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Research Article ((5483 words))

Probing active-site residues of pyranose 2-oxidase from *Trametes multicolor* by semi-rational protein design

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Abstract

D-Tagatose is a sweetener with low caloric and non-glycemic characteristics. It can be produced by an enzymatic oxidation of D-galactose specifically at C2 followed by chemical hydrogenation. Pyranose 2-Oxidase (P2Ox) from *Trametes multicolor* catalyzes the oxidation of many aldopyranoses to their corresponding 2-keto derivatives. Since D-galactose is not the preferred substrate of P2Ox, semi-rational design was employed to improve the catalytic efficiency with this poor substrate. Saturation mutagenesis was applied on all positions in the active site of the enzyme, resulting in a library of mutants, which were screened for improved activity in a 96-well microtiter plate format. Mutants with higher activity than wild-type P2Ox were chosen for further kinetic investigations. Variant V546C was found to show a 2.5-fold increase of k_{cat} with both D-glucose and D-galactose when oxygen was used as electron acceptor. Because of weak substrate binding, however, k_{cat}/K_M is lower for both sugar substrates compared to wild-type *TmP2Ox*. Furthermore, variants at position T169, i.e., T169S and T169N, showed an improvement of the catalytic characteristics of P2Ox with D-galactose. Batch conversion experiments of D-galactose to 2-keto-D-galactose were performed with wild-type *TmP2O* as well as with variants T169S, T169N, V546C and V546C/T169N to corroborate the kinetic properties determined by Michaelis-Menten kinetics.

1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) is occurring frequently among lignocellulose-degrading basidiomycete fungi [1,2]. It plays a role in the decomposition process of this complex material by providing the co-substrate hydrogen peroxide for lignin-degrading peroxidases [3,4]. The enzyme was first isolated from *Polyporus obtusus* [5] followed by various fungal sources, of which *Trametes multicolor* is the best studied [6].

P2Ox from *Trametes multicolor* (*Trametes ochracea*) [6] is a homotetrameric 270-kDa flavoenzyme. It is a member of the glucose-methanol-choline (GMC) structural family of flavin-adenine-dinucleotide (FAD) dependent oxidoreductases [7,8]. Each subunit contains an FAD molecule covalently bound to H167 in the active centre [9,10]. The subunits are symmetrically arranged around a pore, into which the substrates have to enter to reach the active sites in the direct vicinity of the FAD. The active centre is protected by an active-site loop, which blocks the entrance to the substrate-binding pocket and can undergo dynamic changes enabling the substrate to enter the active site from the internal void [10].

P2Ox catalyzes the C2-oxidation of several aldopyranoses commonly, preferentially D-glucose, to their corresponding 2-keto derivatives [11]. In the reductive half reaction electrons from the oxidized substrate are transferred to the co-factor. Re-oxidation of the FADH₂ follows in the oxidative half reaction by reducing molecular oxygen to hydrogen peroxide. The reaction mechanism is of the type Ping Pong Bi Bi typically found in flavoprotein oxidoreductases [12,13].

P2Ox was the key biocatalyst in the Cetus process, in which pure crystalline D-fructose was produced from D-glucose via the intermediate 2-keto-D-glucose. By using the same process D-galactose can be oxidized to 2-keto-D-galactose and subsequently hydrogenated to D-tagatose [14]. This sugar has interesting properties for the food industry such as low caloric and nonglycemic characteristics. Additionally, D-tagatose can be used as prebiotic food additive. 2-Keto-D-glucose is also the key intermediate of a secondary metabolic pathway leading to the antibiotic corticosterone [15].

