D-glucose to D-fructose (Leitner et al. 2001a) and the currently used chemical synthesis has a number of flaws. An alternative could be the oxidation of lactose at C-2 to keto-lactose, followed by a selective reduction at C-1 to lactulose. A similar conversion of 1,6-disaccharides such as allolactose or isomaltose to allolactulose or isomaltulose via the keto-disaccharide using pyranose 2-oxidase and an NAD(P)H-dependent aldose reductase from *Candida tenuis* has been described (Leitner et al. 2001a). P2O, however, does not catalyze the C-2 oxidation of 1,4-linked disaccharides such as lactose.

P2O is a suitable enzyme for the production of 2-keto-glucose or 2-keto-galactose, intermediates towards fructose and tagatose; galactose, however, is a significantly poorer substrate, with conversion rates of approximately 8 % as compared to glucose (*T. multicolor* P2O: $K_{m,Glc}$ 0.74 mM, $k_{cat,Glc}$ 54 s^{-1}; $K_{m,Gal}$ 9.2 mM, $k_{cat,Glc}$ 3.1 s^{-1}).

Cellobiose dehydrogenase has been employed for the conversion of lactose to lactobionic acid by C-1-oxidation (Ludwig et al. 2004a), but is limited to C-1 oxidation and does not catalyze C-2 oxidation of either monosaccharides (galactose) or disaccharides (lactose). It is therefore suitable for the development of a process for lactobionic acid production, but unable to synthesize either 2-keto-galactose or 2-keto-lactose.

Pyranose dehydrogenases from fungal species of *Agaricaceae* and *Coprinaceae* represent an enzyme family of very similar proteins suitable for a wide range of carbohydrate transformations – the enzymes from, e. g., *A. bisporus* or *A. xanthoderma* appear to be suitable for the production of 2-keto-galactose, as their relative activity with galactose is in the range of activity with glucose or higher (kinetic constants for *A. xanthoderma* PDH: $K_{m,Glc}$, 0.78 mM, $k_{cat,Glc}$, 94 s^{-1}; $K_{m,Gal}$, 11 mM, $k_{cat,Gal}$, 250 s^{-1}; $K_{m,Lac}$, 120 mM, $k_{cat,Lac}$, 90 s^{-1}), whereas an enzyme from *A. xanthoderma* can be used for production of – predominantly - 2-keto-lactose (the concomitantly produced lactobionic acid is of commercial interest as well and can be easily separated from 2-keto-lactose by using ion

exchangers), and PDH from *A. campestris* for production of pure lactobionic acid. A biocatalytic process for carbohydrate conversion with PDH, using the same redox mediator regeneration system and the same general process conditions, should therefore be a highly versatile and easily adaptable tool for synthesizing a large number of commercially attractive compounds.

Besides having a broader sugar substrate spectrum and much more favorable kinetics for the turnover of some of the envisaged substrates than the catalytically comparable P2O, pyranose dehydrogenase offers another advantage over other technologically interesting oxidoreductases: In contrast to P2O, PDH is a true dehydrogenase and shows no discernible activity with oxygen, hence the formation of hydrogen peroxide, which is formed as a second product when electrons are transferred to oxygen, is completely avoided. H_2O_2 as a highly reactive compound is known to inactivate enzymes, especially during substrate turnover by these oxidases. This problem of stability is of general interest and has been observed with several technologically attractive flavin oxidases such as glucose oxidase (Bourdillon et al. 1988), D-amino acid oxidase (Ju et al. 2000), or pyranose oxidase (Treitz et al. 2001). During substrate turnover P2O is rapidly inactivated ($t_{1/2}$ of approx. 1 h) unless hydrogen peroxide is decomposed by e.g. catalases (Leitner et al. 1998). However, even in the presence of an excess of catalase activity P2O is not sufficiently stable both in its free (Leitner et al. 1998) and immobilised forms (Treitz et al. 2001). Hence we expect a biocatalytic system based on PDH/laccase to be much more stable under operational conditions than comparable biotransformation systems employing flavin oxidases. Since many of these oxidases show also significant activity with alternative electron acceptors (such as quinones or chelated transition metal ions) the results obtained in this proposed project can be valuable for other biotransformations using these H_2O_2-producing oxidases as well.

PDH is a comparably novel enzyme, and published information is scarce. A number of reactions that are catalyzed by PDH are described, but there is no established process employing PDH, and no work on reaction engineering of PDH has been performed thus far. In light of the versatility of PDH and the multitude of potential applications, such investigations will constitute significant progress and be of great interest for biotechnology and applied enzymology.

Fig. 2. Conversion of lactose to (a mixture of) lactobiono-1,5-lactone and 2-keto-lactose, and of galactose to 2-keto-galactose, as catalyzed by PDH from *A. xanthoderma*, coupled with reoxidation of the redox mediator by laccase.

Figure 2 shows a reaction scheme for the biocatalytic conversion of interest: lactose is converted to lactobiono-1,5-lactone (C-1-oxidation) and 2-keto-lactose (C-2-oxidation), and galactose to 2-keto-galactose (C-2-oxidation). This reaction is the key step in the proposed enzymatic production of lactobionic acid, lactulose and tagatose. Lactobiono-1,5-lactone is spontaneously hydrolyzed to lactobionic acid; 2-keto-lactose and 2-keto-galactose can be transformed into lactulose and tagatose, resp., by C-1-specific reduction. This second step can be done enzymatically as well, using the above mentioned aldose reductase, or chemically, using a platinum- or palladium-catalyst. Both methods are established or under investigation at (Leitner et al. 2001a).

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Chapter 1

Pyranose 2-oxidase from *Phanerochaete chrysosporium*—Expression in *E. coli* and biochemical characterization

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Pyranose 2-oxidase from *Phanerochaete chrysosporium*—Expression in *E. coli* and biochemical characterization

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ABSTRACT

The presented work reports the isolation and heterologous expression of the *p2ox* gene encoding the flavoprotein pyranose 2-oxidase (P2Ox) from the basidiomycete *Phanerochaete chrysosporium*. The *p2ox* cDNA was inserted into the bacterial expression vector pET21a(+) and successfully expressed in *Escherichia coli*. We obtained active, fully flavinylated recombinant P2Ox in yields of approximately 270 mg/l medium. The recombinant enzyme was provided with an N-terminal T7-tag and a C-terminal His_6-tag to facilitate simple one-step purification. We obtained an apparently homogenous enzyme preparation with a specific activity of 16.5 U/mg. Recombinant P2Ox from *P. chrysosporium* was characterized in some detail with respect to its physical and catalytic properties, both for electron donor (sugar substrates) and – for the first time – alternative electron acceptors (1,4-benzoquinone, substituted quinones, 2,6-dichloroindophenol and ferricenium ion). As judged from the catalytic efficiencies k_{cat}/K_m, some of these alternative electron acceptors are better substrates than oxygen, which might have implications for the proposed *in vivo* function of pyranose 2-oxidase.

1. Introduction

Pyranose 2-oxidase (P2Ox, pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) belongs to the glucose–methanol–choline (GMC) oxidoreductase family of flavin adenine dinucleotide (FAD)-dependent sugar oxidoreductases (Hallberg et al., 2004). It is localized in the periplasmic space and associated with membranous materials of lignocellulolytic fungi (Daniel et al., 1992) and is believed to play a role in lignocellulose degradation by providing the essential co-substrate hydrogen peroxide for lignin and manganese peroxidases (Daniel et al., 1994). Another biological function under discussion is its role in a secondary metabolic pathway leading from D-glucose via 2-keto-D-glucose to the β-pyrone-antibiotic cortalcerone (Baute et al., 1977). In general, pyranose 2-oxidase is a 270 kDa homotetramer that catalyzes regioselectively the C-2 oxidation of several aldopyranoses to the respective 2-dehydro derivates, transferring electrons to molecular oxygen to yield H_2O_2. However, for some substrates, the enzyme also displays specificity for oxidation at C-3 (Freimund et al., 1998; Giffhorn, 2000; Eriksson et al., 1986). P2Ox can also transfer electrons to acceptors other than oxygen, e.g., quinones, organic radical molecules or complexed metal ions, but this property has not been studied and compared in any detail for different enzymes (Shin et al., 1993).

Pyranose 2-oxidase is an enzyme with high potential for biotransformations of carbohydrates; applications in bioprocesses, clinical analytics and in synthetic carbohydrate chemistry have been reviewed (Giffhorn, 2000). Recently, we showed that P2Ox can be electrically wired to graphite electrodes in the presence of suitable osmium redox polymers, and hence could be used advantageously in enzyme electrodes or biofuel cells (Tasca et al., 2007).

A critical point in enzymatic processes is the availability of the biocatalyst and its stability under operational conditions. Production of native enzymes by fungal cultures is often – depending on the source – hampered by slow growth and limited yield. Heterologous expression in a suitable recombinant strain offers an attractive alternative with higher yields, shorter fermentation times and often cheaper fermentation media. Techniques such as site-directed mutagenesis, directed evolution, gene shuffling and combinations of these methods have been shown to be powerful

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tools for enzyme engineering and protein design (Bornscheuer and Pohl, 2001). All of these methods require heterologous expression of the respective encoding gene, with *Escherichia coli* and yeast-based systems being the preferred choice whenever possible due to short generation times, ease of handling and amenability for genetic engineering.

There are several examples of pyranose 2-oxidase genes from basidiomycetous fungi cloned and expressed heterologously, all in *E. coli*-based expression systems. These include *Coriolus* (*Trametes*) *versicolor* (Nishimura et al., 1996), *Trametes hirsuta* (Christensen et al., 2000), *Tricholoma matsutake* (Takakura and Kuwata, 2003), *Peniophora* sp. (Bannwarth et al., 2004), *Trametes multicolor* (synonymous for *T. ochracea*) (Kujawa et al., 2006; Vecerek et al., 2004) and *Trametes pubescens* (Maresova et al., 2005). Experimentally determined crystal structures of P2Ox are available for only two proteins to date, those from *T. multicolor* (Hallberg et al., 2004) and *Peniophora* sp. (Bannwarth et al., 2004), which have highly similar amino acid sequences. In addition, the structures of P2Ox liganded either with the poor substrate 2-deoxy-2-fluoro-D-glucose or its product 2-keto-D-glucose are published (Bastian et al., 2005; Kujawa et al., 2006).

Phanerochaete chrysosporium is considered the prime model organism among white-rot fungi for lignin degradation and investigation of the respective enzyme systems; its genome was recently sequenced and annotated by the US Department of Energy Joint Genome Initiative (Martinez et al., 2004). The biochemistry and transcriptional regulation of pyranose 2-oxidase from this fungus (PcP2Ox) is reported (Artolozaga et al., 1997; De Koker et al., 2004; Eriksson et al., 1986). A comparison of P2Ox sequences reveals several distinct features in the sequences of the enzymes from *P. chrysosporium*, as well as from *Lyophyllum shimeji* and *T. matsutake*, when compared to those from *Trametes* spp. and *Peniophora* spp., namely deviations from consensus in the first and second FAD-binding subregions (De Koker et al., 2004). PcP2Ox further deviates from consensus in the third FAD-binding subregion. These differences are reflected in the phylogenetic relationship of P2Ox sequences, with the *P. chrysosporium* and the *L. shimeji* sequences grouping in a separate clade from *Trametes* spp. and *Peniophora* spp. Structural investigations of *P. chrysosporium* P2Ox could therefore provide valuable insight into structure-function relationships of GMC oxidoreductases, yet crystallization of wild-type fungal P2Ox from *P. chrysosporium* is unsuccessful to date, probably due to microheterogeneity of the wild-type enzyme preparations reported (Artolozaga et al., 1997; De Koker et al., 2004). Heterologous expression will also be an invaluable help to produce enzyme variants for further detailed studies.

Here we report the successful heterologous expression of PcP2Ox in *E. coli*. Biochemical characterization shows similarity with the reported properties of the wild-type protein for electron-donor sugar substrates, while activity with alternate electron acceptors, in addition to oxygen, is reported for the first time. Enzyme kinetics with benzoquinone suggests a possible role for PcP2Ox in redox cycling during lignocellulose metabolism.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study were of the purest grade available and were purchased from Sigma (St. Louis, MO) unless otherwise stated. Horseradish peroxidase (grade II; EC 1.11.1.7) was obtained from Roche (Mannheim, Germany), various electron acceptors were provided from Aldrich (Steinheim, Germany), and bovine serum albumin (BSA) was from United States Biochemical (Cleveland, OH). Restriction endonucleases, T4 DNA ligase and DNA modifying enzymes were obtained from Promega (Madison, WI).

2.2. Organisms, plasmids and media

E. coli strain DH5α was used for cloning experiments and plasmid propagation. *E. coli* strain BL21(DE3) (Novagen, Madison, WI) was used as a host for the production of the active P2Ox. Plasmid pGEM-T Easy (Promega) was used for cloning of PCR fragments, and the expression vector pET21a(+) (Novagen) for recombinant production of P2Ox.

Production of recombinant PcP2Ox was done in the following media: Luria-Bertani (LB; (Ausubel et al., 1990)), 2× LB, Terrific Broth (TB; Ausubel et al., 1990), and MCHGly medium containing (g/l): glycerol, 10; casein hydrolysate, 10; $Na_2HPO_4{\cdot}12H_2O$, 14.6; KH_2PO_4, 3.0; NaCl, 0.5; NH_4Cl, 1.0; and $MgSO_4{\cdot}7H_2O$, 0.25. Isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose in different concentrations was added to induce the expression system.

2.3. Isolation and expression of P2Ox-encoding cDNA

The cDNA sequence encoding P2Ox of *P. chrysosporium* BKM-F-1767 (GenBank accession no. AY522922 (De Koker et al., 2004) was used to construct the expression vector. Isolation of mRNA and first strand synthesis were carried out as described by Stewart and Cullen (1999) using the primers 5′-CGGAGTCAGAGGACTGCTCCA-3′ and 5′-ATTGTACTTACGCAGCACGAGGTT-3′ (forward and reverse, respectively; mRNA provided by Dan Cullen and Jill Gaskell of Forest Products Laboratory, Madison WI, and assistance with first strand synthesis is gratefully acknowledged). The *p2ox*-coding sequence was amplified using two modified primers based on the genomic sequence: poxMK1for: 5′-CGC**GGATCC**ATGTTTCTTGACACCACACCA-3′; and poxMK1rev: 5′-CAG**AAGCTT**GCCGCGGTGACGACCAAA-3′ containing BamHI (poxMK1for) and HindIII (poxMK1rev) restriction sites, respectively (bold print), to facilitate subsequent insertion into the expression vector.

The PCR reaction mixture contained 34 μl sterile nanopure water, 5 μl 10× Pfu buffer (Promega), 4 μl 2.5 mM dNTPs (2.5 mM each), 1 μl template cDNA, 3 μl of each primer (10.5 pmol/l) and 0.5 μl Pfu proofreading thermostable DNA polymerase (Promega).

The resulting PCR product of approximately 1.9 kb was purified from an agarose gel using the Wizard SV Gel & PCR CleanUp System and ligated into a pGEM-T Easy Vector (both from Promega). The A-tailing procedure, ligation and transformation into *E. coli* DH5α were performed according to the manufacturer's recommendation. After sequence analysis, the *p2ox* cDNA insert was isolated by digestion with BamHI and HindIII and ligated into the respective sites of pET21a(+) (Novagen) in frame with the N-terminal T7-tag and C-terminal His_6-tag encoded by the expression vector. *E. coli* strain BL21(DE3) was the host for expression of the resulting plasmid for protein production.

Several clones were grown overnight at 37 °C in 5 ml LB medium with 100 μg/ml of ampicillin, the cell pellet after centrifugation was resuspended in Start Buffer (20 mM bis–tris buffer, 1 M NaCl, and 10 mM imidazole, pH 6.4), and homogenized using a French press at 100 MPa. The cell homogenate was tested for presence of pyranose oxidase activity. Cultivation on a larger scale to provide material for biochemical characterization was done in 1-l shake flasks. Twenty-five milliliter TB medium supplemented with 100 μg/ml of ampicillin were inoculated with a single colony, and cultivated at 37 °C and 150 rpm for 6 h. TB medium (225 ml) containing 100 μg/ml of ampicillin was inoculated with this starter culture and cultivated for additional 2 h, then lactose was added to a final concentration of 5 g/l. After overnight cultivation at 25 °C and 150 rpm, the cells were harvested by centrifugation at 10,000 × *g* for 20 min and 4 °C. The cell pellet was suspended in start buffer and homogenized in a French press. The homogenate was centrifuged at 30,000 × *g* for

30 min and 4 °C, and the supernatant was recovered as a crude extract.

2.4. Enzyme purification

recP2Ox was purified by one-step immobilized metal affinity chromatography (IMAC), on a column (40 ml) packed with Profinity IMAC Ni-Charged Resin (Bio-Rad Inc., Hercules, CA) specific for recombinant His-tagged proteins. The column was pre-equilibrated with start buffer (see above), and the crude extract was applied to the column at a rate of 2.5 ml/min. After an additional washing step with Start Buffer (two column volumes) the enzyme was eluted at a rate of 2.5 ml/min by using a linear gradient (0–1 M imidazole in Start Buffer) in 10 column volumes. Active fractions (10 ml each, a total of 70 ml) were pooled, desalted and concentrated in 50 mM potassium phosphate buffer (pH 6.5) using Amicon Ultra Centrifugal Filter units (Millipore, Billerica, MA), filter sterilized, and stored at 4 °C.

2.5. Enzyme assay

Unless otherwise specified, pyranose oxidase activity was determined spectrophotometrically at 420 nm and 30 °C by measuring the formation of H_2O_2 for 3 min with a peroxidase-coupled assay using ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (ε_{420} = 43,200 M^{-1} cm^{-1}) as the chromogen. The standard assay mixture (total volume 1 ml) contained 1 μmol of ABTS in 50 mM potassium phosphate buffer (pH 6.5), 2 U of horseradish peroxidase, 100 μmol of D-glucose, and a suitable amount of P2Ox sample. One unit of P2Ox activity was defined as the amount of enzyme necessary for oxidation of 2 μmol of ABTS (equivalent to the oxidation of 1 μmol of D-glucose) per minute under the conditions described above (Danneel et al., 1992). Protein contents were determined by the dye-binding method of Bradford, using BSA as the standard.

The effect of pH on different electron acceptors was determined using the following buffers: sodium citrate (pH 3.0–6.0), potassium phosphate (pH 5.5–8.0), Tris–HCl (pH 7.5–9), sodium phosphate (pH 10–12), and sodium borate (pH 8.0–12), each at 100 mM.

2.6. Electrophoretic analysis

Polyacrylamide gel electrophoresis (PAGE) as well as isoelectric focusing was done on a PhastSystem unit (Pharmacia LKB/GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions. Protein bands were visualized by silver staining. The native molecular mass of the enzyme was estimated in 8–25% gradient gels under nondenaturing conditions using a high-molecular-mass protein standard (Amersham, Piscataway, NJ). Subunit molecular mass was determined by sodium dodecyl sulfate (SDS)-PAGE and the low-molecular-mass protein kit (Amersham) as a standard. The marker mix used in isoelectric focusing was for a range of 3.6–6.6 (Sigma).

2.7. Cofactor characterization

FAD absorption spectra were recorded using an 8453 UV–visible Spectrophotometer (Agilent Technologies, Santa Clara, CA) with diode array detection at room temperature. The enzyme was reduced by the addition of D-glucose (20 mM). Homogeneously purified P2Ox was treated with 5% trichloroacetic acid at 100 °C for 10 min; denatured protein was removed by centrifugation and the presence of liberated flavin was measured in the supernatant at 450 nm.

2.8. N-terminal sequencing

The N-terminal sequencing of P2Ox using Edman degradation chemistry was performed on a Procise protein sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's manual.

2.9. Enzymatic in-gel digestion

CBB-stained protein bands were excised from the gel, cut into small pieces and washed with 10 mM DDT, 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). After destaining, the proteins were reduced with 30 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 65 °C for 30 min and alkylated by 30 mM iodoacetamide for 60 min in the dark. The gel pieces were further washed with water, shrunk by dehydration in MeCN and reswollen in water. The supernatant was removed and the gel partly dried in a SpeedVac concentrator. The gel pieces were then reconstituted in a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% MeCN and sequencing grade trypsin (20 ng/μl; Promega) or Asp-N protease (10 ng/μl; Roche). After overnight digestion, the resulting peptides were extracted to 40% MeCN/0.1% TFA.

2.10. MALDI-TOF mass spectrometry

A solution of α-cyano-4-hydroxycinnamic acid (Sigma) in aqueous 50% MeCN//0.1% TFA (5 mg/ml) was used as a MALDI matrix. The sample (0.5 μl) was deposited on the MALDI target and allowed to air-dry at room temperature. After complete evaporation, 0.5 μl of the matrix solution was added. MALDI mass spectra were measured on an Ultraflex III instrument (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam solid-state laser and LIFT technology for MS/MS analysis. The spectra were acquired in the mass range of 700–5000 Da and calibrated internally using the monoisotopic $[M+H]^+$ ions of autoproteolytic fragments of trypsin (842.5 and 2211.1 Da).

2.11. Steady-state kinetic measurements

Unless otherwise stated, all measurements were performed at 30 °C in 50 mM potassium phosphate buffer (pH 6.5). Measurements of kinetic constants for various electron donors (sugar substrates) were done with oxygen (air saturation) as electron acceptor and the routine ABTS-peroxidase assay performed as described above. Kinetic constants for various electron acceptors were determined using 20 mM D-glucose as the electron donor, and measurements were performed at the pH optimum of the respective acceptor as well as at pH 6.5. The kinetic constants for the electron acceptors 1,4-benzoquinone, 2,6-dichloroindophenol (DCIP), and the ferricenium Fc^+ ion (using ferricenium hexafluorophosphate $Fc^+PF_6^-$ (Aldrich, Steinheim, Germany)) were measured as previously described (Kujawa et al., 2007). In short, 10 μl of appropriately diluted enzyme was added to 990 μl of the appropriate buffer containing the sugar substrate D-glucose and which had been flushed with nitrogen for the removal of oxygen. 1,4-Benzoquinone was varied from 0.01 to 2.0 mM, and the absorbance change at 290 nm (ε = 2.24 mM^{-1} cm^{-1}) was followed for 180 s. DCIP was varied from 0.01 to 0.3 mM, and the absorbance change was measured at 520 nm (ε = 6.8 mM^{-1} cm^{-1}) for 180 s. $Fc^+PF_6^-$ was varied from 0.005 to 1.0 mM, and the absorbance change at 300 nm (ε = 4.3 mM^{-1} cm^{-1}) was followed for 180 s. All kinetic constants were calculated by non-linear least-squares regression, fitting the observed data to the Henri–Michaelis–Menten equation. Turnover numbers (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) were

Table 1
Purification of recombinant P2Ox from *Phanerochaete chrysosporium* by immobilized metal affinity chromatography (IMAC).

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	1260	3490	2.77	100	1
IMAC	161	2660	16.5	76.2	5.96

calculated using a molecular mass of 65 kDa for the P2Ox subunit.

3. Results

3.1. Isolation and heterologous expression of P2Ox-encoding cDNA

A full length cDNA encoding pyranose oxidase (P2Ox) from *P. chrysosporium* (GenBank accession number AY522922 (De Koker et al., 2004)) was successfully expressed in *E. coli* BL21(DE3). The nucleotide sequence contains an open reading frame of 1,863 bp encoding a polypeptide of 621 amino acids. Based on the cDNA sequence, two modified oligonucleotide primers containing restriction sites for in-frame ligation of the gene into pET21a(+) were designed. These primers were used to re-amplify the cDNA and construct the expression vector containing *p2ox* cDNA under the control of a lactose- or IPTG-inducible T7 promoter fused in frame with the N-terminal T7-tag as well as a C-terminal His_6-tag encoded by the vector. The resulting vector was transformed into the production strain BL21(DE3), and ampicillin-resistant clones were tested for the presence of P2Ox activity after small-scale cultivation. The bacterial clone with the highest activity (1.11 U/mg) was selected for further studies.

Recombinant P2Ox production by *E. coli* BL21(DE3) was done in LB, 2× LB, TB and MCHGly medium. Specific activities of 2.22 U/mg (using IPTG with a final concentration of 0.1 mM) and 1.66 U/mg (IPTG final concentration 1 mM), respectively, were obtained on TB and MCHGly medium, while specific activities on LB and 2xLB medium were significantly lower (data not shown). Routinely, approx. 270 mg/l (as calculated from 4500 U/l and a specific activity of 16.5 U/mg) of active, soluble recombinant P2Ox were obtained in shake-flask cultivations under the growth conditions outlined above.

3.2. Enzyme purification

A typical purification procedure of recP2Ox from *P. chrysosporium* is summarized in Table 1. The enzyme was purified six-fold from the crude extract with a yield of 76% and a specific activity of 16.5 U/mg under standard assay conditions when glucose and oxygen (air) were used as substrates. The one-step purification procedure yielded an apparently homogenous protein, as judged by SDS-PAGE (Fig. 1).

3.3. Physical properties

The molecular mass of native recombinant P2Ox was approximately 250 kDa as estimated by PAGE under non-dissociating conditions (Fig. 2). SDS-PAGE of recP2Ox resulted in a single band at 65 kDa (Fig. 1), indicating a homotetrameric structure of the active enzyme, as was described for pyranose oxidase from the fungal wild-type as well as for the enzymes from other sources. Isoelectric focusing under native conditions showed a major band of p*I* 5.2 and two minor isoforms of recP2Ox ranging from p*I* 5.1 to 5.3 (Fig. 3).

Purified recP2Ox was bright yellow and produced a typical flavoprotein spectrum with absorption maxima at 357 and 459 nm. When the enzyme was reduced by addition of glucose in the absence of oxygen, the peak at 459 nm disappeared (Fig. 4). Treat-

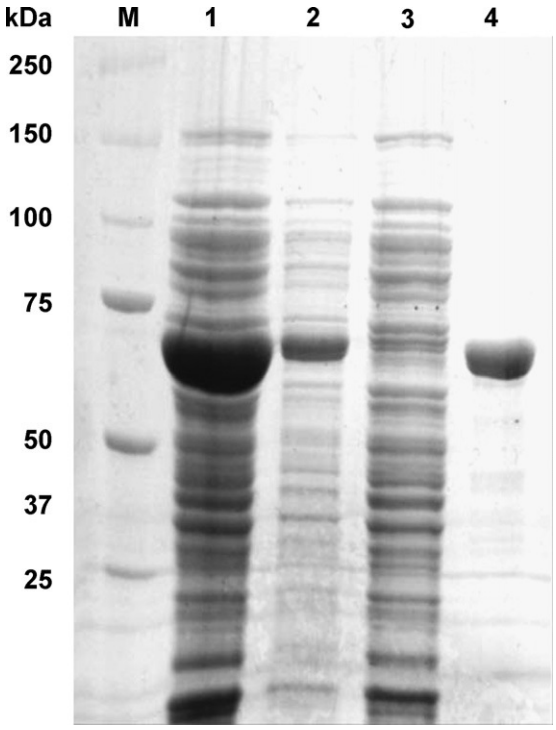

Fig. 1. SDS-PAGE analysis of rP2Ox from *P. chrysosporium*. Lane M, low-molecular-mass protein standards, Amersham; lane 1, crude cell extract undiluted; lane 2, crude cell extract diluted 1:10; lane 3, flow-through from IMAC; lane 4, purified pyranose oxidase after IMAC.