Recently, the crystal structure of *T. multicolor* P2Ox (*TmP2Ox*) in complex with one of its slow carbohydrate substrates, 2-fluoro-2-deoxy-D-glucose, was reported [16]. 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. As was shown by the structural studies on *TmP2Ox*, the active-site loop undergoes dynamical changes in the presence of a carbohydrate substrate, and the sugar can dock into the active site. Two amino acids, H548 and N593, are thought to control the catalytic event by supporting the electron transfer from the substrate C2 (or C3) to the N5 of the flavin. According to the hypothetical binding mode for the C2-oxidation [16] (Fig. 1), side chains of D452, an amino acid of the active-site loop, and R472 form hydrogen bonds with the O6 of the substrate, with additional hydrogen bonds being formed by Q448 and V546. T169 has been proposed to play an important role in the re-oxidation of the reduced flavin. The hydroxyl group of threonine forms a hydrogen bond to the N5-O4 of the FAD and supports the electron transfer to the acceptor [16]. The position also seems to partially explain the poor reactivity with D-galactose. D-Galactose differs from D-glucose by an axial rather than equatorial orientation of the C4 hydroxyl group, resulting in a steric clash with the side chain of T169 and unfavorable binding of D-galactose [17].

We chose the individual active-site residues of the *TmP2Ox* interacting with the bound sugar substrate as targets for saturation mutagenesis to probe the effect of mutations on the activity of this enzyme with both D-glucose and D-galactose, in order to better understand structure/function relationships of this enzyme as well as to improve the catalytic efficiency with D-galactose as a substrate. Improved variants were used in a batch conversion experiment to display their characteristics.

2 Material and Methods

Organisms and plasmids: P2Ox from *T. multicolor* was heterologously expressed in *E.coli* BL21* (DE3) (Fermentas, St. Leon-Roth, GER) using the pET21-d(+) expression vector (Novagen; Madison, WI, USA) [16]. The recombinant P2Ox has a C-terminal His₆-tag for one-step purification via immobilized metal affinity chromatography (IMAC).

Saturation mutagenesis: Saturation mutagenesis was done by the overlap extension method (OEM). OEM is based on four primers using two flanking (T7 primer) and two internal primers (Table 1). Both internal primers must contain the mutation of interest and have overlapping nucleotide sequences [19]. Internal primers with one random codon each (NNN or NNS; N=A, U, G, C; S=G, C) were designed, and two fragments were amplified by PCR using one flanking and one internal primer. These two overlapping fragments were then used as template for a third PCR reaction using the flanking primers to amplify the full-length gene. The mutagenized full-length gene was subsequently inserted into the expression vector pET 21-d(+) as described previously [16].

Microtiter-plate cultivation and induction: Depending on the type of the mutagenic codon (NNS or NNN), the size of the library to be screened was determined to be at least 190 (NNN) or 95 (NNS) colonies to achieve a high probability of “success” (appearance of Trp and Met) of either 90% or 95% [20]. Microtiter (96-well) master plates (MP) were prepared with 200 µL LB-Amp (5 g/L yeast extract, 10 g/L peptone from casein, 10 g/L sodium chloride, 100 µg/ml ampicillin) medium and inoculated with single colonies using sterile toothpicks. Six wells, randomly spread over each plate, were inoculated with clones expressing wild-type P2Ox. The MP were shaken at 140 rpm overnight at 25 °C in a closed tray with water-soaked paper towels to prevent desiccation. Screening plates (SP) were prepared with 200 µL of inducing LB-Amp + IPTG (isopropyl-β-D-thiogalactopyranoside) media (0.1 mM IPTG) and inoculated from the MPs using sterile dry replicators, MPs were stored at 4 °C and SPs were shaken at 140 rpm overnight at 25 °C as described above.

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Cell lysis: The screening plates were centrifuged in a 96-well plate centrifuge (Eppendorf) at 3700 rpm for 15 min, and supernatants were discarded by inverting the plates on dry paper towels. Cells were lysed by adding 100 μ L of 0.5-fold BugBuster Protein Extraction Reagent (Novagen) in KH_2PO_4 buffer (pH 7, 25 mM) per well and shaking at room temperature for 1 h. After lysis the plates were centrifuged again as above, and the supernatant was used for further determination.