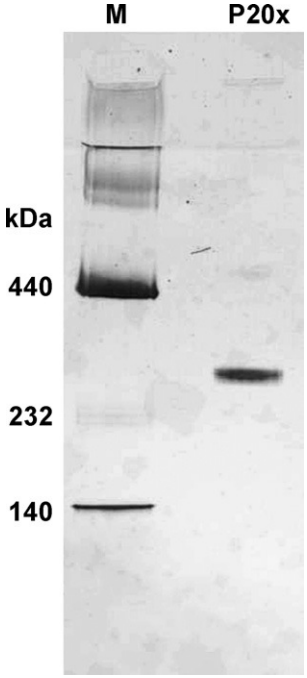

Fig. 2. Native PAGE analysis of rP2Ox from *P. chrysosporium*. Lane M, high-molecular-mass marker protein kit, Amersham; rP2Ox, purified recombinant pyranose oxidase.

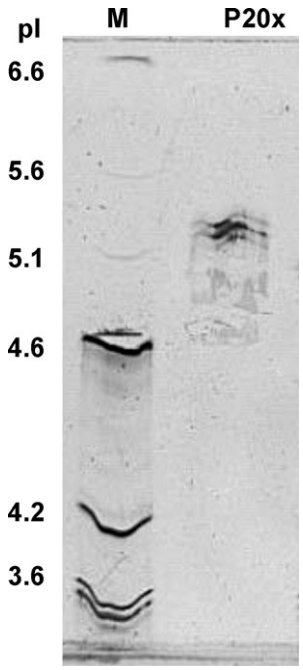

Fig. 3. Isoelectric focusing of rP2Ox from *P. chrysosporium*. Lane M, isoelectric marker range 3.6–6.6 (Sigma); rP2Ox, purified recombinant pyranose 2-oxidase.

ment of the enzyme with 5% trichloroacetic acid at 100 °C for 10 min did not release the flavin moiety from the polypeptide, suggesting a covalent linkage to the protein.

3.4. N-terminus

The N-terminus of the purified protein was determined using Edman-chemistry and was found to be MASMTGGQQMG, the sequence of the vector-encoded T7-tag. However, an additional protein was also detected as secondary peaks in the sequencing runs (not shown). This protein represented approx. 15% of the total amount, and the sequence was that of the theoretical translation of the P2Ox cDNA (MFLDTTPFRAD). MS/MS peptide sequencing of the digested purified protein samples gave sequence coverages of 63% (Trypsin) and 50% (AspN protease), respectively, with a total coverage of 77%. The only sequences not belonging to the translation of the P2Ox cDNA could be assigned to the T7-tag.

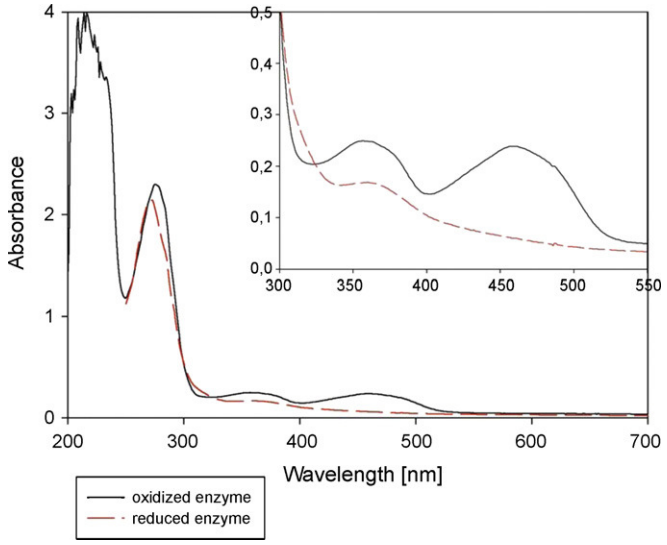

Fig. 4. FAD absorption spectrum. The spectrum of the oxidized enzyme displays maxima at 357 and 459 nm (black line). In the reduced enzyme, the 459 nm maximum disappears (red interrupted line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

3.5. pH dependence of activity

pH optima for the purified recombinant P2Ox were determined for the electron acceptors oxygen, 1,4-benzoquinone, the ferricenium ion (Fc^+), and 2,6-dichloroindophenol (DCIP). The enzyme exhibited rather sharp pH profiles for Fc^+ and DCIP with pH optima of 8.0 and 6.5, respectively. When Fc^+ was the electron acceptor we found a strong dependence of activity on the buffer used and that P2Ox retained 60% of its maximal activity even at pH 9.5. Bell-shaped profiles with an optimum pH range between 6.0 and 9.0 were observed when oxygen was used as the electron acceptor while 1,4-benzoquinone displayed an even broader range from pH 4.0 to 9.0 (Fig. 5).

3.6. Kinetic properties

Various sugars were tested as possible substrates of recP2Ox, and the activities relative to those with D-glucose were determined under standard assay conditions at a sugar substrate concentration of 100 mM. The enzyme showed approximately 30% relative activity with L-sorbose and D-xylose and 5% relative activity with D-galactose, while D-fructose was hardly oxidized at all. Subsequently, the kinetic constants were determined for these sugars using oxygen (air) as electron acceptor (Table 2). The highest catalytic efficiency (k_{cat}/K_m) was found for D-glucose (98.9 $mM^{-1}\ s^{-1}$) whereas the corresponding values for L-sorbose, D-xylose, and D-galactose were ~40–50-fold lower (Table 2). The lowest catalytic efficiency was measured for D-fructose as a substrate as a result of both an unfavourable Michaelis constant K_m and turnover number k_{cat}.

In addition to oxygen, recP2Ox from *P. chrysosporium* is able to transfer electrons to other acceptors. Kinetic constants were determined for the two-electron acceptors 1,4-benzoquinone, various substituted quinones and DCIP as well as for one-electron acceptors, including the ferricenium ion Fc^+ or the ABTS cation radical. These measurements were performed with D-glucose as the saturating substrate and both at the pH value of the standard assay, pH 6.5, and at the pH optimum of the respective electron acceptor substrates (benzoquinone, pH 4.5; DCIP, pH 6.5; Fc^+, pH 8.0). Results are summarized in Table 3. 1,4-Benzoquinone shows a k_{cat}/K_m value of ~$10^7\ M^{-1}\ s^{-1}$ when the reaction is carried out at pH 4.5 while at pH 6.5 the value is reduced to about one third of this value (Table 3); this is mainly the effect of a considerably increased Michaelis constant at the higher pH value. Variously substituted 1,4-benzoquinones showed K_m values comparable to that of 1,4-benzoquinone, whereas the turnover numbers determined for these substrates were decreased dramatically. The pH optimum for DCIP was found to be similar to the standard assay conditions. DCIP as an electron acceptor of *Pc*P2Ox is characterized by a very low Michaelis constant in the micromolar range, but also by k_{cat} values that are significantly lower than that of, e.g., 1,4-benzoquinone. P2Ox showed significant activity with several one-electron acceptors. For Fc^+ the pH of the reaction does not affect the K_m value significantly, while at the optimal pH of 8.0 and in the presence of borate buffer the turnover number is increased 2.4-fold compared to pH 6.5, resulting in an ~3-fold higher catalytic efficiency ($1.9 \times 10^6\ M^{-1}\ s^{-1}$). In comparison, the catalytic efficiency for the other one-electron acceptors tested, the ABTS cation radical and ferricyanide, were considerably lower.

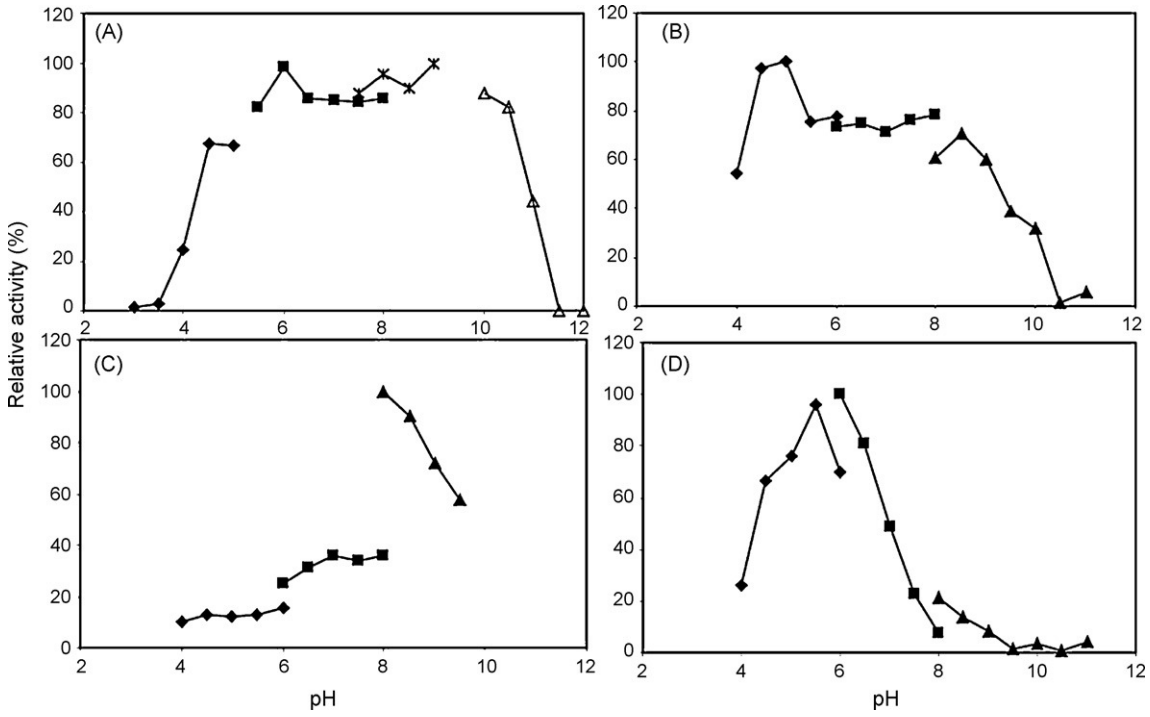

Fig. 5. Effect of pH on the activity of recP2Ox from *P. chrysosporium* in the presence of different electron acceptors. (A) Oxygen, (B) 1,4-benzoquinone, (C) ferricenium ion, and (D) 2,6-dichloroindophenol. The buffers used were 100 mM sodium citrate (♦), 100 mM potassium phosphate (■), 100 mM borate (▲), 100 mM Na_3PO_4 (△), 100 mM Tris–HCl (*).

Table 2
Apparent steady-state kinetic constants of recombinant pyranose 2-oxidase from *P. chrysosporium* kinetic constants were determined for several electron donors at 30 °C by using oxygen (air saturation) as electron acceptor under the standard ABTS assay conditions.

Substrate	v_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
D-Glucose	19.9 ± 0.74	0.84 ± 0.15	83.1 ± 3.1	98.9
L-Sorbose	14.1 ± 0.81	23.5 ± 3.4	58.8 ± 3.4	2.50
D-Xylose	10.8 ± 0.48	20.9 ± 2.7	44.9 ± 2.0	2.15
D-Galactose	1.17 ± 0.03	2.94 ± 0.35	4.87 ± 0.13	1.66
D-Fructose	0.45 ± 0.068	103 ± 46	1.88 ± 0.28	0.0183

4. Discussion

We report here on the successful expression in *E. coli* of a *P. chrysosporium* cDNA encoding pyranose 2-oxidase, as well as its biochemical and kinetic characterization. The respective gene had previously been identified in the *P. chrysosporium* genome as encoding the P2Ox enzyme purified from mycelium grown on wheat bran supplemented with corn steep liquor (De Koker et al., 2004). cDNA was synthesized from mRNA obtained under carbon-limiting conditions, as this was shown to promote transcription of the *p2ox*-gene (De Koker et al., 2004), and ligated into an expression plasmid for *E. coli*, a host–vector system that was already shown to work effectively for other, related pyranose oxidase enzymes (Bastian et al., 2005; Heckmann-Pohl et al., 2006; Kotik et al., 2004; Maresova et al., 2005; Takakura and Kuwata, 2003). We were able to obtain yields of 4500 U of P2Ox activity and 270 mg of active P2Ox protein per liter of production medium in shake-flask cultures without optimization of the expression system and cultivation conditions. This is significantly higher than the yields reported thus far for P2Ox from, e.g., *Trametes* spp., which typically range from 50 to 100 mg/l (Kotik et al., 2004; Maresova et al., 2005), or *Peniophora gigantea*

Table 3
Apparent kinetic constants of recP2Ox from *P. chrysosporium* for several electron acceptors Kinetic constants were determined at 30 °C by using 20 mM D-glucose as electron donor under standard assay conditions (pH 6.5) unless otherwise indicated.

Electron acceptor	v_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Oxygen (pH 8.0)[a]	26.1 ± 0.2	1.22 ± 0.02	109 ± 0.8	89.2
1,4-Benzoquinone	96 ± 3.5	0.11 ± 0.01	400 ± 14	3640
1,4-Benzoquinone (pH 4.5)	115 ± 8.7	0.042 ± 0.013	477 ± 36	11900
$Fc^+PF_6^-$[b]	54.8 ± 35.8	0.33 ± 0.35	228 ± 149	691
$Fc^+PF_6^-$ (pH 8.0)	132 ± 8.6	0.29 ± 0.05	549 ± 36	1890
DCIP[c]	25.9 ± 3.9	0.051 ± 0.031	108 ± 16	2150
2-Chloro-1,4-benzoquinone	0.037 ± 0.004	0.31 ± 0.08	0.15 ± 0.02	0.48
2-Methoxy-1,4-benzoquinone	9.65 ± 0.76	0.21 ± 0.05	40.1 ± 3.16	191
2-Methyl-1,4-benzoquinone	0.029 ± 0.001	0.51 ± 0.05	0.12 ± 0.004	0.23
Ferricyanide	0.074 ± 0.046	3.43 ± 2.51	0.30 ± 0.19	0.087
2,6-Dimethyl-1,4-benzoquinone	2.09 ± 0.32	1.59 ± 0.34	8.69 ± 1.33	5.46
Tetrafluoro-1,4-benzoquinone	1.59 ± 0.16	0.64 ± 0.16	6.61 ± 0.66	10.3
ABTS cation radical	0.32 ± 0.061	0.09 ± 0.044	1.33 ± 0.25	13.4

[a] Data taken from Artolozaga et al. (1997); measured at pH 8.0 and 25 °C in the presence of 100 mM D-glucose.
[b] $Fc^+PF_6^-$, ferricenium hexafluorophosphate.
[c] DCIP, 2,6-dichloroindophenol.

reported at 170 mg/l (Bastian et al., 2005). It can be expected that the use of a bioreactor and an optimized fermentation protocol can substantially improve upon these yields of *Pc*P2Ox, which will make a large-scale industrial production of this enzyme feasible. Furthermore, experiments to obtain high-quality protein crystals for X-ray-structure determination, which were unsuccessful thus far, would no longer be hampered by limited availability of pure protein.

The protein was expressed with both the N-terminal T7-tag as well as the C-terminal His-tag. Previous experience with P2Ox from *T. multicolor/ochracea* has shown that Ni-based affinity chromatography resins may leak Ni ions into the eluate, which has a detrimental effect on enzyme activity (our unpublished information). An overnight incubation in a buffer containing 100 mM EDTA followed by a buffer exchange can remedy that, but may also lead to enzyme loss. Therefore the T7-tag was added as well, in order to provide a possible alternative for detection and purification. The translational fusion with T7 may also be reason for the high levels of protein production; the tag is derived from the most abundant phage T7 gene product (Stevens, 2000).

MS/MS peptide sequencing of the recombinant *Pc*P2Ox expressed in *E. coli* gave 77% sequence coverage in total, indicating that the protein expressed and purified indeed corresponds to the proposed *p2ox* gene. In addition, no peptide sequences not belonging to the P2Ox-encoding gene were discovered with the exception of a short sequence region that could be assigned to the T7-tag. When the N-terminal sequence was determined by Edman degradation of the purified protein, it was found that a fraction of the sample, estimated to approximately 15% of the total, did not contain the vector-encoded T7-tag preceding the coding sequence, but started with the methionine of the original cDNA (Met-1). Presumably, the encoding ATG is also used – to a certain degree – as an alternative translational start point. Proteins that are translated from this start codon, however, also possess the C-terminal His_6-tag, which was used for the one-step-purification of the recombinant protein, and are therefore co-purified. Use of affinity chromatography employing antibodies against the T7-tag may avoid this co-purification of the fraction missing the T7-tag. The two ATGs contained in the sequence encoding the T7-tag apparently are not used as an alternative start codon, or if so, only to such a minor fraction as to be undetectable in the N-terminal sequencing. The difference of only 12 amino acids between the two proteins did not result in a sufficiently altered electrophoretic mobility to be detected in SDS-PAGE as only one sharp band was observed for the purified sample, but may account for the observed minor bands in isoelectric focusing, especially since this short tag contains one charged amino acid and the His_6-tag contains additional charged residues. The phenomenon of several distinct bands was observed for the native protein purified from the original source, where doublet bands were observed on SDS-PAGE as well as microheterogeneity in isoelectric focusing (De Koker et al., 2004; Volc and Eriksson, 1988), but the reason for this remains unclear. P2Ox was found to be encoded by only one gene in the *P. chrysosporium* genome, therefore the co-purification of closely related isoforms can reasonably be ruled out. A possible explanation for this heterogeneity of the enzyme preparation from the natural, fungal source could be partial degradation by endogenous proteases or by proteases that are released from autolysing fungal mycelium during prolonged cultivation. Here, the expression in *E. coli* is certainly of advantage since proteolytic modification can be largely avoided by short cultivation times and low-protease expression hosts.

Previous attempts at determining the N-termini of purified pyranose oxidases have yielded ambiguous results, with the first amino acid obtained in sequencing being as far as 66 amino acid residues into the conceptual translations of the respective ORFs (Artolozaga et al., 1997; Nishimura et al., 1996; Takakura and Kuwata, 2003). It has been speculated that processing of P2Ox, i.e., removal of a putative (unidentified) signal sequence is responsible for these discrepancies (Nishimura et al., 1996; Takakura and Kuwata, 2003). Yet, this removal of parts of the N-terminal region is especially pronounced for *Pc*P2Ox isolated from its original fungal source, for which the N terminus QFGPGQVPIPGYSKNNEIEYQKDIDRFVNVI was reported (Artolozaga et al., 1997). This N-terminus starts at amino acid position 66 (Fig. 6), within a 22-amino acid insert not found in P2Ox of the *Trametes*/*Peniophora* group and only after the canonical ADP-binding βαβ motif xhxhGxGxxGxxxhxxh(x)$_8$hxhE(D), where x is any amino acid, and h is a hydrophobic residue, which is present in a large number of flavin and nicotinamide-dependent enzymes (Dym and Eisenberg, 2001; Wierenga et al., 1986). Proteolytic activity associated with ageing and partly autolysing mycelium as mentioned above could be a possible explanation. At present, it is not known how the removal of an essential part of the flavin-binding region as observed for *Pc*P2Ox isolated from its natural source is affecting the prop-

Fig. 6. Sequence coverage of recP2Ox (with T7-tag on N-terminus). Peptides identified by MS/MS sequencing are underlined, the native N-terminus is marked with an '↓', the N-terminus described in Artolozaga et al. (1997) is marked with an '×', canonical amino acids in the boxed ADP-binding βαβ-motif are in bold print.

erties of the enzyme, however, it was speculated that this might influence stability (De Koker et al., 2004). For P2Ox from *Trametes* spp. N-termini were described starting at amino acid 29 (Leitner et al., 2001), within a stretch of amino acids that is not present in, e.g., the *Phanerochaete* enzyme, and before the above-mentioned ADP-binding motif. Although suggestive of a signal sequence, neither the removed parts nor the cleavage sites conform to the respective established consensus motifs.

The activity profiles and kinetic properties of recP2Ox from *P. chrysosporium* described here are in excellent agreement with data that were reported previously for the native enzyme purified from its original source (e.g., fungal wild-type *Pc*P2Ox: $k_{cat,Glc} = 98.8\ s^{-1}$, $K_{m,Glc} = 1.09$ mM; recombinant *Pc*P2Ox: $k_{cat,Glc} = 83.1\ s^{-1}$, $K_{m,Glc} = 0.84$ mM) (Artolozaga et al., 1997; Volc and Eriksson, 1988), as well as on pyranose oxidases from related sources (Leitner et al., 2001; Takakura and Kuwata, 2003), indicating that the addition of the two tags does not interfere with the kinetic properties of the enzyme. A subject that has not been investigated to date is the ability of P2Ox from *P. chrysosporium* to utilize electron acceptors other than molecular oxygen. We have found that the recombinantly produced enzyme is indeed able to transfer very efficiently electrons derived from sugar oxidation onto alternative acceptors, both one- and two-electron acceptors. Some of these alternative electron acceptors studied for *Pc*P2Ox, including 1,4-benzoquinone or the ferricenium ion, are far better substrates than the presumed *in vivo* substrate oxygen (Artolozaga et al., 1997; Daniel et al., 1994), as judged by both the catalytic constant k_{cat} and the catalytic efficiency k_{cat}/K_m (Table 3). This latter bimolecular steady-state constant reached values of, e.g., $3.6–12 \times 10^6\ M^{-1}\ s^{-1}$ for 1,4-benzoquinone, $2.15 \times 10^6\ M^{-1}\ s^{-1}$ for the benzoquinone imine DCIP, or $0.69–1.9 \times 10^6\ M^{-1}\ s^{-1}$ for Fc^+, an organically complexed iron, indicating very rapid and efficient catalysis, also in comparison with oxygen showing a k_{cat}/K_m of $0.87 \times 10^6\ M^{-1}\ s^{-1}$ (Artolozaga et al., 1997). These data are in excellent accordance with those obtained for the only other P2Ox studied pertaining to its activity with alternative electron acceptors, P2Ox from *T. multicolor* (Leitner et al., 2001), which also showed higher activity with several one- and two-electron acceptors, most prominently various quinoid substrates, than with oxygen. The introduction of various substituents at position 2 of 1,4-benzoquinone resulted in a dramatic decrease of the catalytic efficiency of *Pc*P2Ox mainly by affecting k_{cat}, regardless whether an electron-withdrawing or an electron-donating group was introduced. A correlation between the reactivity of electron acceptors and their redox potential was also not possible. For example, 2-chloro-1,4-benzoquinone has a redox potential that is comparable to that of 1,4-benzoquinone [$E(Q/QH_2)$ of 0.304 and 0.298 V versus SHE, respectively (Cape et al., 2006), yet a k_{cat} that is lower by three orders of magnitude. The one-electron acceptors Fc^+, ABTS cation radical and ferricyanide have high redox potentials ($E^{0'}$ of 0.430, 0.520 and 0.358 V, respectively). Fc^+ is an excellent substrate of P2O with a catalytic efficiency of $691\ mM^{-1}\ s^{-1}$ at pH 6.5, while both the ABTS cation radical and ferricyanide are reduced significantly slower (13.4 and $0.087\ mM^{-1}\ s^{-1}$).

Based on the reactivity with alternative electron acceptors and the kinetic data determined it seems plausible that pyranose oxidase not only utilizes oxygen under natural conditions but also various quinones resulting from lignin metabolism and/or complexed metal ions as its substrates. Variously substituted benzoquinones are generated by the peroxidase-catalyzed oxidation of lignin, and are intermediates in the degradation of aromatic compounds by wood-decay fungi, as recently reviewed (Martinez et al., 2005). These benzoquinones are reduced to hydroquinones and undergo further intracellular metabolism. A 1,4-benzoquinone reductase, which is an FMN-containing flavoprotein using NAD(P)H as its second substrate, has been proposed for this activity in intracellular aromatic metabolism (Akileswaran et al., 1999; Brock and Gold, 1996; Brock et al., 1995). Alternatively, extracellular redox cycling of quinones may contribute to the generation of reactive oxygen species (ROS) for non-enzymatic attack of lignocellulose (Hammel et al., 2002). The periplasmic location of P2Ox (or even extracellular under certain growth conditions), together with the quinone reducing activity as demonstrated here, suggests that the oxidase could play a role in this extracellular metabolism.

An analysis of the *Pc*P2Ox amino acid sequence as derived from the coding sequence of the encoding gene as well as a comparison with P2Ox sequences from other sources, including their phylogenetic relationships, has already been reported (De Koker et al., 2004). That study focused on identifying amino acid residues that are strictly conserved not only among pyranose 2-oxidase proteins but also throughout the GMC-family of flavin-dependant enzymes. The availability of the crystal structure of *T. multicolor* P2Ox complexed with its slow substrate 2-deoxy-2-fluoro-D-glucose allows a more detailed comparison focusing on certain amino acids in the immediate vicinity of the active site. T169 was postulated to form a hydrogen bond with the flavin's $N_5–O_4$ during the oxidative half-reaction, supporting the electron transfer to the acceptor (Kujawa et al., 2006), as well as partially accounting for the substrate specificity due to steric hindrances between the amino acid side chain and the axially oriented C4 hydroxyl group of D-galactose – the equatorially oriented C4 hydroxyl group of D-glucose can be better accommodated. Side chains of D452, an amino acid of the active-site loop, and R472 are postulated to form hydrogen bonds with the O6 of the substrate, with additional hydrogen bonds being formed by Q448 and V546, while H548 and N593 putatively control the catalytic event by supporting the electron transfer from the substrate C2 (or C3) to the N5 of the flavin. The alignment of several P2Ox amino acid sequences as reported previously (De Koker et al., 2004) shows the conservation of all these amino acids in *Pc*P20x (Q448/Q454, R472/R478 and N593/N596) as well as their immediate vicinities, such as the motif THWTC (166–170 in *Tm*P20x, 157–161 in *Pc*P20x, containing the flavin-anchoring residue H167/H158) and the active site loop DAFSYG (452–457 in *Tm*P20x, 458–463 in *P. chrysosporium*). The only exception is V546 (the valine at this position is common in *Trametes* and *Peniophora* sequences), which corresponds to A551 in *P. chrysosporium* and A538 in *L. shimeiji*. In *Tm*P20x, V546 is interacting with the C1 hydroxyl group of the bound sugar substrate by forming a hydrogen bond through its carbonyl oxygen rather than interacting through its side chain, hence an alanine at this position should not alter the function of this active site residue. This high overall similarity with respect to the active site and to amino acids that were identified as crucial for substrate binding and oxidation is reflected in the catalytic properties of *Pc*P2Ox, which do not dramatically differ from *Tm*P2Ox, as mentioned above.

We have also searched the genomes of other fungi for the presence of P2Ox-encoding genes, including *Laccaria bicolor*, *Postia placenta* (both at http://genome.jgi-psf.org/) and *Coprinopsis cinerea* (http://fungal.genome.duke.edu/). *Laccaria* (Agaricales, Tricholomataceae) is an ectomycorrhizal symbiotic fungus (Martin et al., 2008); *Postia* (Polyporales, Coriolaceae) is physiologically classified as a wood-degrading brown-rot fungus, which selectively degrades cellulose while leaving the lignin fraction largely untouched (Wei et al., 2008), whereas *Coprinopsis* is a litter-decomposing fungus belonging to the Agaricales (Psathyrellaceae). No genes encoding enzymes with significant similarities to the P2Ox amino acid sequence could be identified in these genomes. This is in agreement with a previous study (Volc et al., 2001) where 76 basidiomycetous fungi were screened for activity of P2Ox and/or the catalytically and structurally related pyranose dehydrogenase (PDH, (Kujawa et al., 2007; Sygmund et al., 2007)). The authors reported PDH to be limited to litter-decomposing Agaricaceae and Lycoperdaceae (*C.*

cinerea is a close relative), whereas P2Ox was only produced by the white-rot fungi included in the study. Several fungi produced neither activity under the conditions tested. It has to be noted, however, that there is a number of oxidoreductases with similar catalytic activities and conceivably similar or overlapping biological functions *in vivo,* such as aryl alcohol oxidases or cellobiose dehydrogenases, many of which show significant sequence similarities with each other. The *C. cinerea* genome contains a number of similar genes encoding such putative oxidoreductases, which are not experimentally confirmed or catalytically characterized to date (Kittl et al., 2008). Phylogenetically, all known *p2ox* genes are descendants of a *Firmicutes* gene encoding a 2-keto-gluconate dehydrogenase (GenBank accession AAY60294), suggesting horizontal gene transfer from *Firmicutes* (Kittl et al., 2008). In this context it is interesting to note that a gene in the genome of the phylogenetically distant ascomycete *Aspergillus nidulans* was annotated to encode a pyranose 2-oxidase (glucose 2-oxidase, protein accession no. Q5B2E9). This indicates a rather early transfer event, after which the introduced gene has "survived" in a number of phylogenetically diverse groups, but was lost in others – there are species with and without *p2ox* genes among the Tricholomataceae as well as the Coriolaceae – as well as an evolution of these genes to eventually fulfil diverse biological functions in physiologically diverse fungi such as *A. nidulans*, which does not degrade lignocellulose.

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Chapter 2

Identification, heterologous expression and biochemical characterization of novel pyranose 2-oxidases from the ascomycetes *Aspergillus nidulans* and *Aspergillus oryzae*

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Abstract

A gene encoding a pyranose 2-oxidase (POx; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10) was identified in the genome of the ascomycete *Aspergillus nidulans.* Attempts to isolate POx directly from *A. nidulans* cultures were unsuccessful, and the gene was homologously overexpressed under control of the constitutive *gpd*A promoter. The cDNA encoding POx was expressed in *Escherichia coli* and the enzyme was subsequently purified and characterized. The purified enzyme was shown to be a flavoprotein consisting of subunits of 65 kDa and contains a covalently bound flavin adenine dinucleotide as a cofactor. From all substrates, the highest catalytic efficiency was found with D-glucose. In addition the enzyme catalyzes the two-electron reduction of 1,4- benzoquinone, several substituted benzoquinones and 2,6-dichloroindophenol. As judged by the catalytic efficiencies (k_{cat}/k_m), some of these quinine electron acceptors are better substrates for pyranose oxidase than oxygen, which might have implications for the proposed *in vivo* function of pyranose 2-oxidase. During the course of this work, a putative pyranose 2-oxidase-ecoding gene was identified in the genome of *Aspergillus oryzae*. The coding sequence was synthetically produced and expressed in *E. coli*, and the resulting protein was found to be highly similar to the *A. nidulans* enzyme. Distinct differences in the stability of the two enzymes may be attributed to intriguing sequence variations.

Introduction

Pyranose 2-oxidase (POx, pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) belongs to the glucose-methanol-choline (GMC) oxidoreductase family of flavin adenine dinucleotide (FAD)-dependent sugar oxidoreductases (Hallberg et al., 2004a). In general, pyranose 2-oxidase is a 270 kDa homotetramer that catalyzes regioselectively the C-2 oxidation of several aldopyranoses to the respective 2-dehydro derivates, transferring electrons to molecular oxygen to yield H_2O_2. POx can also transfer electrons to acceptors other than oxygen, e.g., quinones, organic radical molecules or complexed metal ions (Leitner et al., 2001, Pisanelli et al., 2009)

Pyranose 2-oxidase is an enzyme with high potential for biotransformations of carbohydrates; applications in bioprocesses, clinical analytics and in synthetic carbohydrate chemistry have been reviewed (Giffhorn, 2000). Recently, we showed that POx can be electrically wired to graphite electrodes in the presence of suitable osmium redox polymers, and hence could be used advantageously in enzyme electrodes or biofuel cells (Tasca et al., 2007).

Pyranose 2-oxidase is so far only described in basidiomycetous fungi, several examples were purified and encoding genes were cloned and expressed heterologously, all in *E. coli*-based expression systems. These include *Coriolus (Trametes) versicolor* (Nishimura et al., 1996), *Tricholoma matsutake* (Takakura and Kuwata, 2003), *Peniophora* sp. (Bannwarth et al., 2004), *Trametes multicolor* (synonymous for *T. ochracea*) (Kujawa et al., 2006, Vecerek et al., 2004), *Trametes pubescens* (Maresova et al., 2005) and *Phanerochaete chrysosporium* (Pisanelli et al., 2009). Experimentally determined crystal structures of P2Ox are available for only two proteins to date, those from *T. multicolor* (Hallberg et al., 2004a) and *Peniophora* sp. (Bannwarth et al., 2004), which have highly similar amino acid sequences.

All sources of POx thus far are ligninolytic fungi employing the white-rot type of lignocellulose degradation, and POx is widely believed to constitute a component of the ligninolytic system by providing the essential co-substrate hydrogen peroxide for lignin and manganese peroxidases (Daniel et al., 1994). There are distinct deviations from consensus in the first and second FAD-binding subregions in the sequences of the enzymes from *P. chrysosporium, Lyophyllum shimeji* and *Tricholoma matsutake* when compared to those from *Trametes* spp. and *Peniophora* spp. (de Koker et al., 2004). The enzyme from *P. chrysosporium* further deviates from consensus in the third FAD-binding subregion, which is also reflected in the phylogenetic relationship of P2Ox sequences. Recently, a number of gene sequences obtained from genome sequencing projects were annotated as potentially encoding POx, among them ascomycetous fungi such as *Aspergillus nidulans*. These fungi do generally not degrade lignocellulose, suggesting different biological functions for these sugar oxidoreductases, and the catalytic nature of these hypothetical proteins is not confirmed to date. There is, however, a similar enzyme described in *A. niger* and other fungi, glucose oxidase (EC 1.1.3.4.), which oxidizes glucose at C-1 to the corresponding lactone. The biological function of these enzymes is largely unknown.

Here we report the successful heterologous expression in *E. coli* of cDNA sequences from two *Aspergillus* species putatively encoding POx. Biochemical characterization confirms their activity and the regioselectivity of oxidation at C-2 as well as similar substrate specificities for both electron-donor sugar substrates and electron acceptors in addition to oxygen. Significant variations in comparison to known sequences suggest a diverse evolution of these enzymes in ascomycetous fungi.

Materials and methods

Chemicals. Chemicals used in this study were of the purest grade available and were purchased from Sigma (St. Louis, MO) unless otherwise stated. Horseradish peroxidase (grade II; EC 1.11.1.7) was obtained from Roche (Mannheim, Germany), various electron acceptors were provided from Aldrich (Steinheim, Germany), and bovine serum albumin (BSA) was from United States Biochemical (Cleveland, OH). Restriction endonucleases, T4 DNA ligase and DNA modifying enzymes were obtained from Fermentas (St.Leon-Rot, Germany).

Strains, plasmid and growth conditions. *A. nidulans yA2, ΔniaD*, *pantoB100*, *riboB2*, *argB2* strain was used to homologously overexpress POx. A total of 10^6 spores per ml were inoculated into liquid minimal medium with the appropriate supplements plus 1% glycerol as the carbon source and ammonium tartrate as the nitrogen source. Mycelia were grown for at 37°C on a rotary shaker with continuous shaking at 180 rpm and then harvested by filtration. *E. coli* strain DH5α was used for cloning experiments and plasmid propagation. *E. coli* strain BL21(DE3) (Novagen, Madison,WI) was used as a host for the production of the active POx. Plasmid pJET 1.2 (Fermentas) was used for cloning of PCR fragments, and the expression vector pET21a(+) (Novagen) for recombinant production of POx. Production of recombinant POx to provide material for biochemical characterization was done in 1-liter shake flasks. Twenty-five ml Terrific broth (TB, (Ausubel et al., 1990) medium supplemented with 100 μg/ml of ampicillin were inoculated with a single colony, and cultivated at 37°C and 150 rpm for 6 hours. TB medium (225 ml) containing 100 μg/ml of ampicillin was inoculated with this starter culture and cultivated for additional 2 hours, then lactose was added to a final

concentration of 5 g/l. After overnight cultivation at 25°C and 150 rpm, the cells were harvested by centrifugation at 10,000 × g for 20 min and 4°C. The cell pellet was suspended in Start Buffer and homogenized in a French press. The homogenate was centrifuged at 30,000 × g for 30 min and 4°C, and the supernatant was recovered as a crude extract.

Nucleic acid manipulations. PCR, restriction enzyme digestions and agarose gel electrophoresis were performed according to standard methods (Sambrook et al., 1989). Genomic DNA extraction was performed according to the method described by (Liu et al., 2000). Total RNA was extracted from *Aspergillus* strains using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Identification of the pyranose 2-oxidase. The complete genomes of *Aspergillus nidulans* and *Aspergillus oryzae* are available at http://www.broad.mit.edu/annotation/genome/aspergillus_nidulans/MultiHome.html. To identify a putative pyranose oxidase in the *A. nidulans* and *A. oryzae* genome, a Blast search (Altschul et al., 1997) was performed using the sequence of POx from *Trametes ochracea* (*To*POX; accession number gi|31044224|). Structural models of POx proteins were done using the program PyMOL (Delano Scientific LLC, San Carlos, CA).

Overexpression of POx in *A. nidulans*. The POx-encoding gene (including downstream sequences) was amplified from *A. nidulans* genomic DNA by PCR using the forward primer AspP2Ofw: 5´-ATGCAATACAGCAGAATGACCGCAACC-3´ and the reverse primer XbaIASPrv: 5´-CTGCCAGCCGACTCTAGAGCTAGTAG-3´ containing an *Xba*I restriction site. A 1.0 kb fragment from the *A. nidulans gpd*A promoter (AN 8041.3; Contig 139,

Chromosome II), was isolated by PCR with the forward primer (including an *Xba*I restriction site) XbaIASPfw: 5´-CCTAGCTGATTCTAGAGTGACCCAG-3´and reverse primer ChimAspP2O:

5´-CTGGTTGCGGTCATTCTGCTGTATTGCATACGACCGAAGCCGTTGATTCCGA-3´

containing 26 nt from the POx coding sequence. The two fragments were fused by overlap extension PCR using the flanking primers XbaIASPfw and XbaIASPrv. The complete fragment was inserted into the XbaI site of pBluescript KS(+) (Stratagene, La Jolla, CA) resulting in plasmid pIP21.

Fungal transformation. *A. nidulans* strain *yA2, ΔniaD*, *pantoB100*, *riboB2*, *argB2* was cotransformed with plasmid pFB39 (containing *argB*) and plasmid pIP21. Transformations were performed as described previously (Tilburn et al., 1983). Transformants were selected on minimal media with appropriated supplements in the absence of arginine.

Isolation of POx-encoding cDNA. Isolation of total RNA was carried out as described above and cDNA first strand synthesis was done using the First Strand cDNA Synthesis kit (Fermentas) and the anchor primer 5´-GGCCACGCGTGACTAGTACTTTTTTTTTTTTTT-3´. The POx cDNA was amplified using the primers: AspP2Ofw 5´-ATGCAATACAGCAGAATGACCGCAACC- 3´ and AspP2Orv 5´-TCAAGCGTCATCGTCCGTATCAGTGTC -3´ and inserted into pCR II TOPO (Invitrogen).

Construction of POx expression vectors. The POx-coding sequence was re-amplified using the two modified primers AspP2O-NdeIfw (5'-GCCGACGCCCATATGCAATACAGCAGAATG-3') and AspP2O-NotIrv (5'-

GGCGATGCGGCCGCTCAAGCGTCATCGTCC-3') containing *Nde*I and *Not*I restriction sites for insertion into the expression vector pET21a(+) in frame with the C-terminal His_6-tag encoded by the expression vector. The resulting plasmid was called pIP21AC. The cDNA encoding the *Aspergillus oryzae* hypothetical pyranose 2-oxidase (GeneID: 5990680, AO090001000158 hypothetical protein [Aspergillus oryzae RIB40]) was synthesized by Mr Gene (Regensburg, Germany) after optimization of the codon usage to *E. coli* using the proprietary software of the supplier. An *Nde*I restriction site at the 5´ end and a *Not*I restriction site at the 3´ end were added to the sequence of the optimized gene for insertion into the vector pET21a(+) analogously to *An*POx.

Enzyme purification. recPOx was purified by one-step immobilized metal affinity chromatography (IMAC), on a column (40 ml) packed with Profinity IMAC Ni-Charged Resin (Bio-Rad Inc, Hercules, CA) specific for recombinant His-tagged proteins. The column was pre-equilibrated with Start Buffer (KH_2PO_4 0.1M, NaCl 1M, Imidazole 0.01M, pH 6.5) and the crude extract was applied to the column at a rate of 2.5 ml/min. After an additional washing step with Start Buffer (2 column volumes) the enzyme was eluted at a rate of 2.5 ml/min by using a linear gradient (0 to 1 M Imidazole in Start Buffer) in 10 column volumes. Active fractions were pooled and stored at 4°C.

Enzyme assay. Unless otherwise specified, pyranose oxidase activity was determined spectrophotometrically following the standard procedure already described (Leitner et al., 2001). The effect of pH on oxygen was determined using the following buffers: sodium citrate (pH 2.0 to 5.0), potassium phosphate (pH 5.5 to 7.5) and sodium borate (pH 8.0 to 10), each at 100 mM.

Analysis of D-glucose reaction products. D-glucose (1 g/l) was converted by purified *An*POx or *Ao*POx (3.5 U/ml, 50 µl) in a total volume of 1 ml at 25°C for 20 hrs. Thin-layer chromatography of the reaction products was conducted on HPTLP aluminium sheets LiChrospher Si 60 (Merck) using 140:100:33:80 1-BuOac-AcOH-Me_2CO-H_2O and diphenylamine-aniline-phosphoric acid detection reagent (Kocourek et al., 1966).

Steady-state kinetic measurements. Unless otherwise stated, all measurements were performed at 30°C in 50 mM potassium phosphate buffer (pH 6.5). Measurements of kinetic constants for various electron donors (sugar substrates) were done with oxygen (air saturation) as electron acceptor and the routine ABTS-peroxidase assay performed as described above. Kinetic constants for various electron acceptors were determined using 20 mM D-glucose as the electron donor, and measurements were performed at pH 6.5. The kinetic constants for the electron acceptors were measured as previously described (Leitner et al., 2001, Pisanelli et al., 2009).

Results and Discussion

Identification and isolation of a POx-encoding gene in *A. nidulans*

In a comparative search using already described sequences of POx, the genome of *Aspergillus nidulans* was found to contain a gene encoding a hypothetical pyranose 2-oxidase (AN5281.3). Our attempts to isolate POX from *A. nidulans* grown on minimal medium with 1% glycerol were unsuccessful, although fungal growth appeared to be adequate. Failure to detect activity could be due to the low transcription level of the gene. Therefore, primers AspP2Ofw and AspP2Orv were used to amplify the full-length gene,

resulting in a 2232 nt fragment as predicted from the genome annotation. For homologous overexpression the plasmid pIP21AC was constructed fusing the *A. nidulans* *gpd*A promoter with the *pox* gene and the native terminator. *A. nidulans* protoplasts were cotransformed with this construct and the plasmid pFB39 containing the *argB* selectable marker to complement the arginine auxotrophy of the used strain. Fifteen arginine-protrotrophic transformants were obtained and screened for integration of the plasmid into the host genome by PCR using the primers XbaIASPfw and XbaIASPrv. The full-size *gpd*A- *pox*-fusion (4.0 kb) was found in only one transformant strain.

Heterologous expression of POx in *E. coli.* *An*POx cDNA was synthesized from total RNA of the transformant fungal strain as described. The cDNA contains an open reading frame of 1,806 bp encoding a polypeptide of 602 amino acids. The coding sequence was re-amplified with primers containing suitable restriction sites for insertion of the gene into the expression vector pET21a(+), in frame with a C-terminal His_6-tag encoded by the vector.

During the course of this work, a hypothetical protein putatively encoding a pyranose 2-oxidase was found in the genome of *A. oryzae*. The conceptual translation of *Ao*POx consists of an open reading frame of 1,890 bp encoding a polypeptide of 630 amino acids. A DNA fragment encoding this protein was synthetically produced by a commercial supplier and inserted via added restriction sites into the expression vector pET21a(+), analogously to *An*POx.

Recombinant *E. coli* BL21(DE) harboring the expression plasmids were cultivated in 1L shake flasks and TB media with lactose 0.5 % as the inducer. Routinely, approx. 790 mg/l (calculated from 918 U/l and a specific activity of 1.16 U/mg) of active, soluble

recombinant *An*POx and 57 mg/l of active, soluble recombinant *Ao*POx were obtained, as calculated from 33 U/l and a specific activity of 0.58 U/mg.

Enzyme purification. POx variants were purified from the crude extracts by immobilized metal affinity chromatography (IMAC) followed by ultrafiltration. This two-step purification procedure resulted in proteins that were apparently homogenous (> 98%) as judged by native PAGE and SDS/PAGE (Fig.2, 3). *An*POx was purified six-fold from the crude extract with a yield of 54% and a specific activity of 1.16 U/mg under standard assay conditions when glucose and oxygen (air) were used as substrates (Table 1) while *Ao*POx was purified up to 200 times with a specific activity of 0.6 U/mg. (Table 2).

Reaction products of D-glucose. In order to confirm the regioselectivity of the reaction of the recombinant enzymes, D-glucose was enzymatically converted as described, and the reaction products were identified as 2-keto-glucose using TLC as described in Material and Methods (Fig.4). The obtained pattern is consistent with oxidation at C-2 of D-glucose as described for POx from various basidiomycetous sources (Leitner et al., 2001, Pisanelli et al., 2009, Volc et al., 1996). The investigated genes therefore encode a pyranose 2-oxidase, and not, e.g., a glucose oxidase as it is known from *A. niger* (EC 1.1.3.4.), which oxidizes glucose at C-1.

Physical properties. The molecular mass for both recombinant *An*POx and *Ao*POx was approximately 260 kDa as estimated by PAGE under non-dissociating conditions (Fig.2). SDS-PAGE of POx resulted in a single band at 65 kDa (Fig.3), indicating a

homotetrameric structure of the active enzyme, as was described for pyranose oxidase from basidiomycetous fungi (Leitner et al., 2001).

pH dependence of activity. pH optima for the purified recombinant POx were determined using oxygen as the electron acceptor. Bell-shaped profiles with an optimum pH range between 6.5 and 8.5. were observed (Fig.1).

Kinetic properties. Various sugars were tested as possible substrates of *An*POx and the kinetic constants were determined using oxygen (air) as electron acceptor (Table 3). The highest catalytic efficiency (k_{cat}/K_m) was found for D-glucose (1167.68 mM^{-1} s^{-1}) whereas the corresponding values for L-sorbose, D-xylose, and D-galactose were dramatically lower. β-1,4-linked disaccharides such as maltose and lactose did not result in measurable activity, as is known for POx from other sources (Leitner et al., 2001, Pisanelli et al., 2009). The lowest catalytic efficiency was measured for D-fructose as a result of both an unfavourable Michaelis constant K_m and turnover number k_{cat}. In addition to oxygen, POx from *A. nidulans* is able to transfer electrons to other acceptors. Kinetic constants were determined for the two-electron acceptors 1,4-benzoquinone and DCIP as well as for a one-electron acceptor, the ferricenium ion Fc^+. These measurements were performed with D-glucose as the saturating substrate and both at the pH value of the standard assay, pH 6.5, and at the pH optimum of the respective electron acceptor (benzoquinone, pH 4.5; DCIP, pH 6.5; Fc^+, pH 8.0). Results are summarized in Table 5. 1,4-Benzoquinone shows a k_{cat}/K_m value of ~10^7 M^{-1} s^{-1} when the reaction is carried out at pH 4.5 while at pH 6.5 the value is reduced to about one third of this value; this is mainly the effect of a considerably increased Michaelis constant at the higher pH value. For Fc^+ the pH of the reaction does not affect the K_m value significantly, while at the optimal pH of 8.0 and in

the presence of borate buffer the turnover number is increased 2.4-fold compared to pH 6.5, resulting in an ~3-fold higher catalytic efficiency. The pH optimum for DCIP was found to be similar to the standard assay conditions. DCIP as an electron acceptor of PcPOx is characterized by a very low Michaelis constant in the micromolar range, but also by k_{cat} values that are significantly lower than those of the other electron acceptors tested.

Steady-state kinetic constants for the recombinant *Ao*POx were determined for the three sugars substrate D-glucose, L-sorbose, D-xylose, and D-galactose. Table 4 provides a summary of kinetic data. For the presumed natural substrate of POx, D-glucose, *Ao*POx showed slightly increased K_m value while the V_{max} value decreased 8 times compared to the corresponding values for *An*POx, resulting in a significant lowering of the catalytic efficiency k_{cat}/K_m (0.52 mM^{-1} s^{-1}). L-sorbose, D-xylose, and D-galactose are poor substrate of *Ao*POx and all showed notably reduced values compared to *An*POx for k_{cat}/K_m., due to higher K_m and lower V_{max} values (all in the range of 0.002-0.015 mM^{-1} s^{-1}). Furthermore, the kinetic constants were determined for 1,4-Benzoquinone (a two electron H^+ acceptor) and ferricenium ion (Fc^+; a one electron acceptor) with D-glucose as substrate in saturating concentration (Table 6). These values are generally well in the range of other described POx. An alignment of several POx amino acid sequences was reported previously (de Koker et al., 2004) and revealed a strong conservation of several amino acids in most analyzed sequences, which play various important roles in sugar binding and putatively electron transfer (Hallberg et al., 2004, Kujawa et al., 2006). Fig. 5 shows such an alignment including the sequences of *An*POx and *Ao*POx. The characteristic motif THWTC (166 – 170 in *To*POx, containing the flavin-anchoring residue H167) is also present in both *An*POx and *Ao*POx (140 – 144 and 174 – 178, respectively), as are Q448 (Q396 and Q458), R472 (R420 and R482), H548 (H495 and H557) and N593

(N548 and N608), as well as the active site loop DAFSYG (452 – 457 in *Tm*POx, 400 – 405 in *An*POx, 462 – 467 in *Ao*POx). V546, common in *Trametes* and *Peniophora* sequences, is replaced by alanine in *P. chrysosporium* and *L. shimeiji* (A551 and A538, respectively, (Pisanelli et al., 2009)), and also in the two *Aspergillus* sequences (A495 and A555). Alanine was described as likely maintaining the function of the valine (interaction with the C1 hydroxyl group of the sugar substrate), therefore this exchange does not alter the reactivity significantly. *Aspergillus* spp. POx therefore appear to have conserved the essential biochemical properties, which still are compatible with various theories concerning the biological function of these enzymes, as discussed for white-rot fungi thus far. Variously substituted benzoquinones are intermediates in the degradation of aromatic compounds by wood-decay fungi, as recently reviewed (Martinez et al., 2005). These benzoquinones are reduced to hydroquinones and undergo further intracellular metabolism, putatively by a 1,4-benzoquinone reductase, an FMN-containing flavoprotein using NAD(P)H as its second substrate (Akileswaran et al., 1999, Brock and Gold, 1996, Brock et al., 1995). Alternatively, extracellular redox cycling of quinones may contribute to the generation of reactive oxygen species (ROS) for non-enzymatic attack of lignocellulose (Hammel et al., 2002). It remains unclear what function these enzymes fulfill in *Aspergillus* spp., which do not degrade lignocellulose. A horizontal gene transfer of a *Firmicutes* gene (encoding a 2-keto-gluconate dehydrogenase; accession nr. AAY60294) into the kingdom Fungi was suggested recently (Kittl et al., 2008). This introduced gene apparently has “survived” in a number of phylogenetically diverse groups, but was lost in others – there are species with and without *pox* genes among the Tricholomataceae as well as the Coriolaceae – and may have taken over biological functions that are as yet unknown.

Comparison of *An*POx and *Ao*POX. Despite their catalytical similarity, a comparison of the translated amino acid sequences of *An*POx and *Ao*POX reveals a marked difference: the *An*POx sequence has a deletion of approximately 30 amino acids in the domain constituting the oligomerization arm (Fig.). A structural model of the enzyme shows a significantly stunted arm domain. This may influence the monomeric interactions in the active tetramers, both in the initial formation of the dimers as well as in tetramerization (Hallberg et al., 2004b), and may account for the observed reduced stability of the enzyme. Isolation of active monomeric POx was unsuccessful to date, and abolishment of the tetramerization by mutagenesis of selected amino acid residues in the interacting domains of *Trametes multicolor* (*ochraceae)* POx never resulted in active soluble protein (our unpublished information). In contrast, *Ao*POX has an insertion of approximately 20 amino acids around position 330 compared to other sequences. Modeling of this additional sequence is of course tentative, since no significant similarity to known structures exists. The structural model according to PyMOL seems to suggest a "second arm domain" that may interact with the base of the "head" domain during formation of the dimers and tetramers. Even if the inserted sequence does not form a second arm, it may provide extra amino acid residues for interaction of the monomers. Whether this additional area of interaction results in the formation of more stable tetramers is a subject of ongoing investigation.