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Enzyme assay: The activity of P2Ox was determined spectrophotometrically at 420 nm and 30 $^{\circ}\text{C}$ by a coupled chromogenic assay. Enzyme variants were screened for D-galactose and D-glucose activity to compare differences in substrate specificity. Eighty μ L of ABTS reagent (0.7 mg/mL ABTS, 0.035 mg/mL horseradish peroxidase type II [181 U/mg], KH_2PO_4 buffer: 50 mM pH6.5) were pipetted into every well of the 96-well microtiter plate followed by 10 μ L of the enzyme crude extract. The reaction was started with 10 μ L of the sugar substrate (1 M). D-Glucose oxidation was immediately measured spectrophotometrically in a plate reader (Sunrise Remote; Tecan Austria, Grödig) for 180 sec at room temperature. For D-galactose two one-point measurements were done after 15 min and 24 hours. Mutants with a higher activity compared to wild-type P2Ox were cultivated over night in 3 mL of liquid LB-Amp media at 37 $^{\circ}\text{C}$ and 150 rpm. Plasmids were isolated using the GenElute Plasmid Miniprep Kit (Sigma) and sequenced (AGOWA GmbH, Berlin, Germany) to identify the mutations.

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Cultivation: Selected mutant strains were pre-cultivated in four small shaking flasks in 30 mL of LB-Amp medium, transferred after 4 h into baffled shaking flasks containing 250 mL of LB-Amp + 5% Lactose, and grown overnight at 25 $^{\circ}\text{C}$ and 110 rpm. Cells were harvested by centrifugation for 15 min at 4000 rpm (Sorvall, Evolution RC, SLC-6000 Rotor), resuspended in a threefold volume of Buffer A (50 mM KH_2PO_4 , 20 mM imidazole, 0.5 M sodium chloride) and homogenized in a continuous homogenizer (APV Systems, Denmark) adding PMSF as a serine-protease inhibitor. The homogenates were ultra-centrifuged (Beckmann, L-70 ultra

centrifuge) at 35,000 x g for 30 min at 4 °C, and the recombinant protein was purified from the supernatants.

Purification: One step purification was done in an Äkta Purifier System (Amersham Pharmacia Biotech, Uppsala, Sweden) using a 100 mL Ni²⁺ immobilized column with Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) as a chromatographic support. After washing the column with Buffer A, bound protein was eluted with an imidazole gradient using Buffer A and Buffer B (50 mM KH₂PO₄, 0.5 M sodium chloride, 1 M imidazole). Active fractions were pooled and concentrated in an Amicon Ultra Centrifugal Filter Device with a 10-kDa cut-off membrane (Millipore, Billerica, MA). Enzyme preparations were checked for homogeneity by SDS-PAGE using the Precision Plus Protein Dual Color Kit (BioRad, Hercules, CA) as molecular mass standard (Fig. 2).

Characterization: Kinetic constants P2Ox variants for different sugar substrates were determined using the routine ABTS-peroxidase assay and air saturation as described previously [6]. D-glucose and D-galactose were varied over a range of 0.1–50 mM and 0.1–200 mM, respectively, and protein concentrations were determined using a Bradford assay [21] (Table 2).

Conversion experiments: Batch conversions of D-galactose with recombinant wild-type *TmP2Ox* and selected variants (T169N, T169S, V546C, T169N/V546C) were performed in 20 mL flasks at 30 °C in a water bath. The reaction mixture (total volume of 10 mL) contained 250 mM of D-galactose dissolved in KH₂PO₄ buffer (50 mM pH 6.5), 3500 units of bovine liver catalase (Sigma) and 26.7 mg of P2Ox (both for wild-type and engineered enzyme variants). This protein concentration corresponds to 7 U of wild-type *TmP2Ox*. Pure oxygen was supplied continuously to the reaction mixture through a sparger. Samples were taken every 30 min, and the reaction was stopped by heating (2 min at 95 °C). Sugar analysis was done

by HPLC (Dionex) using an Aminex HPX87-P column (Bio-Rad) at 60 °C, a flow rate of 0.5 mL/min and water as eluent.

3 Results and Discussion

Based on structural data of *TmP2Ox* with its bound sugar substrate [10, 16] we decided to perform saturation mutagenesis of amino acid residues directly positioned in the active site and interacting with the sugar substrate. The following positions were chosen for this semi-rational approach: T169, Q448, D452, R472, V546, H548, and N593 (Fig. 1). All of these residues are located in highly conserved regions of the *p2ox* gene, and are supposedly involved in the reduction half-reaction, i.e., sugar oxidation, either by taking part in the electron transfer (H548, N593) or by forming hydrogen bonds to the sugar substrate (Q448, D452, R472, V546).

The *p2ox* gene was mutagenized in three steps by an overlap extension method and ligated into the expression vector. Colonies (total number of 360) were picked for every single position to be screened for substrate specificity by using a new microtiter plate-based activity assay based on the ABTS assay. Depending on the amino acid position mutated and studied, varying numbers of active mutants were found and used for further studies. Variants showing activity with both sugar substrates in the initial screening were selected for DNA sequence analysis to identify the mutation and to exclude undesired mutations in other regions. Variants with higher or comparable activity relative to recombinant wild-type *TmP2Ox* were cultivated, purified to homogeneity determined by SDS-PAGE (Fig. 2) and characterized in terms of kinetic constants by using the standard spectroscopic ABTS assay under standard conditions (Table 3).

Determination of catalytic constants of the active mutants revealed one variant with noticeable properties, V546C. V546 is positioned directly in the active site, interacting with the C1 hydroxyl group of the sugar substrate by forming a hydrogen bond through its carbonyl oxygen [16]. When replacing V546 by a cysteine, k_{cat} values for both D-glucose and D-galactose, were approximately 2.5-fold increased compared to wild-type P2Ox (Table 2).

However, this increase in the turnover number comes at the cost of substrate binding, since the introduction of Cys also lowered the ability of *TmP2Ox* to bind or interact with 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. The high number of active variants at this position (73.9%) found in the screening assay indicates that position 546 does not have to be strictly conserved, which is also obvious from the sequence alignment shown in Figure 3. Several of the naturally occurring *p2ox* genes, e.g., from *Phanerochaete chrysosporium* or *Lyophyllum shimeji*, carry an alanine residue in this position rather than the valine, which is typical for *p2ox* genes of *Trametes* or *Peniophora* species. Two other variants at this position, showing significant activity in the screening, were also selected and further characterized (V546G and V546P). These did not show the increase in turnover number as observed for V546C but again showed elevated K_M values for both sugar substrates. Therefore, position 546 has a strong impact on substrate binding and affinity of the enzyme for its sugar substrates. Interestingly, the V546A variant, naturally occurring in the above-mentioned enzymes, was not identified among the isolated active variants in this saturation mutagenesis study of *TmP2Ox*.

Q448 is another active-site residue that has a large effect on the Michaelis constant and hence on the formation of the enzyme-substrate complex. In this case, however, substrate binding as expressed by the K_M values as well as turnover numbers k_{cat} changed drastically for the worse with both substrates for all three amino acid substitutions studied at this position (Q448N, Q448S, Q448C). Especially the K_M values were increased 40- to 150-fold when compared to the wild-type enzyme.

As mentioned above, the side chain of threonine at position 169 clashes with the axially orientated C4-OH group of D-galactose. This amino acid position had been studied previously by our group using rational protein design, introducing glycine and alanine to create additional space, as well as serine as a conservative substitution that retains the hydroxyl functionality in the side chain [17]. To study whether other, less obvious, amino acid substitutions at this position can have a positive effect, saturation mutagenesis was again