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Tables

Table 1. Purification of recombinant POx from *Aspergillus nidulans* by immobilized metal affinity chromatography (IMAC)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	4405	800	0.181	100	1
IMAC	395	459	1.16	54	6.4

Table 2. Purification of recombinant POx from *Aspergillus oryzae* by immobilized metal affinity chromatography (IMAC)

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	1920	4.8	0.0025	100	1
IMAC	9	4.8	0.58	100	232

Table 3. Apparent steady-state kinetic constants of recombinant pyranose 2-oxidase from *A.nidulans* for several electron donors, as determined at 30°C by using oxygen (air saturation) as electron acceptor under the standard ABTS assay conditions.

Substrate	v_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
D-Glucose	8.18 ± 0.12	1.77 ± 0.11	35.44 ± 0.52	20.02
L-Sorbose	2.23 ± 0.16	32.6 ± 6.36	9.66 ± 0.69	0.3
D-Xylose	0.32 ± 0.03	66.9 ± 19.5	1.39 ± 0.13	0.02
D-Galactose	0.75 ± 0.02	19.2 ± 2.07	1.95 ± 0.052	0.1
D-Fructose	1.28 ± 0.27	2558 ± 669	5.55 ± 1.17	0.0022

Table 4. Apparent steady-state kinetic constants of recombinant pyranose 2-oxidase from *A.oryzae* for several electron donors, as determined at 30°C by using oxygen (air saturation) as electron acceptor under the standard ABTS assay conditions.

Substrate	v_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
D-Glucose	0.34 ± 0.01	2.86 ± 0.50	1.48 ± 0.051	0.52
D-Galactose	0.006 ± 0.0003	13.0 ± 2.20	0.02 ± 0.001	0.002
L-Sorbose	0.36 ± 0.0212	176 ± 22	1.59 ± 0.091	0.009
D-Xylose	0.062 ± 0.003	18.1 ± 3.45	0.27 ± 0.013	0.015

Table 5. Apparent kinetic constants of POx from *A. nidulans* for several electron acceptors, as determined at 30°C by using 20 mM D-glucose as electron donor under standard assay conditions (pH 6.5) unless otherwise indicated

Electron acceptor	v_{max} (μmol min^{-1} $mg^{-1)}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1,4-Benzoquinone	42.9 ± 3.73	0.15 ± 0,04	186 ± 16.2	1240
$Fc^+PF_6^-$ [a]	36.5 ± 12.6	0.67 ± 0.35	158 ± 54.6	235
DCIP [b]	3.03 ± 0.42	0.39 ± 0.11	13.1 ± 1.82	33.6
2-Chloro-1,4-benzoquinone	3.75 ± 0.57	0.48 ± 0.15	16.3 ± 2.47	34
2-Methoxy-1,4-benzoquinone	10.9 ± 1.07	0.42 ± 0.09	47.2 ± 4.64	112
2-Methyl-1,4-benzoquinone	2.32 ± 0.13	0.11 ± 0.02	10.1 ± 0.56	91.8
2,6-Dimethyl-1,4-benzoquinone	0.01 ± 0.06	0.40 ± 0.07	0.04 ± 0.26	0.1
Tetrafluoro-1,4-benzoquinone	29.8 ± 14.1	9.42 ± 5.25	129 ± 61.1	13.7

[a] $Fc^+PF_6^-$, ferricenium hexafluorophosphate

[b] DCIP, 2,6-dichloroindophenol

Table 6. Apparent kinetic constants of POx from *A. oryzae* for two electron acceptors, as determined at 30°C by using 20 mM D-glucose as electron donor under standard assay conditions (pH 6.5) unless otherwise indicated

Electron acceptor	v_{max} (μmol min^{-1} $mg^{-1)}$	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1,4-Benzoquinone	0.70 ± 0.019	0.32 ± 0.025	3.04 ± 0.08	9.4
$Fc^+PF_6^-$ [a]	0.40 ± 0.09	1.12 ± 0.48	1.7 ± 0.40	1.5

[a] $Fc^+PF_6^-$, ferricenium hexafluorophosphate

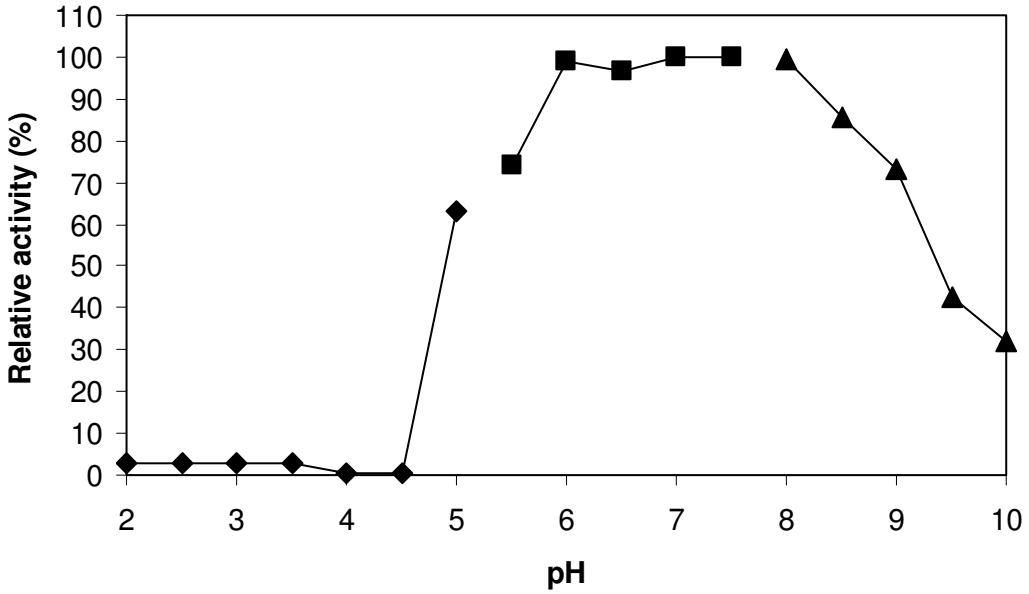

Fig. 1 Effect of pH on the activity of recPOx from *A. nidulans* in the presence of oxygen. The buffers used were 100 mM sodium citrate (♦), 100 mM potassium phosphate (■), 100 mM borate (▲)

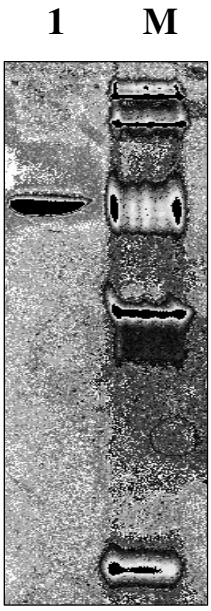

Fig. 2 Native PAGE analysis of rPOx from *A.nidulans***.** Lane M, high-molecular-mass marker protein kit, Amersham; Lane 1, !rPOx, purified recombinant pyranose oxidase.

M 1 2 3

Fig. 3 SDS-PAGE analysis of rPOx from *A. nidulans***.** Lane M, Precision Plus Protein™ Standards Dual Color (BioRad); lane 1, purified pyranose oxidase after IMAC; lane 2, flow-through from IMAC ; lane 3, crude cell extract undiluted.

1

2

3

4

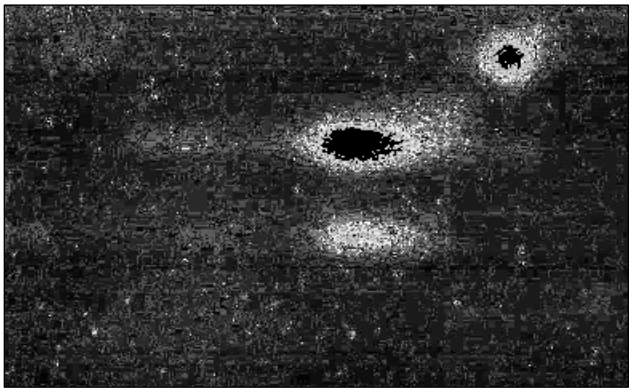

Fig. 4 Characteristic TLC pattern of reaction products in transformations of D-glucose by pyranose oxidase. Lane 1, control for D-glucose without enzyme ; Lane 2 control for 2-keto-glucose without enzyme; Lane 3, C-2 oxidation of D-glucose catalyzed by pyranose oxidase from *A. nidulans;* Lane 4, control without sugar.

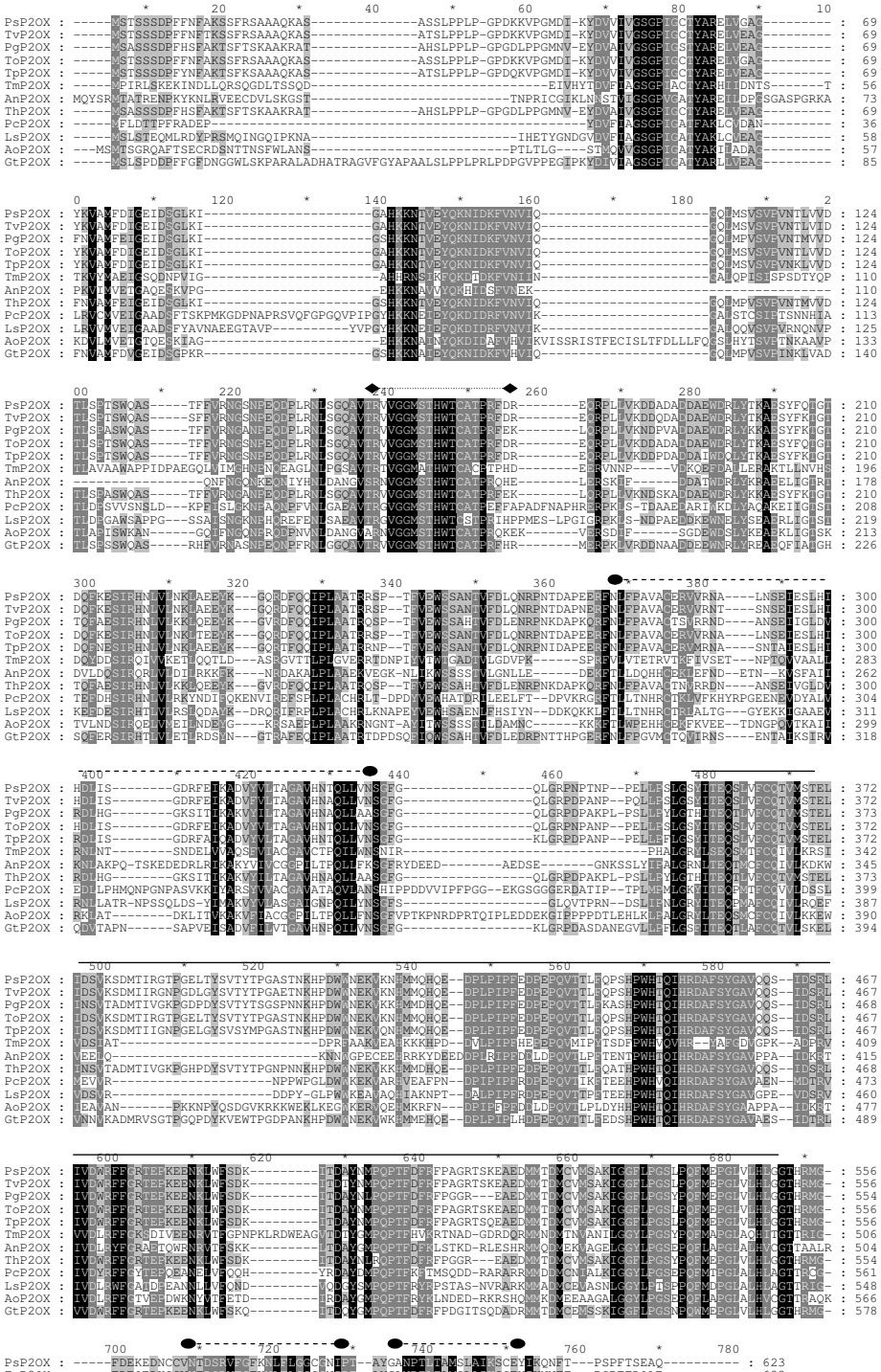

Fig.5 Alignment of POxs and putative POx sequences from various organisms; *Peniophora sp. SG* (*Ps*POx; accession no. gi|27436422|), *Trametes versicolor* (*Tv*POx; accession no. gi|1845549|), *Phlebiopsis gigantea* (*Pg*POx; accession no. gi|34452037|), *Trametes ochracea* (*To*POx; accession number gi|31044224|), *Trametes pubescens* (*Tp*POx; accession no. gi|57867849|), *Tricholoma matsutake* (*Tm*POx; accession no. gi|25553433|), *Aspergillus nidulans* (*An*POx; hypothetical protein AN5281.2, accession no. gi|40743251|); *Trametes hirsuta* (*Th*POx; accession no. gi|25091016|), *Phanerochaete chrysosporium* (*Pc*POx; accession no. gi|46405851|), *Lyophyllum shimeji* (*Ls*POx; accession no. gi|44886073|), *Aspergillus oryzae* (*Ao*POx; hypothetical protein, accession no. gi|169768478|), *Gloeophyllum trabeum* (*Gt*POx; accession no. gi|213876461|).Grey shaded amino acids indicate homology between the POx sequences. Dashed lines above sequences indicate FAD-binding domains; dotted lines above the sequences indicate flavin attachment loops; solid lines above the sequences indicate substrate binding domain.

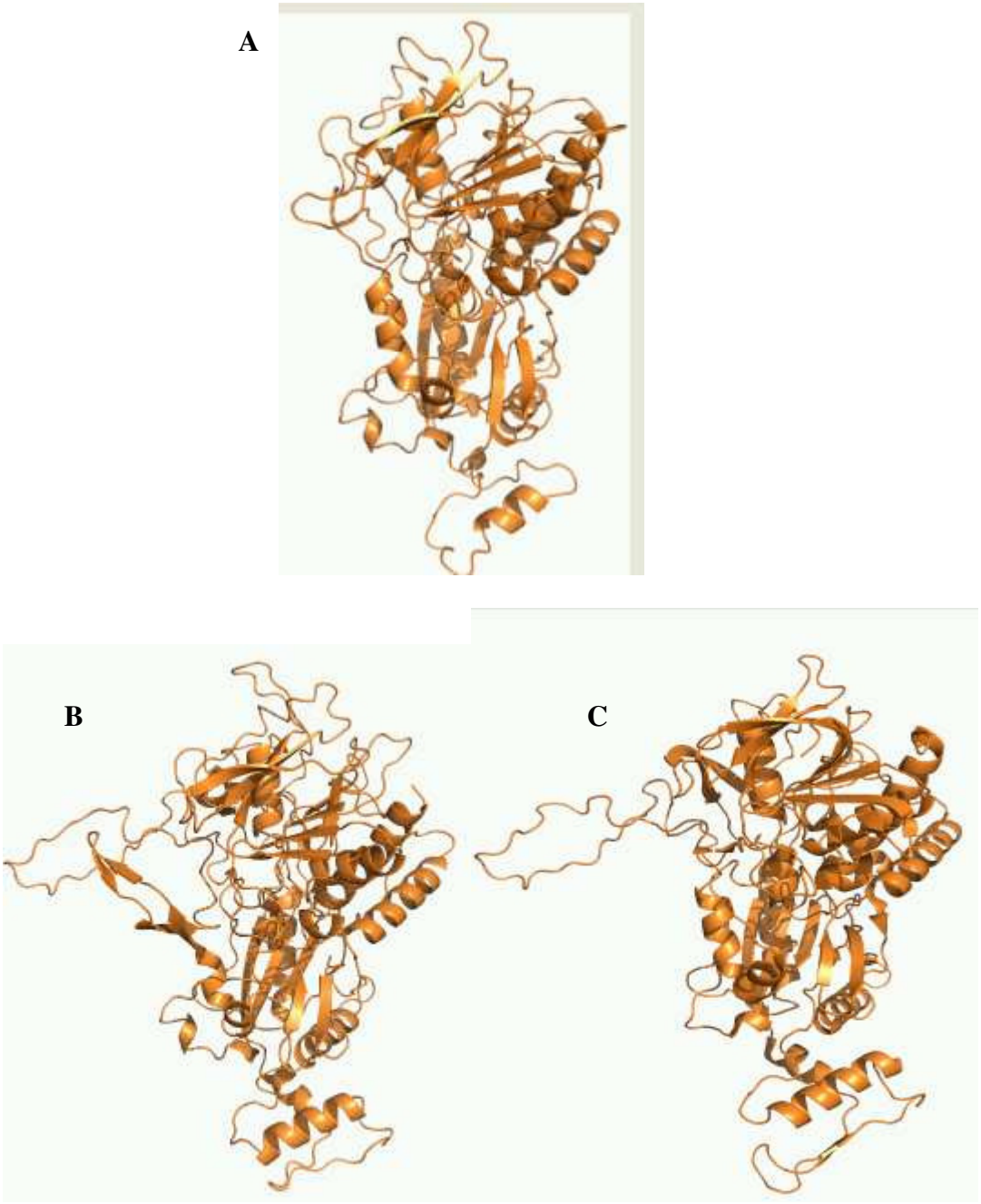

Fig. 6 Structural models of POxs subunits. A, *Aspergillus nidulans* POx; B, *Aspergillus oryzae* POx; C, *Trametes multicolor* POx.

Chapter 3

Engineering of pyranose 2-oxidase: Improvement for biofuel cell and food applications through semi-rationalprotein design

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ABSTRACT

Pyranose 2-oxidase (P2Ox) has several proposed biotechnological applications such as a bio-component in biofuel cells or for carbohydrate transformations. To improve some of the catalytic properties of P2Ox from *Trametes multicolor*, we selected a semi-rational approach of enzyme engineering, saturation mutagenesis of active-site residues and subsequent screening of mutant libraries for improved activity. One of the active-site mutants with improved catalytic characteristics identified was V546C, which showed catalytic constants increased by up to 5.7-fold for both the sugar substrates (D-glucose and D-galactose) and alternative electron acceptors (1,4-benzoquinone, BQ and ferricenium ion, Fc^+], albeit at the expense of increased Michaelis constants. By combining V546C with other amino acid replacements, we obtained P2Ox variants that are of interest for biofuel cell applications due to their increased k_{cat} for both BQ and Fc^+, e.g., V546C/E542K showed 4.4- and 17-fold increased k_{cat} for BQ compared to the wild-type enzyme when D-glucose and D-galactose, respectively, were the saturating substrates, while V546C/T169G showed approx. 40- and 50-fold higher k_{cat} for BQ and Fc^+, respectively, with D-galactose in excess. This latter variant also shows significantly modulated sugar substrate selectivity, due to an increase in k_{cat}/K_M for D-galactose and a decrease in k_{cat}/K_M for D-glucose when oxygen is the electron acceptor, as well as improved catalytic efficiencies for D-galactose, regardless of the electron acceptor used. While the wild-type enzyme strongly prefers D-glucose over D-galactose as its substrate, V546C/T169G converts both sugars equally well as was shown by the kinetic constants determined as well as by biotransformation experiments.

1. Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10) is a flavin adenine dinucleotide (FAD) dependent enzyme widespread in wood-degrading basidiomycetes (Volc et al., 1985; Danneel et al., 1992; Leitner et al., 1998). The reaction catalyzed by P2Ox is of the Ping Pong Bi Bi type typically found in flavoprotein oxidoreductases (Ghisla and Massey, 1989; Artolozaga et al., 1997) and consists of the reductive half-reaction, in which an aldopyranose substrate reduces the flavin adenine dinucleotide (FAD) cofactor to yield $FADH_2$ and 2-dehydroaldose (2-ketoaldose) as the result of oxidation of the sugar at position C-2 (Freimund et al., 1998), and the ensuing oxidative half-reaction, which involves the reoxidation of $FADH_2$ by the electron acceptor. During this oxidative half-reaction, a C-4a-hydroperoxyflavin intermediate is formed when oxygen is used, which is the first evidence of such an intermediate for a flavoprotein oxidase (Sucharitakul et al., 2008). In addition to molecular oxygen, P2Ox can also use various quinones, complexed metal ions and radicals as its electron acceptor (Leitner et al., 2001). As judged from the catalytic efficiency some of these alternative electron acceptors are in fact better substrates for the enzyme than oxygen, suggesting that P2Ox can play an important role in the reduction of quinones during the process of ligninolysis (Ander and Marzullo, 1997; ten Have and Teunissen, 2001). This excellent reactivity of P2Ox with alternative electron acceptors and a range of sugar substrates can be employed in various attractive applications. One possible field of application is as a bio-element in sensors and biofuel cells, where it could replace glucose oxidase, which is typically used but shows certain disadvantages. In these applications, the enzyme communicates with an electrode through small redox-active compounds, so-called mediators, in a process referred to as mediated electron transfer (MET). Recently, we could show that P2Ox can be electrically wired to graphite electrodes through the use of osmium

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redox polymers (Tasca et al., 2007), which gather the electrons from the enzyme and transfer them to the electrode. Additional mediators that have been successfully tested for providing contact between P2Ox or other oxidases and an electrode include ruthenium ion complexes and modified ferrocenes as well as various quinones (Zhu et al., 2006; Nazaruk and Bilewicz, 2007; Tamaki et al., 2007).

In this study we used P2Ox from *Trametes multicolor* (*Tm*P2Ox), which was first isolated and characterized by Leitner et al. (2001). The crystal structure of *Tm*P2Ox was determined at 1.8 Å resolution (Hallberg et al., 2004). It is a homotetrameric enzyme with a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one FAD covalently bound (Halada et al., 2003; Hallberg et al., 2004). Recently, the crystal structure of *Tm*P2Ox in complex with one of its slow carbohydrate substrates, 2-fluoro-2-deoxy-D-glucose, was reported (Kujawa et al., 2006). This structure gave detailed information about residues interacting with the sugar substrate in the active site. As a consequence, site-directed mutagenesis at these residues allows analysis of structure–function relationships, and also possible improvements by (semi-)rational protein design. Mutations of active-site residues are known to often dramatically change the properties of an enzyme. Frequently, these changes are simply unfavorable or even inactivating, however, the altered enzymes can also show improved properties such as broadened substrate specificities or increased activity (Toscano et al., 2007).

The monosaccharide D-glucose is the preferred substrate of *Tm*P2Ox, whereas D-galactose performs poorly with only 5.7% relative activity compared to D-glucose (Leitner et al., 2001). Oxidation of D-galactose at position C-2 is interesting from an applied point of view because the product obtained in this transformation, 2-keto-D-galactose (2-dehydro-D-galactose, galactosone), can be reduced easily at position C-1 to yield D-tagatose (Haltrich et al., 1998), which is a ketose sugar with significant potential as a non-cariogenic, low caloric sweetener in food applications. Lactose, which is available in large quantities as a by-product of dairy industry, can be hydrolyzed by β-galactosidases and thus provides an ample supply of D-galactose (Nakayama and Amachi, 1999). For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose, however, is too low, leading to either very long conversion times or disproportionate amounts of required enzyme. Also, an increased catalytic activity with the alternative electron acceptors such as the ferricenium ion or quinones is desirable for biofuel cell applications. Based on the crystal structure we decided to use saturation mutagenesis to exchange all of the active-site residues one by one, and screen for improvements in the catalytic activity with D-galactose and alternative electron acceptors. In this work, we report on one of these active-site variants, V546C, together with its combinations with other mutations, which show significantly improved catalytic properties and which are attractive for various biotechnological applications.

2. Materials and methods

2.1. Plasmids, microorganisms and media

The construction of the pET21d$^+$/P2Ox vector (pHL2), which expresses the His-tagged P2Ox gene from *T. multicolor* under the control of the T7 promoter, has already been described (Kujawa et al., 2006). The plasmids carrying the T169G as well as the E542K mutation have also been described (Spadiut et al., 2008a,b).

Active, recombinant *Tm*P2Ox and P2Ox mutants were expressed in *E. coli* strain BL21 Star DE3 (Invitrogen, Carlsbad, CA, USA). TB_{amp}-medium (yeast extract 24 g/L, peptone from casein 12 g/L, glycerol 4 mL/L; KH_2PO_4-buffer 1 M, pH 7.5) was used to cultivate *E. coli* cells for protein expression under appropriate selective conditions (ampicillin was added to 0.1 g/L). LB_{amp}-medium (yeast extract 5 g/L, peptone from casein 10 g/L, NaCl 5 g/L, ampicillin 0.1 g/L) was used to cultivate *E. coli* cells in 96-well screening plates. For induction of protein expression cells were grown in 2× LB_{amp}/lactose (yeast extract 10 g/L, peptone from casein 20 g/L, NaCl 5 g/L, lactose 5 g/L; ampicillin 0.1 g/L). All chemicals used were purchased from Sigma (Vienna, Austria) and were of the highest grade available.

2.2. Generation of mutants

The P2Ox gene was mutated by a two-step saturation mutagenesis using PCR and digestion with DpnI (Li and Wilkinson, 1997). Plasmid pHL2 was used as template for saturation mutagenesis at position V546 with primers P2OxV546_fwd (5′-GCCTGGTCTTNNSCTTCACCTTGGTG-3′) and P2OxV546_rev (5′-AAGACCAGGCTCCATGAATTGC-3′). The forward primer carried the variable position NNS, where N = A, G, C or T and S = G or C. To create double mutants, combining the Val → Cys substitution at position 546 with known mutations affecting the kinetic properties of *Tm*P2Ox, the plasmids carrying the mutations E542K and T169G were used as templates for the PCR reactions. Mutation V546C was introduced with primers V546C_E542K_fwd (5′-GAAGCCTGGTCTTTGCCTTCACCTTGGTGG-3′) and V546C_E542K_rev (5′-AAGACCAGGCTTCATGAATTGCGGGAGG-3′). Variant V546C/T169G was constructed by using the primers V546C_fwd (5′-GCCTGGTCTTTGCCTTCACCTTGGTG-3′) and V546C_rev (5′-AAGACCAGGCTCCATGAATTGCGG-3′). The mutant V546C/T169G was further mutated by using the primers L537W_fwd (5′-ACCCGGCTCCTGGCCGCAATTC-3′) and L537W_rev (5′-GGAGCCGGGTAGGAAGCCACC-3′) to additionally introduce tryptophane at position 537. The PCR reaction mix contained 2.5 U pfu DNA polymerase (Fermentas; St. Leon-Rot, Germany), 100 ng of plasmid DNA, 5 pmol of each primer, 10 μM of each dNTP and 1× PCR buffer in a total volume of 50 μL. The mutagenic PCRs were done using the following conditions: 95 °C for 4 min, then 30 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 16 min, and a final incubation at 72 °C for 10 min. After PCR, the methylated template DNA was degraded by digestion with 10 U of DpnI at 37 °C for 2 h. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, USA). Five μL of the PCR products were transformed into electro-competent *E. coli* BL21 Star DE3 cells. To confirm the presence of the correct mutations and the absence of undesired mutations, the P2Ox-encoding insert was sequenced using primers T7promfwd (5′-AATACGACTCACTATAGGG-3′) and T7termrev (5′-GCTAGTTATTGCTCAGCGG-3′).

2.3. Screening assay for improved P2Ox variants in 96-well plates

Position V546 was mutated by saturation mutagenesis, which allowed the creation of a mutant library containing all possible codons at this target position. The size of the library, which has to be screened to cover all possible mutants, is determined by the mutagenic codon. To mutate the amino acid V546 by saturation mutagenesis we used a primer of the NNS type, which defines the minimum library size with 95 colonies to be screened to statistically cover 95% of all possible substitutions (Georgescu et al., 2003). To cover all possible amino acid substitutions with very high probability, we screened 360 mutants for their catalytic activity with either D-glucose or D-galactose. A screening assay based on 96-well microtiter plates as previously described (Spadiut et al., 2008a) was used to this end. Six-well per plate were inoculated with *E. coli* expressing wild-type enzyme for comparison. The average value of specific activity with either of the two sugar substrates D-glucose

or D-galactose of the wild-type P2Ox wells was determined and compared with the values of the mutants.

2.4. Protein expression and purification

Cultures (1 L) of *E. coli* BL21 Star DE3 transformants were grown in TB_{amp} in shaken flasks at 37 °C and 160 rpm. When OD_{600} of ~0.5 was reached, recombinant protein expression was induced by adding lactose to a final concentration of 5 g/L. After incubation at 25 °C for further 20 h, about 20 g wet biomass per liter were harvested by centrifugation at 10,000 × *g* for 20 min, resuspended in KH_2PO_4-buffer (50 mM, pH 6.5) containing the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF; 1 g/L) and disrupted in a continuous homogenizer (APV Systems; Silkeborg, Denmark). The crude extract was separated from cell debris by centrifugation (70,400 × *g*, 4 °C) and used for protein purification by immobilized metal affinity chromatography (IMAC) with a 10-mL BioRad Profinity IMAC Ni-Charged Resin (BioRad; Vienna, Austria). The column was equilibrated with 10 column volumes (CV) of buffer (0.05 M KH_2PO_4, 0.5 M NaCl, 20 mM imidazole; pH 6.5), and washed with 5 CV of the same buffer after loading. Enzymes were eluted with a linear gradient of 10 CV with a buffer containing 1 M imidazole. The active fractions were combined and imidazole was removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica, MA, USA) with a 10-kDa cut-off membrane. The eluted, concentrated enzymes were washed three times with 10 mL of KH_2PO_4-buffer (50 mM, pH 6.5) and finally diluted in the same buffer to a protein concentration of 10–20 mg/mL.

2.5. Enzyme activity assays

P2Ox activity was measured with the standard chromogenic ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay (Danneel et al., 1992). A sample of diluted enzyme (10 μL) was added to 980 μL of assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg) and KH_2PO_4-buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM). The absorbance change at 420 nm was recorded at 30 °C for 180 s. The molar absorption coefficient at 420 nm (ε_{420}) used was 42.3 mM^{-1} cm^{-1}. One Unit of P2Ox activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per min, which equals the consumption of 1 μmol of O_2 per min, under assay conditions. Protein concentrations were determined by the Bradford assay (Bradford, 1976) using the BioRad Protein Assay Kit with bovine serum albumin as standard.

2.6. Steady-state kinetic measurements

Kinetic constants were calculated by non-linear least-square regression, fitting the data to the Henri–Michaelis–Menten equation. These constants were measured for the two electron donors, D-glucose (0.1–50 mM) and D-galactose (0.1–200 mM) using the standard ABTS assay and oxygen (air saturation). In addition, the catalytic constants were measured for the two-electron proton acceptor 1,4-benzoquinone and the one-electron non-proton acceptor ferricenium ion Fc^+ (ferricenium hexafluorophosphate, $FcPF_6$) at a saturating concentration of 100 mM of either D-glucose or D-galactose. The kinetic parameters for BQ were determined by adding 10 μL of diluted enzyme to 990 μL of assay buffer containing the sugar substrate (100 mM), KH_2PO_4-buffer (50 mM, pH 6.5) and BQ (0.01–2 mM). The absorbance change at 290 nm was recorded at 30 °C for 180 s. The molar absorption coefficient at 290 nm (ε_{290}) used was 2.24 mM^{-1} cm^{-1}. $FcPF_6$ was varied from 0.005 to 1.5 mM and the absorbance change at 300 nm was recorded at 30 °C for 180 s. The molar absorption coefficient at 300 nm (ε_{300}) used was 4.3 mM^{-1} cm^{-1}.

2.7. Electrophoresis

To check the purity and the correct molecular mass of the purified enzymes, electrophoresis was done as described by Laemmli (1970). Both native PAGE and SDS-PAGE were performed using a 5% stacking gel and a 10% separating gel in a PerfectBlue vertical electrophoresis system (Peqlab; Erlangen, Germany). Samples were diluted to 1–2 mg of protein per mL and aliquots of 5 μL were loaded per lane. The High Molecular Weight Calibration Kit (Amersham; NJ, USA) and the Precision Plus Protein Dual Color Kit (BioRad) were used as mass standards for native electrophoresis and SDS-PAGE, respectively. Gels were stained with Coomassie blue.

2.8. Bioreactor cultivations

To produce sufficient amounts of the wild-type and the mutated enzyme V546C/T169G, two batch cultivations were carried out in a 5-L Biostat MD stirred tank reactor with a working volume of 4 L. Both cultivations were done in TB_{amp}-medium. A pre-culture grown in an Erlenmeyer flask containing 200 mL of sterile TB_{amp}-medium (37 °C, 140 rpm) was transferred to the bioreactor at an OD_{600} of ~0.5. The temperature was set to 25 °C, the culture pH was maintained at pH 7.0 by automatic addition of sterile NaOH (4 M), and the dissolved oxygen concentration (DO_2) was set to 30%. DO_2 levels were maintained by supplying filtered air automatically (0–4 L/min) and adjusting the stirrer velocity. The parameters were controlled by the IMCS-2000 digital control unit (PCS AG; Wetzikon, Switzerland). The medium was supplemented with 5 g/L lactose for induction of P2Ox expression from the beginning of the cultivation. Samples were taken every 2 h to measure the optical density, total intracellular protein concentration and P2Ox activity to monitor the enzyme production. When the volumetric activity reached a maximum, cells were harvested by centrifugation for 10 min at 16,000 × *g*. The cells were homogenized and the enzymes were purified as described above.

2.9. Batch conversion experiments

Wild-type P2Ox and the variant V546C/T169G were compared in terms of their ability to concomitantly oxidize D-glucose and D-galactose to the corresponding 2-ketoaldoses with either oxygen or BQ as an electron acceptor. Four batch conversion experiments (300 mL) were performed in parallel in a multi-fermenter (Infors; Bottmingen, Switzerland). Specific activity of wild-type P2Ox with D-glucose is 7.6 U/mg, and of variant V546C/T169G is 0.08 U/mg. For 300-mL batch conversions, 45 U of wild-type P2Ox and 5 U of mutated enzyme were used. Catalase was used in excess (100,000 U) to decompose H_2O_2. When BQ was applied as an electron acceptor in excess (5 mM), laccase was used in a three times higher activity than P2Ox (150 and 15 U, respectively) to reoxidize the formed hydroquinone (Ludwig et al., 2004). Laccase from *Trametes pubescens* MB 89 was prepared according to Galhaup et al. (2002) and purified to yield a specific activity of 64.5 U mg^{-1}. The conversion experiments were conducted in 100 mM sodium citrate buffer (pH 5.0) at 30 °C, 300 rpm and a DO_2 concentration of 20%. As calculated from the volumetric activity of the wild-type enzyme with its substrate D-galactose, both conversion reactions were performed with 0.75 g/L of both D-glucose and D-galactose to ensure complete conversion within reasonable time for the selected activities. For the batch experiments with the mutated variant, 1.4 g/L of each sugar were used, again based on the volumetric activity of the mutant enzyme.

Samples (2 mL) were taken periodically, held at 95 °C for 3 min to inactivate the enzymes and centrifuged. The supernatants were analyzed for their sugar content using high performance anion exchange chromatography with pulsed amperometric detection

(HPAEC-PAD), which was carried out using a Dionex DX-500 system (Dionex; Sunnyvale, CA, USA) and a CarboPac PA-1 column (4 mm × 250 mm) at 27 °C (Splechtna et al., 2006).

3. Results

3.1. Generation of mutants

The presence of different mutations at position V546 in the *Tm*P2Ox gene after saturation mutagenesis was confirmed by DNA sequence analysis of 10 randomly picked clones, proving the successful application of this technique. Six variants, which showed the highest activity with D-galactose in the 96-well plate screening assay, were isolated and were all identified as V546C encoded by either of the two possible codons for the amino acid cysteine (TGT, TGC). Variant V546C was expressed with a C-terminal His-tag added in *E. coli*, purified by IMAC and concentrated by ultrafiltration as previously described (Hallberg et al., 2004; Spadiut et al., 2008b). The purity of the protein preparations was confirmed by native PAGE and SDS-PAGE (Fig. 1). Subsequently, this variant was combined with already described mutations of *Tm*P2Ox to possibly combine their positive effects. T169G shows dramatically decreased K_M values for both sugar substrates D-glucose and D-galactose (Spadiut et al., 2008b), and the variants E542K and L537W are characterized by elevated k_{cat} and reduced K_M values for some electron acceptors and significantly increased stability (Spadiut et al., 2008a). Thus, the P2Ox variants V546C/T169G, V546C/E542K and V546C/T169G/L537W were produced, purified to apparent homogeneity and characterized.

3.2. Kinetic characterization of mutational variants

Initial rates of activity were recorded over a substrate range of 0.1–50 mM D-glucose and 0.1–200 mM D-galactose for wild-type P2Ox and the mutational variants V546C, V546C/T169G, V546C/E542K and V546C/T169G/L537W, using the standard ABTS assay and air oxygen. Kinetic data are summarized in Table 1. The turnover number (k_{cat}) of the mutational variant V546C increased significantly for both substrates, D-glucose and D-galactose, compared to the wild-type enzyme (1.8- and 2.6-fold, respectively). Since the K_M values increased (three- and fivefold, respectively), the mutant resulted in an overall reduced catalytic efficiency, k_{cat}/K_M. In order to lower K_M, we introduced several additional amino acid substitutions that are known to affect the kinetic properties of *Tm*P2Ox into the V546C variant. The Glu → Lys substitution at position 542 is known to decrease the K_M value for sugar substrates considerably while lowering k_{cat} to some extent (Masuda-Nishimura et al., 1999; Spadiut et al., 2008a). This mutational approach was effective since when comparing the V546C/E542K double mutant with V546C, the K_M values for both D-glucose and D-galactose decreased significantly (by approx. 50% compared to V546C) while k_{cat} was hardly affected. Overall, V546C/E542K shows catalytic efficiencies that are comparable to the wild-type enzyme, yet with a twofold increased turnover number for both sugar substrates.

The Thr → Gly substitution at position 169 is known to lower the K_M values dramatically, especially for D-galactose, while k_{cat} is also lowered significantly (Spadiut et al., 2008b). Again, the role of this residue was confirmed when comparing V546C/T169G and V546C/T169G/L537W with V546C (Table 1). Despite this reduction in k_{cat}, variant V546C/T169G shows interesting properties. Wild-type P2Ox as well as V546C strongly prefer D-glucose over D-galactose as their substrate, as is expressed by their substrate specificity, i.e., the ratio of the specificity constants k_{cat}/K_M for the two substrates (Morley and Kazlauskas, 2005). The ratio of k_{cat}/K_M of D-glucose to that of D-galactose are 180 for the wild-type, while it is 1.05 for V546C/T169G. Therefore, K_M and k_{cat} for both sugar substrates D-glucose and D-galactose are almost identical for this mutational variant, indicating that both sugars are equally good substrates.

Furthermore, the kinetic constants were determined for both BQ (a two electron, H^+ acceptor) and ferricenium ion (Fc^+; a one electron, no H^+ acceptor) with either D-glucose or D-galactose in saturating concentrations (Tables 2 and 3). Here the Val → Cys replacement at position 546 of *Tm*P2Ox showed comparable effects as with the electron donors, it significantly increased the turnover number by a factor of two to six depending on the substrates, yet also the Michaelis constant was increased. Overall, V546C showed improved catalytic efficiencies for all substrate combinations (electron acceptor and donor) investigated. Compared to the wild-type enzyme, variant V546C/T169G had a twofold higher catalytic efficiency with BQ and D-glucose and a nearly ninefold increased k_{cat}/K_M with D-galactose, the latter is due to the 39-fold increased k_{cat}. The positive effect of these mutations on the catalytic activity is even more obvious with the electron acceptor Fc^+ and the sugar D-galactose. Here, the turnover number was increased 49-fold resulting in a catalytic efficiency that was increased more than 11-fold compared to the wild-type enzyme (Table 3). Variant V546C/E542K showed higher k_{cat} values for both electron acceptors in combination with both sugars, but overall its catalytic performance was not competitive with the wild-type enzyme because of worsened substrate binding. Mutant V546C/T169G/L537W showed higher k_{cat} values with D-galactose and either of the two electron acceptors, whereas for the reaction with D-glucose k_{cat} was reduced. Regarding the catalytic efficiency, increased values for the reactions with D-galactose are noteworthy, but are not comparable to the improvements of variant V546C/T169G.

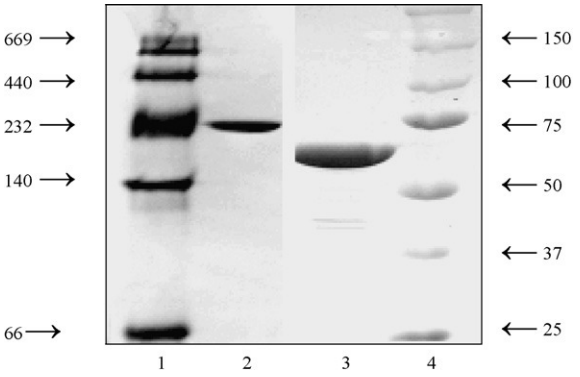

Fig. 1. Native PAGE (lanes 1 and 2) and SDS-PAGE (lanes 3 and 4) analysis of *Trametes multicolor* P2Ox mutational variant V546C. Lanes 1 and 4, molecular mass marker proteins; lanes 2 and 3, V546C after purification by IMAC.

3.3. Enzyme production and substrate conversion experiments

Batch cultivations of *E. coli* BL21DE3 carrying either the wild-type or the mutated enzyme V546C/T169G, which shows comparable kinetic properties for both D-glucose and D-galactose, were carried out to obtain sufficient amounts of protein for the following conversion experiments. Biomass was harvested at an OD_{600} of 8.0, when the volumetric activity of wild-type P2Ox with D-glucose reached a maximum level of 240 U L^{-1}, corresponding to approximately 30 mg protein per liter. Then the purified enzymes were used for batch conversion experiments using an equimolar mixture of D-glucose and D-galactose as substrate, and either oxygen or BQ as electron acceptor (Fig. 2). Six mg of the purified wild-type enzyme, corresponding to 45 D-glucose units under standard assay conditions, and 60 mg of purified mutant protein, corresponding to 5 D-glucose units, were used for each batch conversion. When

Table 1
Kinetic properties of wild-type P2Ox from *Trametes multicolor* and mutational variants with either D-glucose or D-galactose as substrate and O_2 (air) as electron acceptor.

Enzyme	D-Glucose			D-Galactose		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
wtP2Ox	0.94 ± 0.04	48.1 ± 0.5	51.2	8.79 ± 0.54	2.51 ± 0.05	0.29
V546C	3.06 ± 0.14	88.6 ± 1.3	29.0	46.2 ± 3.2	6.57 ± 0.15	0.14
E542K[a]	0.52 ± 0.02	35.9 ± 0.3	68.9	3.87 ± 0.30	2.59 ± 0.04	0.67
T169G[b]	0.69 ± 0.11	0.26 ± 0.01	0.38	2.48 ± 0.94	0.27 ± 0.02	0.11
L537W[a]	0.75 ± 0.02	59.0 ± 0.5	78.8	9.41 ± 0.40	2.90 ± 0.03	0.31
V546C/E542K	1.52 ± 0.13	82.2 ± 1.9	53.9	24.6 ± 1.7	6.13 ± 0.11	0.25
V546C/T169G	0.44 ± 0.02	0.43 ± 0.01	0.99	0.40 ± 0.09	0.38 ± 0.01	0.94
V546C/T169G/L537W	0.49 ± 0.11	0.27 ± 0.01	0.55	0.86 ± 0.28	0.34 ± 0.02	0.40

[a] Spadiut et al. (2008a).
[b] Spadiut et al. (2008b).

Table 2
Kinetic properties of wild-type P2Ox from *Trametes multicolor* and mutational variants with 1,4-benzoquinone as electron acceptor and either D-glucose or D-galactose as saturating substrate.

Enzyme	D-Glucose			D-Galactose		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
wtP2Ox	0.24 ± 0.03	152 ± 6.0	632	0.060 ± 0.003	4.79 ± 0.06	74.2
V546C	0.37 ± 0.08	520 ± 49	1410	0.19 ± 0.05	15.1 ± 1.1	78.9
E542K[a]	0.182 ± 0.025	189 ± 9.2	1040	0.049 ± 0.009	5.52 ± 0.22	113
T169G[b]	0.026 ± 0.011	6.50 ± 0.62	252	0.101 ± 0.046	21.7 ± 3.6	219
L537W[a]	0.134 ± 0.013	205 ± 6	1580	0.036 ± 0.004	5.37 ± 0.13	150
V546C/E542K	0.37 ± 0.07	664 ± 54	1800	1.52 ± 0.13	82.2 ± 1.9	53.9
V546C/T169G	0.043 ± 0.020	42.1 ± 4.9	1040	0.28 ± 0.11	189 ± 54	663
V546C/T169G/L537W	0.072 ± 0.048	24.1 ± 6.9	345	1.18 ± 0.91	103 ± 50.3	87.1

[a] Spadiut et al. (2008a).
[b] Spadiut et al. (2008b).

oxygen was the electron acceptor the wild-type enzyme clearly preferred D-glucose over D-galactose as substrate. Only when D-glucose was completely consumed, D-galactose was converted at a very low rate of 0.02 g L^{-1} h^{-1} during the batch conversion. This conversion rate was 100-fold lower than the value of 2.0 g L^{-1} h^{-1} calculated for D-glucose, again stressing the considerable discrimination of D-glucose oxidation versus that of D-galactose. In contrast, variant V546C/T169G showed identical conversion rates of 0.2 g L^{-1} h^{-1} for both D-glucose and D-galactose under the reaction conditions. The mutant did not prefer any of the sugars as its substrate but oxidized both of them concomitantly in equal rates as was also predicted by the kinetic constants determined.

When BQ was the electron acceptor the catalytic activity of both enzymes with either D-glucose or D-galactose was increased two- to threefold compare to oxygen (air saturation), resulting in significantly faster turnover of the sugars. Again, the wild-type enzyme reached a more than 100-fold higher conversion rate for D-glucose (6.65 g L^{-1} h^{-1}) than for D-galactose (0.047 g L^{-1} h^{-1}), whereas the conversion rates for the sugars were identical (0.54 g L^{-1} h^{-1}) when the mutated variant was used.

4. Discussion

During the last years, both rational design and directed evolution have emerged as powerful methods for engineering, redesigning and improving both functions and properties of enzymes (Penning and Jez, 2001; Williams et al., 2004; Toscano et al., 2007; Jäckel et al., 2008). Analysis of enzyme engineering results indicates that the majority of mutations that affect beneficially enzyme properties such as substrate selectivity or catalytic promiscuity are located in or close to the active site, and in particular near residues that are involved in catalysis or substrate binding. For other properties such as stability or activity, both close and distant mutations seem similarly effective in engineering enzymes (Chica et al., 2005; Morley and Kazlauskas, 2005). In order to improve the enzyme pyranose oxidase from *T. multicolor* with respect to its substrate promiscuity and selectivity (i.e., increased activity with a broader range of electron acceptors and increased activity with its poor substrate D-galactose), we used a semi-rational approach, saturation mutagenesis of active-site residues involved in binding of the carbohydrate substrate of *Tm*P2Ox and subsequent screening in 96-well

Table 3
Kinetic properties of wild-type P2Ox from *Trametes multicolor* and mutational variants with the ferricenium ion Fc^+ as electron acceptor and either D-glucose or D-galactose as saturating substrate.

Enzyme	D-Glucose			D-Galactose		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
wtP2Ox	0.25 ± 0.10	151 ± 35	592	0.070 ± 0.008	5.37 ± 0.22	77.0
V546C	0.93 ± 0.14	866 ± 94.3	933	0.13 ± 0.02	11.95 ± 0.76	93.2
E542K[a]	0.290 ± 0.096	54.4 ± 9.2	187	0.068 ± 0.014	1.44 ± 0.18	21.2
T169G[b]	0.086 ± 0.07	25.9 ± 1.2	302	0.075 ± 0.032	49.4 ± 8.73	660
L537W[a]	0.253 ± 0.093	334 ± 61	1320	0.063 ± 0.017	8.18 ± 0.91	130
V546C/E542K	1.06 ± 0.18	246 ± 32	233	0.15 ± 0.03	5.54 ± 0.49	37.9
V546C/T169G	0.50 ± 0.31	221 ± 98	442	0.31 ± 0.21	261 ± 119.3	854
V546C/T169G/L537W	0.092 ± 0.057	56.4 ± 14.9	619	0.22 ± 0.06	51.9 ± 6.7	239

[a] Spadiut et al. (2008a).
[b] Spadiut et al. (2008b).

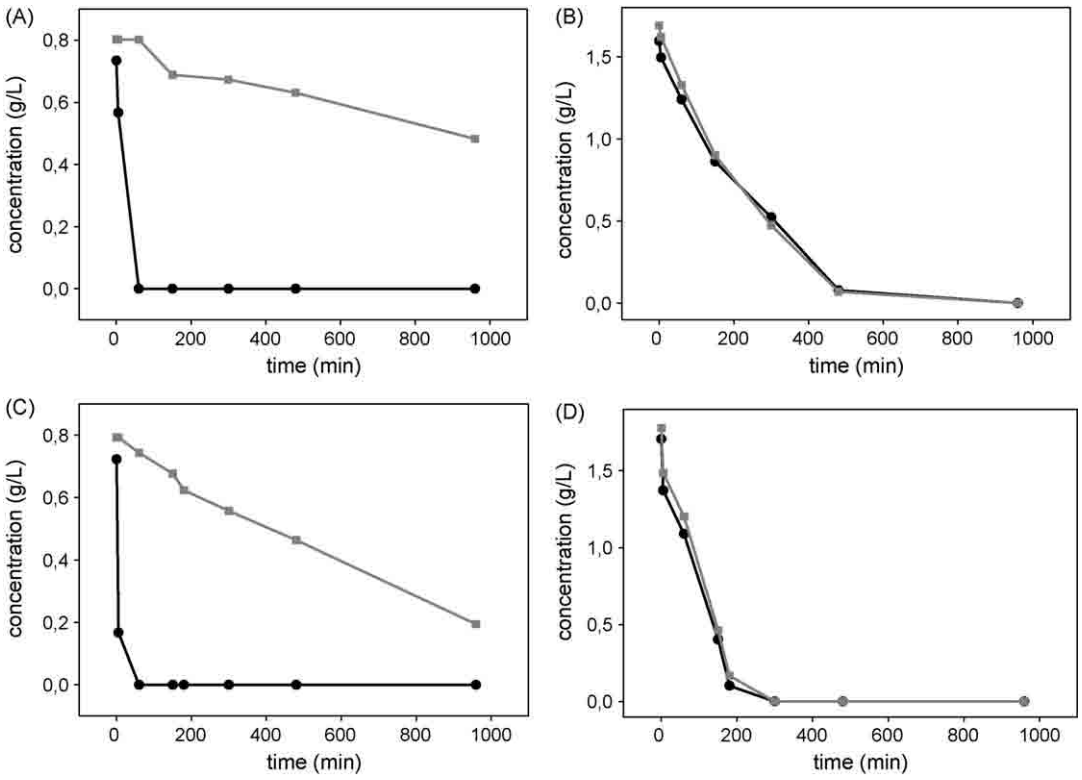

Fig. 2. Batch conversion experiments of an equimolar mixture of the sugars D-glucose and D-galactose and either oxygen or 1,4-benzoquinone as electron acceptor, using wild-type *Tm*P2Ox or the variant V546C/T169G as biocatalyst. (A) wtP2Ox and oxygen; (B) V546C/T169G and oxygen; (C) wtP2Ox and BQ; (D) V546C/T169G and BQ. Symbols: (●) D-glucose; (■) D-galactose.

plates. While the structures of P2Ox with its bound slow substrate 2-fluoro-2-deoxy-D-glucose (Kujawa et al., 2006) and its product 2-keto-D-glucose (Bannwarth et al., 2006) are known, the structure of P2Ox liganded with an electron acceptor such as benzoquinone or a ferrocene is not known to date. In accordance with structural models of glucose oxidase, however, one can assume that at least the less bulky part of these molecules also enter the active site of P2Ox and bind in direct vicinity of the isoalloxazine ring and hence interact with at least some of the active-site residues (Alvarez-Icaza et al., 1995; Wohlfahrt et al., 2004; Leskovac et al., 2005). When screening the libraries of *Tm*P2Ox variants, which were obtained after saturation mutagenesis at position Val546, we found several mutants that reacted faster with D-galactose in the microtiter-plate-based screening assay than does the wild-type. Val546 is positioned at the active site, interacting with the C-1 hydroxyl group of the bound sugar substrate by forming a hydrogen bond through its carbonyl oxygen. In our study the replacement of this small hydrophobic residue, Val, by Cys positively affected the turnover rates for both the sugar substrates and the alternative electron acceptors investigated. The clear reason for this effect is, however, not known to date and waits for further investigation using rapid-kinetics; it is possible that this mutation increases the rate of the flavin reduction by sugar substrates. However, the introduction of Cys also lowered the ability of *Tm*P2Ox to bind these substrates, as indicated by elevated K_M values. Hence, the catalytic efficiency k_{cat}/K_M of V546C for the sugar substrates was lower than that of the wild-type P2Ox.