applied, with a subsequent screening for variants with improved properties for D-galactose oxidation. However, the only variant with high activity found in the screening assay, T169S, has already been proposed and investigated in our previous study [17]. Serine is a smaller amino acid than threonine, but also provides the hydroxyl group and thus can form a hydrogen bond to the FAD. Presumably due to reduced steric hindrances in T169S, this mutant showed improved binding of D-glucose (K_M reduced almost twofold compared to the wild-type) and higher turnover rates with D-galactose (increased twofold compared to wtP2Ox). Two other variants, T169G and T169N, showed somewhat lower activity in the screening, yet were selected for subsequent characterization. Especially the T \rightarrow N substitution at position 169 is of interest for the oxidation of D-galactose as was revealed by this characterization, since it results in a significantly reduced K_M for this sugar, while k_{cat} is hardly affected. Apparently, asparagine can provide the hydrogen bond to the N5 of the isoalloxazine ring of the flavin, which is a recurrent and apparently an essential feature in flavoproteins [24]. N5 of the isoalloxazine takes directly part in substrate dehydrogenation, and any interaction involving this atom can affect catalysis. Fraaije and Mattevi even proposed a more subtle effect of this hydrogen bond. Upon reduction, N5 becomes protonated and so the hydrogen-bond interaction might become energetically less favorable in the reduced than in the oxidized state. Therefore, the proximity of a hydrogen-bond donor is generally expected to increase the oxidative power of the cofactor [25]. The relatively high number of active variants found at position 169 (22.2%) indicates that abolishing this H bond does not result in complete loss of enzyme activity, and that several of the variants retain at least a certain level of activity.

Depending on the type of mutagenic codon employed (NNN or NNS) and the number of codons used for an amino acid one can calculate the theoretical frequency of a given amino acid position to stay unchanged in a saturation mutagenesis experiment, i.e., re-insertion of the original amino acid rather than a different one [20]. For example, asparagine is encoded by two codons, therefore 3.8% of the variants should theoretically contain the original amino acid [20]. During the screening for active variants at position N593 only 2.2% of the totally

257 screened mutant enzymes showed activity, indicating a very conserved position where most
258 changes result in total loss of enzyme activity. Only three active mutants of this position were
259 found, one of them was identified as N593R and subsequently characterized. N593 takes
260 part in hydrogen bonding of the hydroxyl groups at C2 and C3 of the sugar substrate [16].
261 Presumably, arginine can provide these H bonds as no changes in the Michaelis constants
262 for both sugars were observed, however, the turnover number was reduced by a factor of
263 approximately 2, indicating a detrimental effect on the catalytic mechanism of a negative
264 charge at this position in the direct vicinity of the isoalloxazine ring.

265 The arginine residue at position 472 on the active site loop has been proposed to be involved
266 in substrate binding through a H-bond with the hydroxyl group at position C6 and thereby in
267 the correct binding of the sugar substrate for C-2 oxidation [16]. Two of the active variants
268 identified in the screening were R472G and R472L. Interestingly, the Michaelis constants for
269 both sugars stay almost unchanged for these two mutants even though they obviously
270 cannot form the hydrogen bond to C6-OH, and k_{cat} was reduced slightly (Table 2). The
271 relatively high number of active variants for this position (18.4%) indicates a lesser
272 importance of this residue with respect to activity.

273 Some of the interesting mutations of P2Ox that were identified in the saturation mutagenesis
274 experiments were joined in double mutants to possibly combine their individual
275 advantageous effects. These were amino acid substitutions at position 546 (V→C, V→G,
276 V→P), which increased k_{cat} for D-galactose but also increased K_{m} considerably, and position
277 169 (T→G, T→N), which lowered K_{m} for D-galactose albeit also decreased k_{cat} to a varying
278 degree. The introduction of glycine or asparagine at position 169 into the variants V546C,
279 V546G or V546P could in fact decrease the K_{m} again to some extent, yet these double
280 mutants were also characterized by low k_{cat} values for both sugars substrates tested, and
281 therefore the catalytic efficiencies of these double mutants are significantly lower than that of
282 wild-type P2Ox. One exception is V546P/T169G, which showed a Michaelis constant almost
283 10-fold lower than the wild-type, and hence a catalytic efficiency comparable to that of

wtP2Ox for D-galactose, while activity with D-glucose is drastically decreased so that its catalytic efficiencies are comparable for both sugar substrates.