Recombinations of beneficial mutations are often used to improve enzymes in addition to saturation mutagenesis studies (Chica et al., 2005). Therefore, the additional mutations T169G, E542K and L537W, which had shown positive effects on the catalytic properties of *Tm*P2Ox in previous studies (Spadiut et al., 2008a,b), were introduced into V546C for further improvements of the catalytic efficiency. Some of the properties of these new *Tm*P2Ox variants make them highly interesting for various biotechnological applications. As mentioned above, enzymes can transfer electrons from their prosthetic group to an electrode in MET by using so-called mediators, rather small molecules such as organic complexes of iron, osmium or ruthenium as well as benzoquinones, in bioelectrochemistry. In biofuel cells, the current output depends directly on the actual turnover rate of the enzyme, and consequently an enzyme with an increased catalytic constant will also boost the power output of the biofuel cell, which is especially attractive for implantable miniature biofuel cells (Heller, 2004). In this respect, some of the studied variants exhibit promising catalytic properties. For example, V546C shows increased k_{cat} values for all the substrates and substrate couples tested (D-glucose, D-galactose, BQ, Fc^+).

A second area of applications of P2Ox has been proposed in carbohydrate conversions (Giffhorn, 2000), and for this purpose variant V546C/T169G is of interest. Wild-type *Tm*P2Ox clearly prefers D-glucose ($k_{cat}/K_M = 51{,}200\ M^{-1}\ s^{-1}$) over D-galactose ($k_{cat}/K_M = 2900\ M^{-1}\ s^{-1}$), and therefore the substrate selectivity of wild-type *Tm*P2Ox for these two substrates can be calculated as 180. This ratio changes to 1.05 for V546C/T169G, due to an increase in k_{cat}/K_M for D-galactose and a decrease in k_{cat}/K_M for D-glucose. This significant reduction of the selectivity, however, comes at a cost in k_{cat}, which is reduced for both sugar substrates. Thr169 occupies an important position in the *Tm*P2Ox structure. During the oxidative half-reaction when electron-acceptor substrates are bound, the dynamic substrate loop is fully closed (Hallberg et al., 2004) and Thr169 forms a hydrogen bond with the flavin N5 atom. During the reductive half-reaction the substrate, the loop swings out of the active site to accommodate binding of the sugar substrate. In this conformation, the Thr169 side chain adopts a different rotamer and discards of its hydrogen bond to the flavin cofactor (Kujawa et al., 2006). In a recent comparison of

a number of different flavoenzymes, it was noted that a recurrent feature in most of these proteins is a hydrogen bond between N5 of their flavin prosthetic group and a hydrogen-bond donor, typically a backbone or side-chain nitrogen (Fraaije and Mattevi, 2000). This H-bond is expected to increase the oxidative power of the cofactor, and hence the k_{cat}. Therefore, the significant lower k_{cat} value of the double mutant V546C/T169G (Table 1) when compared to those of the wild-type or V546C is likely to be due to the loss of the H-bond of Thr169 and the flavin N5. A removal of this H-bond in P2Ox also results in an almost complete loss of activity (decrease in k_{cat} while K_M is hardly affected) as was previously shown for the *Tm*P2Ox T169A variant (Spadiut et al., 2008b). D-Galactose differs from D-glucose by having the C-4 hydroxyl group in axial position rather than equatorial. As described elsewhere, we presume that the axial C-4 hydroxyl group of D-galactose appears sterically hindered by the side chain of Thr169, thus providing a tentative explanation for the lower turnover and the relatively high K_M of this monosaccharide by P2Ox (Spadiut et al., 2008b). From the modeled structure of D-glucose in the P2Ox active site (Kujawa et al., 2006), it can be seen that the side chain of Thr169 is in close vicinity but does not create any steric hindrance with the hydroxyl group (equatorial) of D-glucose at the C-4 position. It can be envisaged that when the C-4 hydroxyl group is in the axial position as in D-galactose, the side chain of Thr169 may not accommodate the C-4 axial hydroxyl group of D-galactose well. Therefore, the replacement of Thr by Gly creates additional space in the active site and may offer more flexibility for the sugar binding mode, especially for the axial C-4 hydroxy group of D-galactose, resulting in comparable K_M values for D-galactose and D-glucose. Combining T169G with the V546C mutation results in an additional drastic decrease of K_M to 0.40 mM for D-galactose, which is more than 100-fold lower than for the single V546C variant. Apparently, the T169G mutation alleviates some of the unfavorable interactions that cause the increase in K_M for carbohydrate substrates observed for the V546C variant. In contrast to the wild-type enzyme, V546C/T169G catalyzes the concomitant oxidation of both sugars equally well, as was confirmed in small-scale bioconversion experiments.

In conclusion, the semi-rational approach selected for the engineering of P2Ox with respect to its substrate specificity and promiscuity proved very successful. By combining the V546C variant with other mutations, some of which are also located in the active site, we obtained a set of biocatalysts that show clearly improved catalytic properties compared to the wild-type. These variants can be used for example in carbohydrate biotransformations or as the anodic bio-component in biofuel cells. This latter application is currently studied in our laboratories.

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Chapter 4

A thermostable triple mutant of pyranose 2-oxidase from *Trametes multicolor* with improved properties for biotechnological applications

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Research Article

A thermostable triple mutant of pyranose 2-oxidase from *Trametes multicolor* with improved properties for biotechnological applications

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In order to increase the thermal stability and the catalytic properties of pyranose oxidase (P2Ox) from *Trametes multicolor* toward its poor substrate D-galactose and the alternative electron acceptor 1,4-benzoquinone (1,4-BQ), we designed the triple-mutant T169G/E542K/V546C. Whereas the wild-type enzyme clearly favors D-glucose as its substrate over D-galactose [substrate selectivity $(k_{cat}/K_M)_{Glc}/(k_{cat}/K_M)_{Gal}$ = 172], the variant oxidizes both sugars equally well [$(k_{cat}/K_M)_{Glc}/(k_{cat}/K_M)_{Gal}$ = 0.69], which is of interest for food biotechnology. Furthermore, the variant showed lower K_M values and approximately ten-fold higher k_{cat} values for 1,4-BQ when D-galactose was used as the saturating sugar substrate, which makes this enzyme particularly attractive for use in biofuel cells and enzyme-based biosensors. In addition to the altered substrate specificity and reactivity, this mutant also shows significantly improved thermal stability. The half life time at 60°C was approximately 10 h, compared to 7.6 min for the wild-type enzyme. We performed successfully small-scale bioreactor pilot conversion experiments of D-glucose/D-galactose mixtures at both 30 and 50°C, showing the usefulness of this P2Ox variant in biocatalysis as well as the enhanced thermal stability of the enzyme. Moreover, we determined the crystal structure of the mutant in its unligated form at 1.55 Å resolution. Modeling D-galactose in position for oxidation at C2 into the mutant active site shows that substituting Thr for Gly at position 169 favorably accommodates the axial C4 hydroxyl group that would otherwise clash with Thr169 in the wild-type.

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1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10), a flavoprotein found widespread in wood degrading basidiomycetes, catalyzes the oxidation of different aldopyranoses at C2 to the corresponding 2-ketoaldoses, producing H_2O_2 as a by-product [1–3]. It is a homotetrameric enzyme, typically of a molecular mass of ~270 kDa with each of the four 68 kDa subunits carrying one flavin adenine dinucleotide (FAD) covalently bound [4]. The crystal structure of

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Abbreviations: 1,4-BQ, 1,4-benzoquinone; **ABTS,** 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid; **CV,** column volumes; **IMAC,** immobilized metal affinity chromatography; **P2Ox,** pyranose oxidase

P2Ox from *T. multicolor* was determined at 1.8 Å resolution [5]. An intriguing structural feature of P2Ox is its large internal cavity, from which the active sites are accessible. Four solvent channels lead from the surface of the polypeptide into this cavity through which substrates must enter before accessing the narrow active-site channels of P2Ox. The monosaccharide D-glucose is its preferred substrate and it shows high activities with *e.g.*, D-xylose and L-sorbose, whereas D-galactose is a rather poor substrate with only 5.7% relative activity [6]. Oxidation of D-galactose at position C2 is interesting from an applied point of view, since 2-keto-D-galactose can be easily reduced at position C1 to yield D-tagatose [7], which is used as a non-cariogenic, low caloric sweetener in food industry. Lactose, which is available in large amounts as a by-product of the cheese and dairy industry, can be hydrolyzed enzymatically, and thus provides an ample supply of D-glucose and D-galactose. For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose, however, is too low, leading to either very long conversion times or disproportionate amounts of required enzyme. Additionally, a conversion at elevated temperatures is desirable, as catalytic activities increase with higher temperatures and also undesired microbiological growth is avoided during conversions at increased temperatures. Besides applications in food industry, P2Ox is becoming attractive for enzymatic biosensors and biofuel cells. Biofuel cells convert sugars into electrical energy by employing oxidoreductases as an anodic biocomponent, and coupling this with a suitable enzyme on the cathode [8]. In mediated electron transfer, certain mediators such as quinones [9] or osmium redox polymers [10] collect the electrons from the prosthetic group of the enzymes and transfer them to a graphite electrode. To improve the performance of biofuel cells, it is crucial to increase both the catalytic activity and the stability of the enzymes applied. Rational protein design to change substrate specificity and reactivity has already been successfully performed for oxidoreductases, hydrolases, and transferases [11], and thus provides an excellent tool for adapting P2Ox to the above-mentioned biotechnological applications. In order to improve P2Ox from *T. multicolor* with respect to its catalytic activity with the poor substrate D-galactose and the alternative electron acceptor 1,4-benzoquinone as well as thermal stability, we combined different mutations, which had previously shown positive effects with respect to the properties of the respective single mutants [12–14]. The mutation E542K, which is located on the surface of the internal cavity, was found to kinetically stabilize the enzyme as well as to improve its catalytic properties to some extent [12]. Furthermore, Thr169 is thought to affect the substrate specificity of P2Ox to some extent. D-Galactose differs from D-glucose by having the C-4 hydroxyl group in an axial position rather than equatorial. As described elsewhere, we presume that the axial C-4 hydroxyl group of D-galactose appears sterically hindered by the side chain of Thr169. The replacement of Thr by Gly creates additional space in the active site so that the sugar substrates can be accommodated more easily, resulting in lowered K_M values for D-galactose [14].

Here, we report on the detailed biochemical and structural characterization of the resulting P2Ox variant T169G/E542K/V546C, and its possible application for the conversion of D-glucose/D-galactose mixtures at elevated temperatures. To examine the details of the structural effects of the amino-acid substitutions, we also determined the crystal structure of T169G/E542K/V546C in the absence of a ligand at 1.55 Å resolution.

2 Material and methods

2.1 Plasmids, microorganism, and media

The construction of the pET21d$^+$/P2Ox vector (pHL2), which expresses the His-tagged P2Ox gene from *T. multicolor* under control of the T7 promoter, has been described previously [15]. Active, recombinant wild-type P2Ox and the mutant T169G/E542K/V546C were expressed in the *E. coli* strain BL21 Star DE3 (Invitrogen; Carlsbad, CA, USA). TB_{amp}-medium (yeast extract 24 g/L, peptone from casein 12 g/L, glycerol 4 mL/L; KH_2PO_4 buffer 1 M, pH 7.5) was used for cultivation and protein expression under appropriate selective conditions (ampicillin was added to 0.1 g/L). All chemicals used were from Sigma (Vienna, Austria) and were of the highest grade available.

2.2 Generation of mutants

The P2Ox gene was mutated by site-directed mutagenesis using PCR and digestion with *Dpn*I [16]. The plasmid pHL2 as template and primers T169G_fwd (5′-gtcgtcgggggcatgtctacgcacctggggatgcgccacacc-3′), T169Grev (5′-ccagtgcgcgcagcagcctccgtacagatgcgtgacc-3′), E542K_V546C_fwd (5′-gaagcctggtctttgccttcaccttggtgg-3′), and E542K_V546C_rev (5′-aagaccaggcttcatgaattgcgggagg-3′) were used for mutagenic PCRs. The triple mutant T169G/E542K/V546C was generated by sequential mutagenic PCR steps. The PCR reaction mix contained 2.5 U *Pfu* DNA polymerase (Fermentas, Germany),

100 ng of plasmid DNA, 5 pmol of each primer, 10 μM of each dNTP and 1 × PCR buffer (Fermentas) in a total volume of 50 μL. The following conditions were used for mutagenic PCRs: 95°C for 4 min, then 30 cycles of 94 C for 30 s; 56°C for 30 s; 72°C for 16 min, with a final incubation at 72°C for 10 min. After PCR, the methylated template-DNA was degraded by digestion with 10 U of *Dpn*I at 37°C for 3 h. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega, USA). PCR products (5 μL) were transformed into electro-competent *E. coli* BL21 Star DE3 cells. To prove that only the desired mutations had occurred, the P2Ox-encoding gene was sequenced using primers T7promfwd (5′-aatacgactcactataggg-3′) and T7termrev (5′-gctagttattgctcagcgg-3′).

2.3 Protein expression and purification

Cultures (2 L) of *E. coli* BL21 Star DE3 transformants were grown in TB_{amp} in shaken flasks at 37°C and 160 rpm. Protein expression was induced at an OD_{600} of ~0.5 by adding lactose to a final concentration of 0.5% w/v. After incubation at 25°C for further 20 h, approximately 20 g of wet biomass per liter were harvested by centrifugation at 10 000 × g for 20 min and 4°C, and resuspended in KH_2PO_4 buffer (50 mM, pH 6.5) containing the protease inhibitor PMSF (0.1% w/v). After disruption in a continuous homogenizer (APV Systems; Silkeborg, Denmark), the crude cell extract was separated from cell debris by centrifugation (70 400 × g, 4°C) and used for protein purification by immobilized metal affinity chromatography (IMAC) with a 10 mL BioRad Profinity IMAC Ni-Charged Resin (Biorad; Vienna, Austria). The column was equilibrated with 10 column volumes (CV) of buffer (0.05 M KH_2PO_4, pH 6.5, 0.5 M NaCl, 20 mM imidazole). After the protein sample had been applied to the column it was washed with 3 CV of the same buffer, before proteins were eluted with a linear gradient of 10 CV starting buffer from 20 to 1000 mM imidazole. Active fractions were combined and imidazole was removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica, MA, USA) with a 10 kDa cut-off membrane. The concentrated enzymes were washed three times with 10 mL of KH_2PO_4 buffer (50 mM, pH 6.5) and finally diluted in the same buffer to a protein concentration of 5–10 mg/mL.

2.4 Enzyme activity assay

P2Ox activity was measured with the standard chromogenic ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay [6]. A sample of diluted enzyme (10 μL) was added to 980 μL of assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg), and KH_2PO_4 buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM). The absorbance change at 420 nm was recorded at 30°C for 180 s. The molar absorption coefficient at 420 nm (ε_{420}) used was 42.3 mM^{-1}/cm. One unit of P2Ox activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per min, corresponding to the consumption of 1 μmol of O_2 per min, under assay conditions. Protein concentrations were determined by the Bradford assay [17] using the BioRad Protein Assay Kit with BSA as a standard.

2.5 Steady-state kinetic measurements

Kinetic constants were calculated by nonlinear least-squares regression, fitting the data to the Henri–Michaelis–Menten equation. The catalytic constants were measured for the electron donor substrates D-glucose (0.1–50 mM) and D-galactose (0.1–200 mM) using the standard ABTS assay and air saturation. Additionally, catalytic constants were measured for the alternative electron acceptor of this oxidase, 1,4-benzoquinone (1,4-BQ), with 100 mM of either D-glucose or D-galactose as saturating substrate by adding 10 μL of diluted enzyme to 990 μL of assay buffer containing either of the two sugar substrates, KH_2PO_4 buffer (50 mM, pH 6.5), and 1,4-BQ (0.01–2 mM). The absorbance change at 290 nm was recorded at 30°C for 180 s. The chromophore ε_{290} used was 2.24 mM^{-1}/cm. Steady-state kinetics measurements were carried out both at 30 and 50°C.

2.6 Electrophoresis

To check the purity of the purified P2Ox variants, electrophoresis was performed as described by Laemmli *et al.* [18]. SDS–PAGE was performed using a 5% stacking gel and a 10% separating gel. The system used was the PerfectBlue vertical electrophoresis apparatus (Peqlab; Erlangen, Germany). The mass standard used was the Precision Plus Protein Dual Color (BioRad). Gels were stained with Coomassie Brilliant Blue.

2.7 Kinetic stability

Kinetic stability, as expressed by the half life time of thermal inactivation $\tau_{1/2}$, of the purified wild-type enzyme and the triple mutant was determined by incubating the proteins in appropriate dilutions in 50 mM KH_2PO_4 buffer (pH 6.5) at both 60 and 70°C for various time intervals and by subsequent measurements of the residual enzyme activity using the standard ABTS assay and D-glucose as substrate. A thermal cycler (thermocycler T3, Biometra; Göttingen, Germany) and thin-walled PCR tubes were used for all thermostability measurements. Residual activities were plotted *versus* the incubation time and the half life values of thermal inactivation ($\tau_{1/2}$) were calculated using $\tau_{1/2} = \ln 2/k_{in}$, where k_{in} is the observed rate of inactivation.

2.8 Batch conversion experiments

Wild-type P2Ox and the variant T169G/E542K/V546C were compared in terms of their ability to oxidize D-glucose and D-galactose to the corresponding 2-ketoaldoses with oxygen as an electron acceptor at both 30 and 50°C. Four batch conversion experiments (each with a volume of 300 mL) using equimolar amounts of D-glucose and D-galactose were designed in a way to guarantee a complete conversion of D-galactose within 20 h for the mutated enzyme and a complete conversion of D-glucose for the wild-type enzyme within reasonable times. The experiments were performed in parallel in a multifermenter (Infors; Bottmingen, Switzerland); these were initial bioreactor studies proving the applicability of the enzyme variant developed and not aiming at process optimization. The conversion experiments were conducted in 100 mM KH_2PO_4 buffer (pH 6.5) at 400 rpm, a DO_2 concentration of 15%, both at 30 and 50°C. Catalase was used in excess (100 000 U) to decompose H_2O_2. Depending on the catalytic activity of the enzymes with D-galactose, different amounts of an equimolar mixture of the sugar substrates and biocatalyst concentrations were used for the conversion experiments. For batch conversions (total volume of 300 mL) at 30°C, 1600 mU wild-type P2Ox and 400 mU mutated enzyme (measured under standard assay conditions with D-galactose as a substrate) were used. The kinetic characterization of the enzymes at 50°C revealed a specific activity of wild-type P2Ox with D-galactose of 500 mU/mg, of variant T169G/E542K/V546C with 20.4 mU/mg. Conversions at 50°C were conducted with 2400 mU of wild-type and 750 mU of T169G/E542K/V546C, respectively. Samples (2 mL) from the bioconversion experiments were taken periodically, held at 95°C for 5 min to inactivate the enzymes and centrifuged. The supernatants were analyzed for their sugar content using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which was carried out using a Dionex DX-500 system (Dionex; Sunnyvale, CA, USA) and a CarboPac PA-1 column (4×250 mm) at 27°C [19].

2.9 X-ray crystallographic analysis

Crystals of the P2Ox variant T169G/E542K/V546C were produced using the hanging drop vapor diffusion method [20]. Drops were prepared by equal volumes of 5 mg/mL protein and reservoir [10% w/v monomethylether PEG 2000 (mme PEG), 0.1 M Mes (pH 5.2), 50 mM $MgCl_2$, 25% glycerol]. Prior to data collection, the crystals were stabilized using their respective reservoir solution where the PEG concentration had been increased to 50% (stabilizing solution) followed by vitrification in liquid nitrogen. The protein crystallizes in space group $P4_22_12$ with one molecule in the asymmetric unit. Data were collected using synchrotron radiation at MAX-lab (Lund, Sweden), beamline *I*911-3 (λ = 1.0 Å; 100 K). Data were processed using XDS [21]. Phases for the T169G/E542K/V546C structure were obtained by means of Fourier synthesis using the refined model of P2Ox variant H167A as a starting model (PDB code 2IGO [15]). Crystallographic refinement was performed with REFMAC5 [22], including anisotropic scaling, calculated hydrogen scattering from riding hydrogens, and atomic displacement parameter refinement using the translation, libration, screw-rotation (TLS) model. The TLS models were determined using the TLS Motion Determination server (TLSMD; [23]). Corrections of the models were done manually based on σ_A-weighted $2F_o$-F_c and F_o-F_c electron density maps. The R_{free} reflection sets were kept throughout refinement. All model building was performed with the program *O* [24] and *Coot* [25]. Data collection and model refinement statistics are given in Table 1. Structural data are available in the Protein Data Base under the accession number 3FDY.

3 Results

3.1 Generation of mutants

After site-directed mutagenesis was performed as described in the section 2, the presence of the correct and the absence of undesired mutations in the P2Ox gene were confirmed by DNA sequence analysis. Wild-type P2Ox and the variant

Table 1. Data collection and refinement statistics

Data collection[a)]	T169G/E542K/V546C
Cell constants a = b, c (Å); β (°)/ Space group	101.58, 126.85
Resolution range, nominal (Å)	79.3–1.55 (1.60–1.55)
Unique reflections	1,581,896 (96,227)
Multiplicity	16.4 (11.3)
Completeness (%)	99.8 (97.5)
$<I/\sigma I>$	12.8 (2.8)
R_{sym}[b)] (%)	15.9 (88.5)
Refinement	
Resolution range (Å)	60.0–1.55
Completeness, all % (highest bin)	99.8 (98.4)
R_{factor}[c)]/work reflns, all	18.6/93,323
R_{free}/free reflns, all	21.9/2,899
Nonhydrogen atoms	5,078
Mean *B* (Å[b)]) protein all/mc/sc	11.5/10.5/12.6
Mean *B* (Å[b)]) solvent/No. molecules	23.5/475
Rmsd bond lengths (Å), angles (°)	0.022, 1.98
Ramachandran: favored/allowed (%)[d)]	97.7/100

a) The outer shell statistics of the reflections are given in parentheses. Shells were selected as defined in XDS [21] by the user.
b) $R_{sym} = [\Sigma_{hkl} \Sigma_i |I - <I>| / \Sigma_{hkl} \Sigma_i |I|] \times 100\%$.
c) $R_{factor} = \Sigma_{hkl} | |F_o| - |F_c| | / \Sigma_{hkl} |F_o|$.
d) As determined by MolProbity [29].

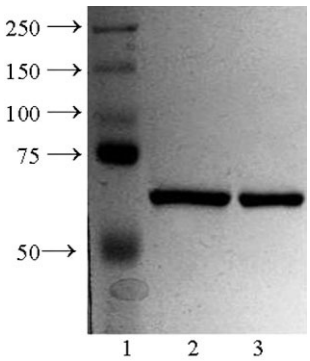

Figure 1. SDS–PAGE analysis of purified wild-type P2Ox from *T. multicolor* and the mutational variant T169G/E542K/V546C. Lane 1, molecular mass marker proteins; lane 2, wild-type P2Ox; lane 3, T169G/E542K/V546C after purification by IMAC.

T169G/E542K/V546C were expressed in *E. coli*, purified to apparent homogeneity and concentrated by ultrafiltration. The purity of the resulting enzyme preparations was confirmed by SDS–PAGE (Fig. 1). Routinely, approximately 20 mg of P2Ox protein were obtained per liter culture medium by this procedure.

3.2 Kinetic characterization

For the determination of the steady-state kinetic constants, initial rates of substrate turnover were recorded over a substrate range of 0.1–50 mM D-glucose and 0.1–200 mM D-galactose for wild-type P2Ox and the mutational variant T169G/E542K/V546C using the standard ABTS assay and oxygen (air saturation), both at 30 and 50°C. Kinetic data are summarized in Table 2. Prior to the determination of the kinetic constants, it was confirmed that the introduction of the amino acid substitutions in the triple mutant did not affect the pH profile of P2Ox activity (data not shown). T169G/E542K/V546C showed an approximately five-fold decreased Michaelis constant K_M for D-galactose compared to the wild-type enzyme when air oxygen was the electron acceptor, whereas K_M for D-glucose was hardly altered. Yet, turnover numbers, k_{cat}, for both sugar substrates were decreased considerably as well, regardless of the temperature used for activity measurements. As a result, the catalytic efficiency k_{cat}/K_M of the mutant was similar to that of the wild-type enzyme with D-galactose but was decreased ≈400-fold with D-glucose, resulting in an enzyme that showed an equal or even higher k_{cat}/K_M value for D-galactose than for D-glucose. In contrast, the wild-type enzyme clearly prefers D-glucose over D-galactose as its sugar substrate as is also expressed by the substrate selectivity values, *i.e.*, the ratio of the catalytic efficiencies k_{cat}/K_M for the two substrates. This value is 172 for the wild-type, while it is 0.69 for T169G/E542K/V546C at 30°C.

Table 2. Steady-state kinetic constants of wild-type P2Ox and variant T169G/E542K/V546C with either D-glucose or D-galactose as substrate and O_2 (air) as electron acceptor at pH 6.5 and at the temperatures indicated

	30°C			50°C		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1}/s)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1}/s)
D-Glucose						
wtP2Ox	0.76 ± 0.05	34.0 ± 0.43	44.7	1.18 ± 0.08	58.9 ± 0.93	50.0
T169G/E542K/V546C	0.64 ± 0.10	0.072 ± 0.003	0.11	1.15 ± 0.12	0.35 ± 0.008	0.30
D-Galactose						
wtP2Ox	7.94 ± 0.39	2.10 ± 0.03	0.26	14.6 ± 1.57	5.51 ± 0.16	0.38
T169G/E542K/V546C	1.66 ± 0.70	0.27 ± 0.02	0.16	2.76 ± 0.34	0.74 ± ± 0.02	0.27

Table 3. Steady-state kinetic constants of wild-type P2Ox and variant T169G/E542K/V546C for 1,4-benzoquinone as electron acceptor with either D-glucose or D-galactose as saturating substrate. Data were determined at pH 6.5 and at the temperatures indicated

	30°C			50°C		
	K_M(mM)	k_{cat}(s^{-1})	k_{cat}/K_M(mM^{-1}/s)	K_M(mM)	k_{cat}(s^{-1})	k_{cat}/K_M(mM^{-1}/s)
D-Glucose						
wtP2Ox	0.40 ± 0.05	349 ± 17.8	863	0.78 ± 0.07	615 ± 32.6	785
T169G/E542K/V546C	0.22 ± 0.10	21.16 ± 3.7	94.5	0.31 ± 0.15	79.3 ± 16.6	258
D-Galactose						
wtP2Ox	0.25 ± 0.03	6.61 ± 0.34	26.3	0.23 ± 0.037	14.6 ± 0.69	62.1
T169G/E542K/V546C	0.093 ± 0.04	59.6 ± 7.59	622	0.19 ± 0.084	171 ± 28.2	911

In addition, the kinetic constants were determined for the alternative electron acceptor 1,4-BQ with either D-glucose or D-galactose in saturating concentrations at 30 and 50°C (Table 3), and it was found that the three amino acid substitutions dramatically affect the catalytic properties. The K_M value of the mutant for 1,4-BQ was reduced two to three times compared to the wild-type enzyme, regardless of the sugar substrate used. While the turnover number with D-glucose as a saturating substrate was reduced significantly, it increased considerably with D-galactose (≈10-fold). Compared to the wild-type enzyme, mutant T169G/E542K/V546C showed a 24- and 15-fold increase in its catalytic efficiency at 30 and 50°C, respectively, for 1,4-BQ and D-galactose as saturating substrate.

3.3 Thermal stability

Kinetic stability (the length of the time in which an enzyme remains active before undergoing irreversible inactivation) of wtP2Ox and of variant T169G/E542K/V546C was determined at 60 and 70°C and a constant pH of 6.5. The inactivation constants k_{in} and the half lives of denaturation $\tau_{1/2}$ were determined (Table 4), and both enzymes showed first-order inactivation kinetics when analyzed in the ln(residual activity) *versus* time plot (Fig. 2). The mutation E542K in combination with T169G and V546C stabilized P2Ox significantly. At 60°C, the half life was increased 76-fold compared to the wild-type enzyme. The effect of the mutations on stability is even more pronounced at 70°C, where $\tau_{1/2}$ was increased 350-fold.

3.4 Enzymatic batch conversion experiments

In order to assess the effects of the selected amino acid substitutions on the biocatalytic performance of P2Ox, batch conversion experiments using equimolar mixtures of D-glucose and D-galactose were performed with oxygen as a electron acceptor (Fig. 3). Reaction conditions were chosen to guarantee reasonable process times in each reactor and were not aimed at process optimization; hence different amounts of enzyme and sugar substrates were used (Table 5). The specific activity of P2Ox when using D-galactose as the substrate and measuring at 30°C was 330 mU/mg for the wild-type and 12.2 mU/mg for variant T169G/E542K/V546C. At 30°C the wild-type enzyme clearly preferred D-glucose compared to D-galactose with a conversion

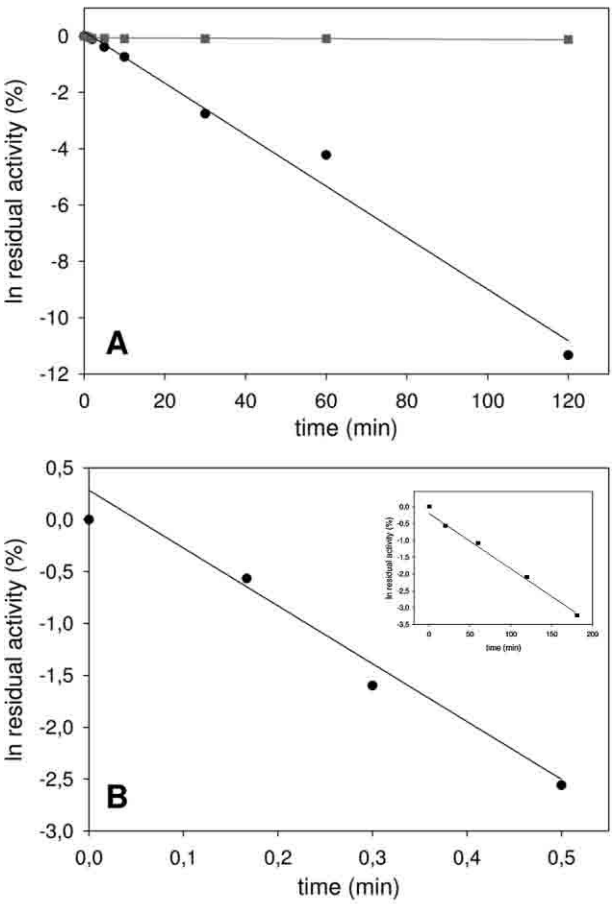

Figure 2. Inactivation kinetics of P2Ox variants from *T. multicolor* at pH 6.5 and various temperatures. A: wtP2Ox and the variant T169G/E542K/V546C at 60°C; B: ●, wild-type P2Ox/variant T169G/E542K/V546C at 70°C, the inset shows the inactivation of the triple mutant. Symbols: ●, wtP2Ox; ■, T169G/E542K/V546C.

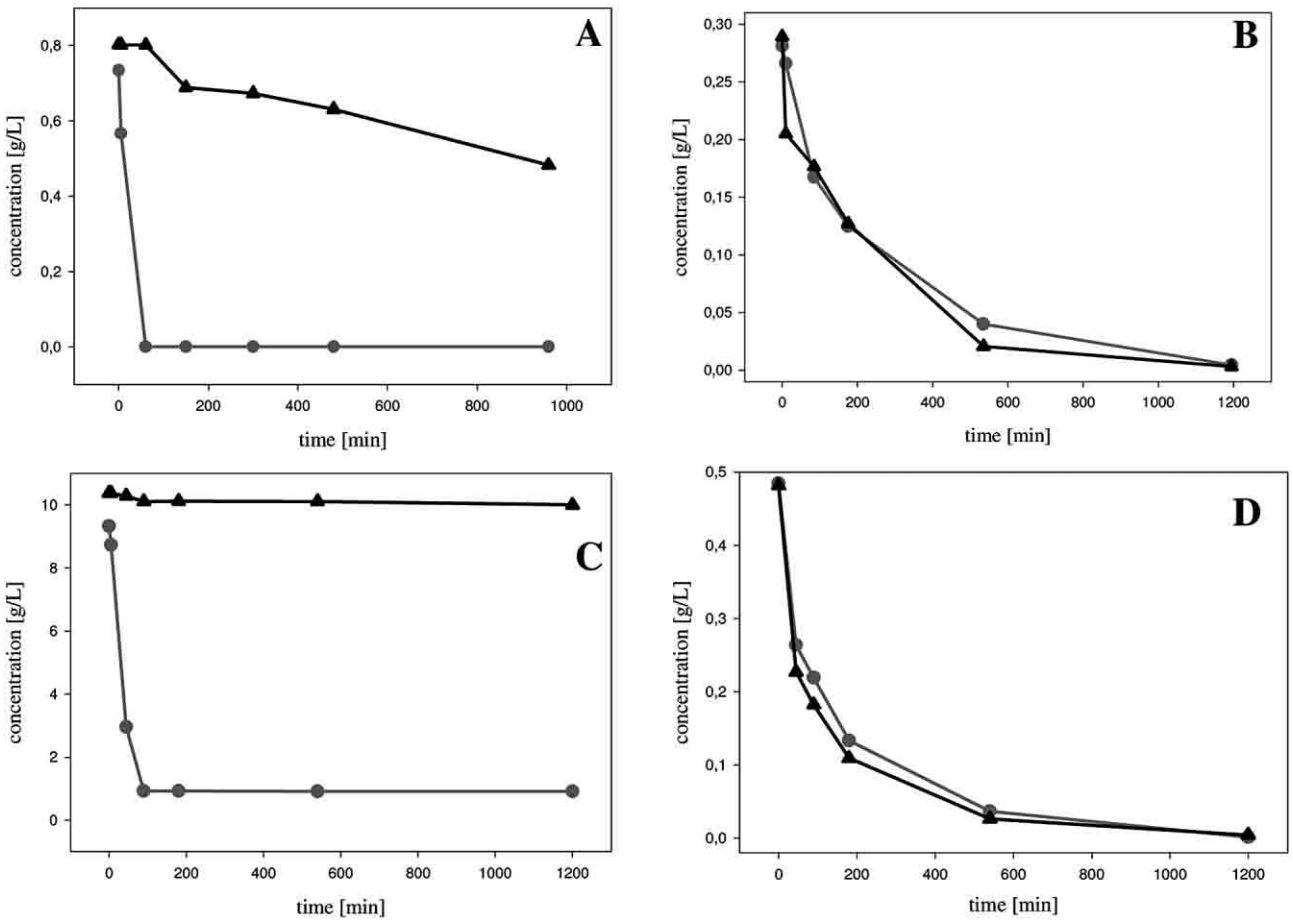

Figure 3. Batch conversion experiments of equimolar mixtures of D-glucose and D-galactose and oxygen as electron acceptor at both 30 and 50°C using wild-type *Tm*P2Ox or the variant T169G/E542K/V546C as a biocatalyst. A, wtP2Ox at 30°C; B, T169G/E542K/V546C at 30°C; C, wtP2Ox at 50°C; D, T169G/E542K/V546C at 50°C. Symbols: ●, D-glucose; ▲, D-galactose.

rate of 2.0 g/L/h. Only when D-glucose was oxidized completely, D-galactose was converted at a very low rate of 0.02 g/L/h. In contrast to that, mutant T169G/E542K/V546C showed similar conversion rates of 0.054 and 0.065 g/L/h for D-glucose and D-galactose. The engineered variant did not prefer either of the sugars as its substrate but converted both of them simultaneously. As is also expressed by the slightly higher k_{cat} for D-galactose, this monosaccharide was converted at a somewhat faster rate than D-glucose. When the conversion experiments were performed at 50°C, the wild-type enzyme oxidized D-glucose initially at a high rate of 8.7 g/L/h for the first phase of the conversion (up to 45 min). Yet, thermal inactivation of the enzyme resulted in a rapid drop of the conversion rate over time and P2Ox activity was completely lost after 90 min, as was evident from residual D-glucose left in the reaction mixture and the complete lack of 2-keto-D-galactose. In contrast, variant T169G/E542K/V546C converted both sugar substrates at an almost equal rate of 0.12 g/L/h resulting in complete conversion of both sugar substrates within 20 h.

Table 4. Kinetic stability of pyranose oxidase from *T. multicolor* at various temperatures. The inactivation constants k_{in} and half life times of inactivation $\tau_{1/2}$ are given for 60 and 70°C

Variant	60°C		70°C	
	k_{in} (min^{-1})	$\tau_{1/2}$ (min)	k_{in} (min^{-1})	$\tau_{1/2}$ (min)
wtP2Ox	-9.15×10^{-2}	7.6	−5.57	0.12
T169G/E542K/V546C	-1.20×10^{-3}	578	$-1.65 \cdot 10^{-2}$	42.0

Table 5. Batch conversion experiments of wild-type pyranose oxidase from *T. multicolor* and the variant T169G/E542K/V546C using equimolar mixtures of D-glucose and D-galactose at 30 and 50°C

Enzyme	Batch A Wild-type	Batch B Variant	Batch C Wild-type	Batch D Variant
Temperature (°C)	30	30	50	50
Enzyme activity applied (mU)	1600	400	2400	750
Initial sugar concentration (g/L)	0.8	0.3	10	0.5
Conversion rate D-glc (g/L/h)	2.0	0.051	8.7/2.7[b)]	0.117
Conversion rate D-gal (g/L/h)	0.02[a)]	0.065	0.00[b)]	0.124

a) D-gal was not converted until D-glc was completely oxidized.
b) During the first 45 min the average conversion rate was high with 8.7 g/L/h, inactivation resulted in a lower average conversion rate of 2.7 g/L/h over the subsequent 45 min period, wild-type enzyme was completely inactived after 90 min.

4 Discussion

Pyranose oxidase is an enzyme of interest for use in biofuel cells and enzyme-based biosensors as well as for applications in food industry. In several previous studies the improvement of P2Ox both in terms of stability and reactivity was reported. The mutation E542K was found to improve both the kinetic and thermodynamic stability of the enzyme as well as its catalytic properties to some extent [12, 26]. Other studies showed the positive effects of the mutations V546C [13] and T169G [14] with respect to kinetic properties, especially for the oxidation of the substrate D-galactose. The replacement of Val by Cys at position 546 in the direct vicinity of the active site of P2Ox resulted in significantly increased turnover rates for both the sugar substrate and the alternative electron acceptor, albeit at the costs of an increased K_M. We determined the crystal structure of the T169G/E542K/V546C mutant at 1.55 Å resolution and performed theoretical modeling of β-D-glucose and β-D-galactose in the active site (Fig. 4). The axial C4 hydroxyl in β-D-galactose cannot be accommodated easily in the active site and clashes with the side chain of Thr169, whereas the β-D-glucose C4 hydroxyl fits well. In the mutant, Gly169 relieves steric hindrance and provides space for the galactose C4 hydroxyl group to give a relative decrease in K_M value. This, at least partly, explains why β-D-galactose is a poor substrate for wild-type P2Ox, and performs relatively better as a substrate for P2Ox T169G/E542K/V546C. By introducing this mutation, we intended to counteract the negative effects on K_M observed for the V546C mutation. By combining these three different mutations, we aimed at creating a thermostable variant of P2Ox, which converts D-galactose and D-glucose concomitantly and at equal rates. This simultaneous conversion of D-glucose and D-galactose is important when *e.g.*, lactose hydrolysates are used as a starting material for the envisaged bioconversion. P2Ox is known to overoxidize its primary reaction product, 2-keto-D-glucose, thus forming 2,3-diketo-D-glucose [27]. Simultaneous conversion of the two sugar substrates will obviously avoid this overoxidation and thus the formation of the undesired by-product. We were further interested in increase in the turnover number for 1,4-benzoquinone, which can be used as an electron mediator in biofuel cells and biosensors, in combination with D-galactose as the saturating substrate. In biofuel cells based on mediated electron transfer, suitable mediators gather electrons from the prosthetic group of an enzyme and transfer them to the electrode. In these applications, the measured current represents the actual turnover rate of the immobilized enzyme, and, consequently, an enzyme with increased turnover rates for the mediator will boost the power output of biofuel cells [8, 10] or improve enzyme electrodes [30].

Kinetic characterization and comparison of variant T169G/E542K/V546C showed that the substrate selectivity was indeed changed significantly for the mutant. Whereas wtP2Ox clearly prefers D-glucose as its substrate, as indicated by a considerably higher k_{cat}/K_M value, T169G/E542K/V546C does not show any clear preference for either sugar substrate as is evident from comparable catalytic efficiencies. This change in substrate selectivity, however, comes at a cost in k_{cat}, which is reduced for the triple mutant for both sugar substrates. The altered sugar selectivity is also obvious when performing small-scale conversion experiments, using equimolar mixtures of D-glucose and D-galactose, as found in lactose hydrolysates, as the starting material.

Here, the variant oxidized both sugars simultaneously, while the wild-type enzyme converted D-galactose only when D-glucose was exhausted from the reaction mixture. Introducing the E542K mutation in the variant also enabled conversions at higher temperatures, which is preferable because

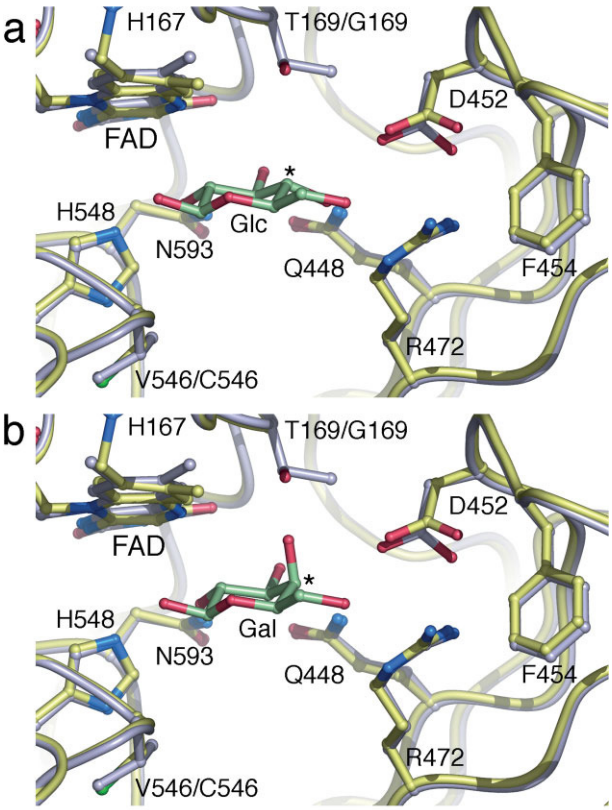

Figure 4. Theoretical models showing the presumed binding of (a) β-D-glucose, and (b) β-D-galactose in the active site of *T. multicolor* P2Ox variant T169G/E542K/V546C based on the crystal structure of P2Ox variant H167A in complex with 2-fluoro-2-deoxy-D-glucose (PDB code 2IGO; [15]). The triple mutant is shown in yellow, and the protein model of 2IGO in light blue (ligand removed). The modeled monosaccharides (glucose and galactose) are shown in light green. For clarity, protein backbone atoms and water molecules have been omitted. The covalent linkage between the FAD 8α methyl group and His167 $N^{\varepsilon 2}$ is indicated. The monosaccharides are oriented for oxidation at C2, and their C4 atoms are marked by an asterisk (*). Modeling was performed using the program O [24], and the picture was made using MacPyMOL v. 0.98 [28].

of higher reaction rates and a decreased possibility of microbial contamination. The triple mutant showed considerably increased thermostability as is evident from the remarkable increase in half-life times, at both 60 and 70°C, which were improved 76-fold and 350-fold, respectively, when compared to the wild-type. Thus, bioconversions based on the thermostable variant will be feasible at temperatures of up to 60°C.

The triple-mutant T169G/E542K/V546C also showed significantly improved catalytic properties for its substrate 1,4-BQ when D-galactose was the saturating sugar. Compared to the wild-type enzyme, the turnover numbers for 1,4-BQ with D-galactose as a saturated substrate at 30 and at 50°C were increased 9-fold and 12-fold, respectively, for the variant. In combination with a lowered K_M value for the electron acceptor the resulting catalytic efficiency was 24 and 15 times higher, respectively, compared to the wild-type enzyme. This property, together with its considerably increased stability, makes this variant particularly promising for applications in biofuel cells. The bioelectrochemical properties of T169G/E542K/V546C are currently studied in our laboratory.

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Chapter 5

Heterologous expression of an *Agaricus meleagris* pyranose dehydrogenase-encoding gene in *Aspergillus spp.* – purification and characterization of the recombinant enzyme

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Abstract

Pyranose dehydrogenase (PDH) is a flavin-dependant sugar oxidoreductase found in Agaricaceae and Lycoperdaceae, basidiomycetes that degrade lignocellulose-rich forest litter, and is catalytically related to the fungal enzymes pyranose 2-oxidase and cellobiose dehydrogenase. It has a broad substrate specificity and displays similar activities with most sugar constituents of lignocellulose, including disaccharides and oligosaccharides, a number of (substituted) quinones and metal ions are suitable electron acceptors rather than molecular oxygen. In contrast to pyranose 2-oxidase and cellobiose dehydrogenase, which oxidize regioselectively at C-2 and C-1, respectively, PDH is capable of oxidation on C-1 to C-4 as well as double oxidations, depending on the nature of the substrate. this makes it a very interesting enzyme for biocatalytic applications, as many of the reaction products are otherwise unaccessible by chemical or enzymatic means. PDH was characterized in detail in a limited number of fungi, and the first encoding genes were isolated only recently. We report here for the first time the heterologous expression of a PDH-encoding gene in the filamentous fungi *Aspergillus nidulans* and *A. niger*.

Introduction

Enzymatic redox transformations of sugars present a great potential for the regioselective synthesis of various aldoketoses. By combining enzymatic and chemical reactions, a number of sugar-derived compounds of applied potential can be constructed (Giffhorn, 2000, Giffhorn et al., 2000). An example is the synthesis of 2-dehydro-D-glucose (2-ketoglucose) and 2-dehydro-D-galactose (2-ketogalactose), which can be chemically or enzymatically reduced at C-1 to obtain pure D-fructose (Leitner et al., 1998, Neidleman et al., 1981) and D-tagatose (Freimund et al., 1996, Haltrich et al., 1998), respectively, with the latter ketose being particularly attractive for food applications (Kim, 2004). The biocatalyst applied for the first step, pyranose 2-oxidase (P2Ox, EC 1.1.3.10), is a ~ 300 kDa homotetrameric flavoprotein with covalently bound FAD. It is common among lignocellulose-degrading basidiomycetes and has been purified from mycelia of several white-rot fungi including *Phanerochaete chrysosporium* (Artolozaga et al., 1997, Volc and Eriksson, 1988), *Pleurotus ostreatus* (Shin et al., 1993), *Trametes multicolor* (Leitner et al., 2001) and *Trametes (Coriolus) versicolor* (Machida and Nakanishi, 1984). P2Ox-encoding nucleotide sequences from a number of fungi are known (among others, *P. chrysosporium,* AY522922; *Peniophora gigantea,* AY370876; and *T. ochracea* (*multicolor*, syn.)*,* AY291124), and the cDNA can be expressed in *E. coli* (Kotik et al., 2004, Pisanelli et al., Vecerek et al., 2004)

The catalytically related pyranose dehydrogenase (PDH, EC 1.1.99.29) was first isolated from *Agaricus bisporus* (Volc et al., 1997). It is a monomeric glycoprotein that is unable to utilize dioxygen as electron acceptor and relies on (substituted) quinones or complexed metal ions as alternatives. The enzyme displays a broad substrate specificity and intriguing variations in regioselectivity; oxidation at C-1, C-2, C-3 or C-4, as well as double oxidation at C-1,2, C-2,3 or C-3,4 were observed (Sedmera et al., 2004, Volc et al., 2000, Sedmera et al., 2006), yielding several reaction products that are inaccessible

by chemical means or other enzymatic transformations. Additionally, PDH shows a significantly higher activity towards galactose compared to P2Ox, and is able to oxidize a number of β-1,4-linked disaccharides and even oligosaccharides, making it a potentially very versatile biocatalyst for sugar transformations (Volc et al., 2003, Volc et al., 2004). PDH from *Agaricus xanthoderma* and *Agaricus meleagris* was recently characterized in detail (Kujawa et al., 2007, Sygmund et al., 2007, Sygmund et al., 2008), including aspects of biocatalytic applications in galactose conversion. Furthermore, the cloning and analysis of the expression of three genes from *A. meleagris* encoding PDH and two other, as yet unknown, proteins with great similarities to PDH, were presented recently (Kittl et al., 2008).

Natural occurrence of P2Ox and PDH has not been observed in the same species to date: among 76 lignocellulose-degrading basidiomycetes, white-rot fungi such as *Phanerochate chrysosporium*, *Phlebia radiata*, and *Trametes* spp. generally produced P2Ox but not PDH. Production of PDH appears to be limited to "litter-decomposing" Agaricaceae and Lycoperdaceae, which do not produce P2Ox (Volc et al., 2001). They mostly grow on lignocellulose-rich forest litter, many are able to mineralize lignin, albeit with less efficiency than white-rot fungi (Steffen et al., 2000). Most species that produce PDH grow slowly in the laboratory, enzyme titers and yields of active protein are generally low, as is the expression level of *pdh*-genes (Kittl et al., 2008). This has long been a significant hindrance for investigations of these enzymes, especially of structural properties or technological applications, where larger enzyme amounts are required. Expression of the encoding genes in *E. coli* was, unlike for P2Ox, not successful (our unpublished information). We here report the successful heterologous expression of a PDH-encoding gene in the filamentous fungi *Aspergillus nidulans* and *A. niger*.

Materials and methods

Chemicals. Chemicals were of the highest purity grade available and were purchased from Sigma (St. Louis, MO) unless otherwise stated. Ferricenium hexafluorophosphate, dimethylaminomethylferrocene and the various substituted quinones were from Aldrich (Steinheim, Germany), bovine serum albumin (BSA; fraction V) was obtained from United States Biochemical Corp. (Cleveland, OH). Superdex 75 HR 10/30 and Source 15 Phe were purchased from Amersham Biosciences (GE Healthcare, Chafont St. Giles, UK), while Fractogel EMD DEAE 650S was from Merck (Darmstadt, Germany). Restriction endonucleases, T4 DNA ligase and DNA modifying enzymes were obtained from Fermentas (St.Leon-Rot, Germany).

Strains, plasmid and growth conditions. *Agaricus meleagris* (strain CCBAS 907) was obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Academy of Science of the Czech Republic Prague. Stock cultures were maintained on glucose-maltose Sabouraud agar plates at 25^{o}C and transferred every 2 months. *A. nidulans* A713 (*yA2, pyroA4, niiA4* (Pateman et al., 1967)) and *A.niger* AB4.1 (a *pyrG* negative mutant of N402; (Van Hartingsveldt et al., 1987)) were used to express *pdh*1. *A. nidulans* A61 (*galD5*, *biA1*, *wA3 (Roberts, 1963)*) was used for the amplification of the nitrite reductase *niiA*. *A. nidulans* strains were obtained from the Fungal Genetic Stock Center (www.fgsc.net) and were maintained on PDA (potato dextrose agar). Strains were cultivated in *Aspergillus* minimal medium (Bennett and Lasure, 1991), supplemented with vitamins if required. Cultivation for enzyme production was done in a total of 2 l minimal medium containing 10 g/l glucose, 0.92 g/l ammonium tartrate and 20 ml/l of 50x salts solution (26 g/l KCl, 26 g/l $MgSO_4 \cdot 7H_2O$, 76 g/l KH_2PO_4) and 50 ml/l trace elements solution (40 mg/l $Na_2B_4O_7 \cdot 10H_2O$, 400 mg/l $CuSO_4 \cdot 5H_2O$, 800 mg/l $Fe_2(SO_4)_3 \cdot 2H_2O$, 800mg/l $Na_2MoO_4 \cdot 2H_2O$, 8 mg/l $ZnSO_4 \cdot 7H_2O$, pH 6.8). After

autoclaving 2 ml of pyridoxin solution (100mg/l) were added. Routinely a total of 10^6 spores were used for inoculation of 200 ml of medium. and the mycelia were grown at 37°C on a rotary shaker with continuous shaking at 180 rpm and harvested by filtration. *E. coli* strain DH5α was used for cloning experiments and plasmid propagation. Plasmid pCR®-Blunt II-TOPO® vector (Invitrogen, Carlsbad, CA) was used for cloning of PCR fragments.

Construction of a *pdh1* expression cassette for *Aspergillus nidulans.* Isolation of fungal genomic DNA was performed by a rapid small scale method (Liu et al., 2000). PCR amplifications, restriction enzyme digestion, agarose gel electrophoresis and other molecular biological techniques were performed according to standard protocols (Sambrook et al., 1989).

A 2.0 kb DNA fragment consisting of the glucoamylase upstream regulatory sequence (plus 76 bp of the glucoamylase signal sequence) was amplified using the primers **pGLA FW**: 5´-TTAGGTACCTCGAGGATTGTCTGAACATT-3´ and **ssGLA RV**: 5´-GCGCTTGGAA ATCACATTTG-3´. The reverse primer contains an overhang for the fusion with the *pdh1* cDNA from *Agaricus meleagris* , a 1.9 kb fragment amplified with the primers **ssGLa/pdh FW**: 5´-CAAATGGATTTCCAAGCGCGCTATCACGTACCACCACCCTGACG-3´and **Term/pdh RV**: 5´-TCTCAGTCACCTCTAGACCCTTAGTTAT AACTCTTTGCTATCAACGC-3´. The downstream region of the glucoamylase gene, a fragment of 600 bp, was amplified with the primers **TermGLA FW**: 5´-GGGTCTAGAGGTGACTGACACCTGGCGGT-3´ and **TermGLA_kpnI_RV: 5´-**AAGGTACCTGACCCGGGAACTGGC-3´. A variant was constructed consisting of the *glaA*-promoter (amplified with the primers **pGLA FW** and **pGLA RV:** 5´-GGCTGAGGTGTAATGATGCTGGGGA-3´), the *pdh1* gene containing its native signal

sequence amplified with the primers **pGLA/pdh FW :**5´-GCATCATTACACCTCAGCCATGCTGCCTCGAGTGACCAAG -3´ and **Term/pdh RV,** and the downstream region of the of the glucoamylase gene. Fragments were amplified in a Biometra Trio thermocycler using a proof-reading thermostable polymerase (Phusion, New England Biolabs, Ipswich, MA) and fused pairwise using terminal primers and fragment mixtures as template. A PCR-product amplified with pGLA FW and **TermGLA_kpnI_RV** was used directly for transformation.

Construction of a *pdh1* expression vector for *Aspergillus niger* The vector (pCP811) was constructed from a modified pBluescript II SK(-) lacking part of the multiple cloning site between the restriction sites *Kpn*I and *Bam*HI. The *glaA* downstream region was amplified using primers **tglaFOR2**: 5´-**GCGGCCGC**GGTGACTGACAC CTGGCGGTAGAC-3´ and **tglaREV2**: 5´-**TCTAGA**GCGACGAATTTGGG GTATCCGCC-3´, and inserted into a pGEX-4T3 vector via the introduced *Not*I and *Xba*I restriction sites (bold print). The *Nco*I restriction site ca. 150 bp downstream of the glucoamylase stop codon was destroyed by digestion with *Nco*I, filling-in the resulting overhangs with the Klenow-fragment of DNA-polymerase I and blunt religation. The selection marker, *A. nidulans pyrG,* was amplified using primers **Ani_pyrG_FOR**: 5´-AGGAAACTCCGCCTTTTGCAGG- 3´ and **Ani_pyrG_REV**: 5´-AAATCGTCGTACCGCATTGATCC-3´. The resulting 1.5 kb fragment was ligated bluntly into the *Sac*I site of the *glaA* downstream region, 200 bp downstream of the stop codon, destroying the restriction site. The fragment was then transferred to the modified pBluescript backbone via the *Not*I and *Xba*I restriction sites. A 1.0 kb fragment from the *glaA* upstream regulatory region was amplified with primers **pglaFOR2**: 5´-**GAGCTC**GTATGCAGAGGAAATCTCCCC-3´ and **pglaREV2**: 5´**GCGGCCGC**CTCGAGCCATGGTGCTGAGGTGTCATGATGCTGGGG-3´ and

ligated into the vector in front of the downstream region using the introduced *Sac*I and *Not*I restriction sites (bold print). *A. meleagris pdh1* was amplified with **AMpdhNcoIfw**: 5´-ACAG**CCATGG**CAATGCTGCCTCGAGTGACC-3´ and **AMpdhHTNotRV**: 5´-ATT**GCGGCCGC**TCAGTGGTGGTGGTGGTGGTGGTTATAACTCTTTGCTATCA A-3´ and ligated into the vector via the *Nco*I and *Not*I sites, The reverse primer also encodes a C-terminal His-tag. The expression cassette can be excised from the plasmid pCP811 with *Sac*I and *Xba*I and be transformed as a linear fragment.

Fungal transformation. Protoplast transformation was performed as described for *A. nidulans* (Tilburn et al., 1983) and for *A. niger* (Punt and Van den Hondel, 1992). As a marker for *A. nidulans* a 5.1 kb fragment containing 1 kb up - and 450 bp downstream region of the nitrate reductase gene *niiA* (M58289) was amplified by *Taq* Polymerase (Promega, Madison, WI) using oligonucleotide primerss NitritredASPfwd (5´-TCAGCCTCGGAGAAACAG-3´) and NitritredASPrev (5´-GCCATGATGA TGACGGTA-3´) from *A. nidulans* A61 genomic DNA and purified by a gel extraction kit (Qiagen). The purified amplicon was used in a cotransformation with the cassettes ssGla and ssPdh to transform *A. nidulans* A713. *A. niger* AB4.1 was transformed with the linearized plasmid pCP811. Strains were selected for growth on minimal medium (with nitrate as sole nitrogen source for *A. nidulans*), single-spore progeny was isolated and transferred at least twice for mitotic stability.

Enzyme production and purification. Recombinant *A. nidulans* strains were grown in 2 l of minimal medium supplied with pyridoxin. Cultures were incubated at 37°C and 180 rpm for 24 h. The mycelia were then filtrated, washed and transferred to fresh medium containing 20 g/l maltose instead of glucose. Cultivation was continued for 12 days. Mycelia were removed by filtration through a sintered glass filter, and proteins in the

filtrate were precipitated by slow addition of $(NH_4)_2SO_4$ (800 g per litre), stirring for 1 h at 4°C and centrifugation (15,000 × *g*; 30 min), and dissolved in a small volume of 20 mM Bis–Tris-HCl buffer, pH 6.0. Purification using a Fractogel EMD DEAE 650S column as well as hydrophobic interaction chromatography on a Source 15 PHE column was done as described in detail in previous works on the purification of PDH from the culture supernatant of *A. xanthoderma* and *A. meleagris* (Kujawa et al., 2007, Sygmund et al., 2008)

Immobilized Metal Affinity Chromatography was done on a 5 ml Hi-Trap column (GE Healthcare) as described previously for a His-tagged recombinant cellobiose dehydrogenase from *Myriococcum thermophilum* (Zamocky et al., 2008). The pH of the culture filtrate was adjusted to 6.5 with 1 M KOH prior to loading the column.

Enzyme assay. Pyranose dehydrogenase activity was determined using a modified procedure by following spectrophotometrically the D-glucose-dependent reduction of the ferricenium ion (Fc^+) to air-stable ferrocene at 300 nm (ε_{300} = 4.3 $mM^{-1}cm^{-1}$) for 3 min at 30°C (Kujawa et al., 2007).

Electrophoresis. Denaturing SDS-polyacrylamide gel electrophoresis (PAGE) was performed with a Hoefer SE 250 (Amersham Biosciences) apparatus using a 3 % stacking and 10 % running standard gel. Isoelectric focusing (IEF) in the range of pH 2.5 to 5 was performed on the Multiphor 2 System (Pharmacia) using dry gels (CleanGelIEF; Amersham-Pharmacia) rehydrated with carrier ampholytes (5.1% Pharmalyte, pH 2.5 to 5, and 1.6% Ampholine, pH 3.5 to 5; Amersham BioSciences). The pI marker protein kit (BioRad, Hercules, CA) was used to determine p*I* values. Proteins were visualized by silver staining; all procedures were done according to the manufacturer's recommendations.

Steady state kinetic measurements. Unless otherwise stated, all measurements were performed at 30°C in phosphate buffer, pH 7.5. For measuring kinetic constants for the various electron donors the routine Fc^+ assay was used (Kujawa et al., 2007). When kinetic constants were determined for the various electron acceptors 25 mM glucose was used as the electron donor, and measurements were performed at the pH optimum of the respective substrate. The pH dependence of PDH activity when using the different electron acceptors was determined with the following buffers: 100 mM citrate (pH 2.5 to 6.0), 100 mM phosphate (pH 6.0 to 8.0), and 100 mM borate (pH 8.0 to 10.0). All kinetic constants were calculated by nonlinear least-squares regression, fitting the observed data to the Henri-Michaelis-Menten equation.

Enzymatic deglycosylation. Enzymatic deglycosylation of PDH with PNGaseF (New England Biolabs) under denaturing conditions was done according to the manufacturer's recommendations.

Results and Discussion

Expression of *pdh1* in *A. nidulans.* The *pdh* gene was expressed in *A. nidulans* under control of the glucoamylase promoter: PDH1-encoding cDNA was fused (by sequential overlap extension PCR) with 1 kb of the upstream regulatory sequences of the *Aspergillus nidulans* glucoamylase (*glaA*) gene as well as the sequences downstream from the glucoamylase stop-codon to generate a linear fragment of approximately 4.5 kb. A second version of this construct was also prepared in which the glucoamylase signal sequence was fused in frame with the sequence encoding the mature PDH1 protein, thereby replacing the native PDH1 signal sequence. Both fragments were co-transformed into *Aspergillus* with a selectable marker (the nitrate reductase gene resulting in the ability to grow on nitrate as the sole nitrogen source). Resulting progeny were transferred

at least twice with intermittent single-spore-isolation to ensure mitotic stability, and the integrationn of the introduced *pdh*-gene into the *glaA* locus of *A. nidulans* was confirmed by colony PCR (results not shown). Progeny from both transformations produced PDH activity.

Production and purification of recombinant *Am*PDH. Selected transformed strains of *A. nidulans* were cultivated as described in Materials and Methods, and the formation of recombinant *Am*PDH was followed. *Am*PDH was secreted and accumulated in the culture medium in all strains. The clone producing the highest PDH activity in small-scale-shake flask cultures was one that contained the native *pdh* signal sequence rather than the *glaA* signal sequence, suggesting that the native signal sequence is recognized properly by the *Aspergillus* secretory system, and that processing of this signal sequence works better than that of the chimeric construct with the *glaA* signal sequence. This recombinant strain was used for further investigation at a larger scale and cultivated on mineral medium using maltose as the carbon source, resulting in volumetric activities of 5.5 U/ml after 7.5 days. The formation of PDH activity (U/ml) during the course of the cultivation is shown in Fig.1, the maximum volumetric activity was reached after 7.5 days (5.5 U/ml). Recombinant PDH from *A. nidulans* was purified 4.1-fold from the culture filtrate using the same purification protocol that was established for purification from the culture fluid of static *Agaricus* cultures (Kujawa et al., 2007, Sygmund et al., 2008) with an overall yield of 4.54% and a specific activity of 55.9 U/mg. The purification procedure yielded a protein preparation that was apparently homogenous, as judged by SDS-PAGE (Fig. 2). The resulting homogenous protein preparation had a specific activity of 56 U/mg, which relates to a yield of recombinant PDH in the culture filtrate of approximately 50 mg/l. It is difficult to correlate this theoretical yield with other results obtained with comparable enzymes, as this is the first report of a fungal flavin-dependant sugar oxidoreductase

expressed in a filamentous fungus. Similar enzymes such as cellobiose dehydrogenase were reported to be expressed in the methylotrophic yeast *Pichia pastoris* to comparable volumetric yields of 2 - 3 U/ml (Stapleton et al., 2004, Zamocky et al., 2008).

Physical properties. The molecular mass of recombinant *Am*PDH from *A. nidulans* was determinated to be 67 kDa by SDS-PAGE (Fig. 2). Isoelectric focusing (using the wild-type enzyme as control) of the recombinant *Am*PDH showed an identical banding pattern in a p*I* range from 4.15 to 4.65 (Fig.3). The recombinant protein contained approximately 7% N-linked carbohydrate moiety as estimated by SDS-PAGE after treatment with PNGase F (Fig. 4). All these properties are essentially identical to those of the wild-type enzyme from *A. meleagris*. This also extended to the glycosylation, which was estimated at approximately 7% of the total molecular weight as judged by the difference in electrophoretic mobility of a native and an enzymatically deglycosylated sample (Fig. 4), suggesting proper glycosylation and a correct processing of the carbohydrat moieties.

Kinetic properties. The kinetic constants of recombinant *Am*PDH were calculated for different sugars and electron acceptors and compared with those of *A. meleagris* PDH. No significant differences were observed between the results concerning the recombinant and the native enzyme (Sygmund et al., 2008).

Expression of *Am*PDH1 in *Aspergillus niger*. The *pdh* gene was also integrated into the genome of *A. niger*, as this fungus is a well-known and important producer of industrial enzymes, both homologously and heterologously. A similar strategy was used, fusing about 1 kb of glucoamylase upstream regulatory sequence with the *pdh* cDNA and the sequence downstream of the glucoamylase coding region. The expression vector pCP811 is based on a modified pBluescript II SK(-) where part of the multiple cloning site (from

*Kpn*I to *Bam*HI) was removed. The final expression vector was assembled stepwise by insertion of the following modules: a 2.8 kb *Not*I-*Xba*I fragment containing the downstream sequences of the *A.niger* glucoamylase gene in which the *Nco*I site 150 bp downstream of the stop codon had been removed, and which contained a 1.5 kb fragment comprising the *A. nidulans pyrG* gene, inserted by blunt-end ligation into the *Sac*I-site 200 bp downstream of the *glaA* stop codon; a 1.0 kb *Sac*I- *Not*I fragment containing the upstream regulatory sequences of the *A. niger* glucoamylase gene and additional *Nco*I and *Xho*I restriction sites; a 2.0 kb *Nco*I- *Not*I fragment representing the coding sequence of the *A. meleagris pdh1* gene. The selectable marker (the *pyrG* gene) was inserted 200 bp downstream of the stop codon, in order to avoid the necessity of co-transformation with a selectable marker on a plasmid, resulting in more laborious screening for prototrophic progeny that also contains the expression construct. The distance between the stop codon and the marker insertion should ensure that the termination and polyadenylation signals of the downstream sequence are kept intact, and leave ample sequence downstream of the marker to facilitate homologous recombination into the *Aspergillus* genome. The vector pCP811 was transformed into the *pyr*-negative recipient strain AB4.1 as a linearized fragment (digestion with *XbaI*), prototrophic progeny was mitotically stabilized by single-spore isolation as described, and the presence of the $_{p}$*glaA-pdh-*$_{t}$*glaA*-construct was verified by PCR. Several strains containing the construct were cultivated as described above for *A. nidulans*, and formation of active PDH in the culture supernatant was monitored. The yield of active PDH was consistently lower by a factor of 10 compared to the highest-producing recombinant *A. nidulans*-strain. Given the established capacity of the *A. niger* secretory machinery and its long history of successful high-yield production of both homologous as well as heterologous industrial enzymes, this is surprising. It is possible that the strain used co-produced particularly high amounts of extracellular proteases that reduced the yield of PDH, or that, in contrast to *A.*

nidulans, the native signal sequence of PDH did not work as efficiently. Additionally, the close proximity of the selectable marker to the *pdh* coding sequence may have interfered with proper transcription termination and polyadenylation, and may thus have negatively influenced mRNA stability and integrity. In this expression construct, a sequence encoding a 6xHis-tag was inserted into the PDH coding sequence before the stop-codon in order to facilitate fast and efficient purification of the resulting recombinant protein (Zamocky et al., 2008). The concentrated and dialyzed culture filtrate was applied to a His-trap column for Immobilized Metal Affinity purification, however, no binding of the tagged protein to the column under standard conditions could be observed, and purification was not successful. It appears possible that the metal-binding sites of the His-tag were occupied by divalent metal cations in the cultivation medium, which does contain trace metals such as magnesium, zinc and cobalt. It is also possible that the C-terminus carrying the His-tag is not freely accessible but rather hidden in the enzyme´s interior, and thus cannot bind to the immobilized metal ligands of the column. The crystal structure of PDH is not known to date, and the tentative model based on the *A. niger* GOx sequence is not reliable enough to judge this matter conclusively. Further investigations towards positioning of an appropriate tag for efficient one-step purification of recombinant PDH are currently under way.

This report of the first heterologous expression of a cDNA encoding a pyranose dehydrogenase ensures sufficient enzyme supply for further studies of this biotechnologically interesting enzyme.

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TABLE 1. Purification of *Am*PDH from *Aspergillus nidulans*

No PDH activity was lost during harvest, removal of the mycelia by filtration, ammonium sulphate precipitation and desalting by dialysis prior to the first chromatographic step.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture filtrate	204	2760	13.5	100	1
EMD DEAE 650 S	19.5	608	31.2	22.2	2.3
Source 15 PHE	2.24	125	55.9	4.54	4.1

TABLE 2. Apparent kinetic constants of recombinant *Am*PDH produced by *Aspergillus nidulans* for several electron donors.

Kinetic data were determined at 30°C with 0.4 mM ferricenium as the electron acceptor

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
D-Glucose	0.86±0.06	48.06±1.5	55,880
D-Galactose	1.02±0.27	46.61±2.67	45,690
D-Xylose	1.03±0.17	41.49±1.76	40,280
L-Arabinose	0,74±0.24	45.72±2.26	61,790
Lactose	113.94±7.48	31.89±8.42	279

TABLE 3. Apparent kinetic constants of recombinant *Am*PDH produced by *Aspergillus nidulans* for several electron acceptors.

Kinetic data were determined at 30°C using 25 mM D-glucose as the substrate.

Electron acceptor	pH optimum[1]	Kinetic constants		
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
1,4-Benzoquinone	2.0	1,030±65	59±1.17	57.28
2-Chloro-1,4-benzoquinone	5.0	615±107	12.86±1.19	20.9
$Fc^+PF_6^-$ [1]	8.5	223±27	322±38	144.4

[1] $Fc^+PF_6^-$, ferricenium hexafluorophosphate.

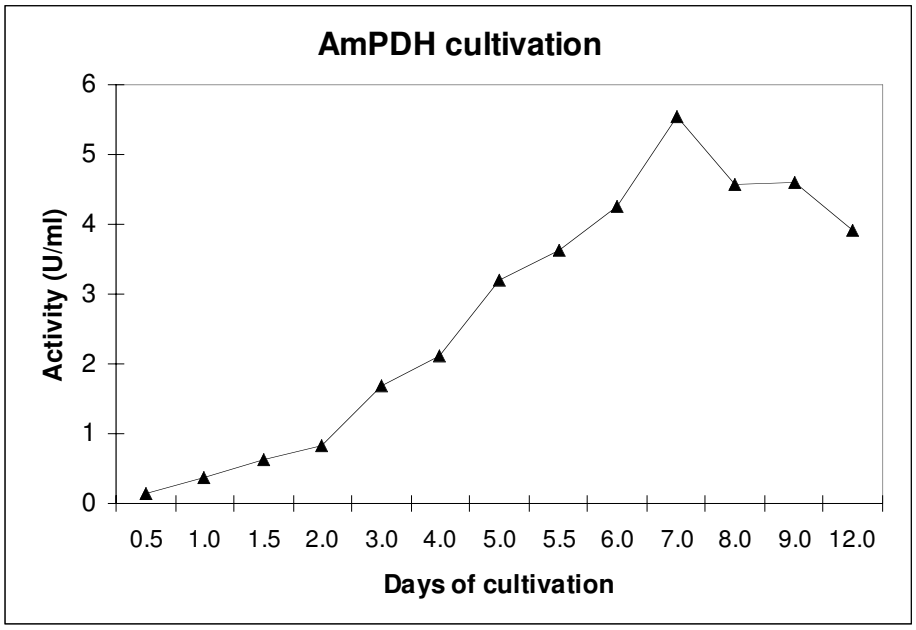

Figure 1. Production of recombinant *Am*PDH from *A.nidulans.*

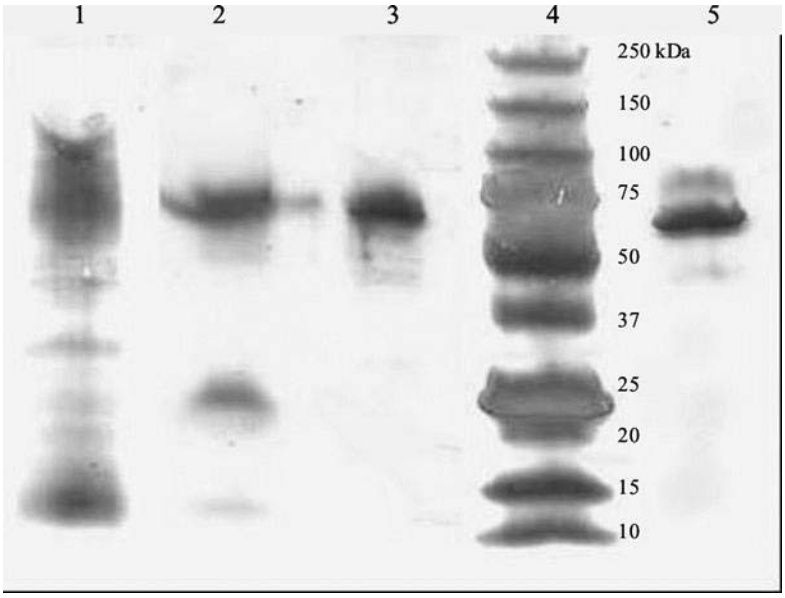

Figure 2. SDS-PAGE analysis of recombinant *Am*PDH from *A. nidulans* and wild-type PDH from *Agaricus meleagris*, protein bands were stained with silver.

Lane 1, crude culture filtrate; lane 2, sample after the AIEX; lane 3, sample after the final purification step; lane 4, molecular-mass marker proteins (Precision Plus Protein Standards, Bio-Rad); lane 5, wild-type PDH from *Agaricus meleagris*

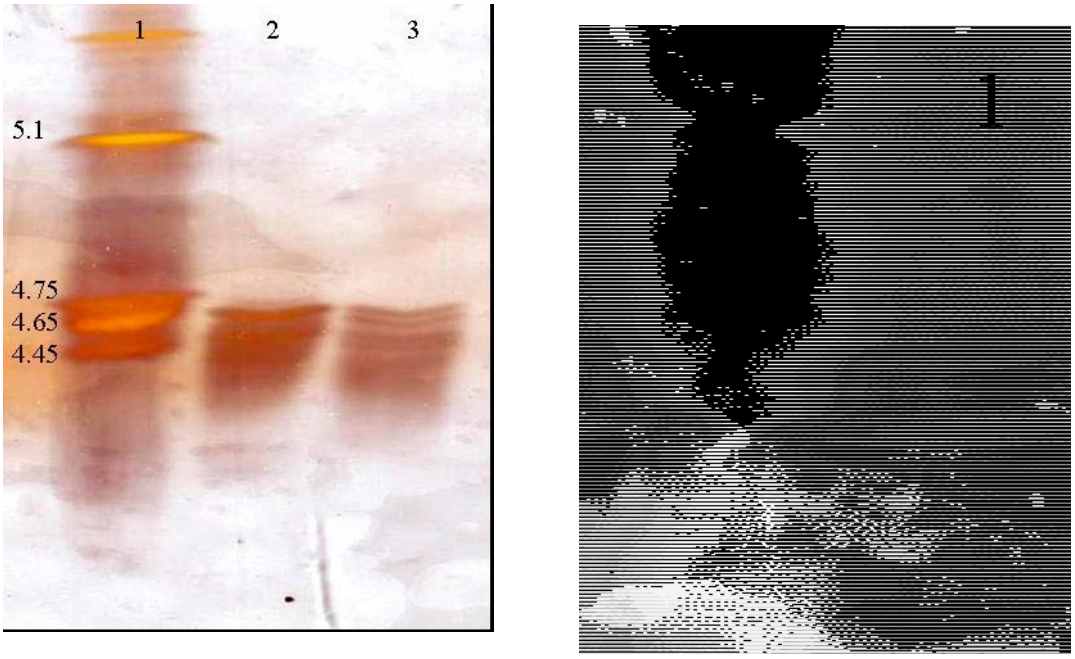

Figure 3. Analytical IEF of recombinant *Am*PDH from *A. nidulans* and wild-type PDH from *A. meleagris* from the final purification step, protein bands were stained with silver. Lane1, Bio-Rad pI standards: Phycocyanin(4.45, 4.65, 4.75); Lactoglobulin B(5.1); lane 2, wild-type PDH; lane 3, recombinant pyranose dehydrogenase from the final purification step.

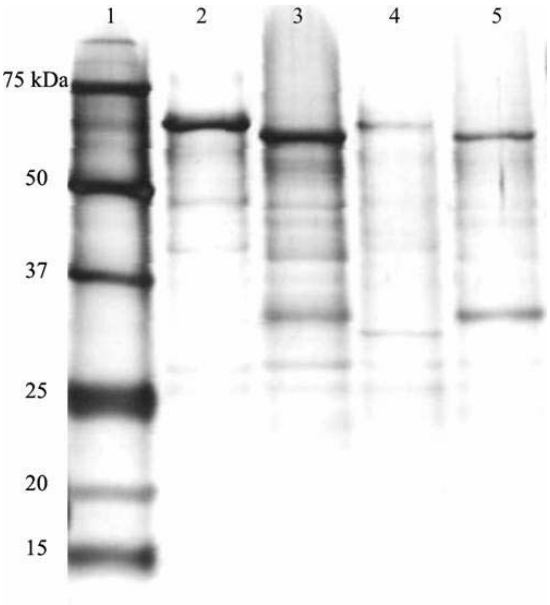

Figure 4. SDS-PAGE of purified and deglycosylated PDH; protein bands were stained with silver.

Lane1, molecular-mass marker proteins (Precision Plus Protein Standards, Bio-Rad); lane 2, wild-type PDH; lane 3, wild-type PDH after deglycosylation with PNGase F; lane 4, recombinant PDH; lane 5, recombinant PDH after deglycosylation with PNGase F.

Figure 5. Effect of pH on the activity of *Am*PDH from *A. nidulans* in the presence of glucose as the electron donor and different electron acceptors:

— (solid line) 1,4-benzoquinone

… (dotted line) ferricenium hexafluorophosphate ($Fc^+PF_6^-$),

--- (dashed line) 2-Chloro-1,4-benzoquinone

□ 100 mM citrate

○ 100 mM phosphate

Δ 100 mM borate