Wild-type pyranose oxidase and selected variants (T169S, T169N, V546C, V546C/T169N) were subsequently applied in preliminary biotransformation experiments to compare their performance for the oxidation of 250 mM D-galactose. Experiments were conducted using constant amounts of P2Ox protein (2.7 mg/mL, which for wtP2Ox corresponds to 0.7 U/mL D-galactose-oxidizing activity), sparging with pure oxygen, and an excess of catalase activity to efficiently remove hydrogen peroxide. The initial pH was 6.5, and it was not adjusted during substrate turnover. Results are shown in Figure 4. Best results for the conversion of D-galactose were obtained for variant T169S, which oxidized this sugar significantly faster than wtP2Ox (Figure 4A). In addition, T169S converted D-galactose more completely than wtP2Ox during the reaction period of 8 h, with 16.5% unconverted D-galactose as compared to 31% for wtP2Ox. These results confirm the data obtained in the kinetic characterization of the enzyme in biocatalytic experiments, namely the higher turnover number k_{cat} determined for T169S (5.53 s^{-1} compared to 2.73 s^{-1} for wtP2Ox) together with a comparable Michaelis constant for these two proteins (Table 3). Interestingly, V546C ($k_{\text{cat}} = 6.57 \text{ s}^{-1}$) performed considerably worse than wtP2Ox despite of its higher turnover number (Figure 4A) as judged from slower sugar oxidation and higher levels of residual D-galactose after 8 h (46.5%); this can be attributed to the very unfavourable K_{M} -value of 46.2 mM determined for this sugar.

The conversion of D-galactose was not complete in all of these biocatalytic experiments. The reason for this is most probably inhibition of the enzyme and not inactivation during substrate turnover, since after a reaction time of 8 h approx. 75–90% of the initially applied P2Ox activity was measured using the standard assay (data not shown). 2-Ketosugars are known to be rather instable, especially under alkaline conditions and in the presence of hydrogen peroxide [29], which will be formed in the conversion experiments despite the addition of an excess of catalase activity. For example, 2-keto-D-glucose is known to decompose in the presence of hydrogen peroxide into various acids, including arabinonic and formic acid [30]. This latter compound has been described as an inhibitor of P2Ox with a K_i of 19 mM; other

acids such as acetic acid are even stronger inhibitors with a K_i of 9.2 mM [10]. This formation of acids during the oxidation of D-galactose was evident from the time course of the pH-value, which initially was set at 6.5. At the end of the conversion (8 h) it had dropped to values of 4.5–5.0 in each of the different experiments, and after a prolonged incubation overnight (18 h) it approached values of 4.0, indicating the formation of acids due to the degradation of the primary oxidation product 2-keto-D-galactose. To avoid this decomposition, short reaction times should be aimed at in the conversion of D-galactose by P2Ox, e.g. through the use of increased enzyme loadings.

Conclusion. An analysis of enzyme engineering results indicates that the majority of mutations that beneficially affect 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 [26, 27]. Using this approach of probing active-site residues of P2Ox by saturation mutagenesis we could identify several amino acid substitutions that show improved activity of this biocatalytically interesting enzyme with its poor substrate D-galactose. Further studies and combinations of these mutations will show the appropriateness of a semi-rational approach for the improvement of P2Ox with regard to the conversion of D-galactose [28].

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420 Table 1. Oligonucleotide primers used in this study.

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T7term	5`- gctagtattgctcagcgg -3`
T7prom	5`- aatacgactcactatagggg -3`
T169wobble_fwd	5`- ctacgcactggnnntgcgccacac -3`
T169wobble_rev	5`- gtgtggcgcannnccagtgcgtag -3`
Q448wobble_fwd	5`- ccgtggcacactnnnatccaccgc -3`
Q448wobble_rev	5`- gcggtggatnnnagtgtgccacgg -3`
D452wobble_fwd	5`- cagatccaccgcnnngcttcagttacgg -3`
D452wobble_rev	5`- ccgtaactgaaagcnnngcggtgatctg -3`
R472wobble_fwd	5`- atcgtggactggnnsttcttcggc -3`
R472wobble_rev	5`- gccgaagaaennccagtccacgat -3`
V546wobble_fwd	5`- agcctggtcttnnncttcaccttggtgg -3`
V546wobble_rev	5`- ccaccaaggtgaagnnnaagaccaggct -3`
H548wobble_fwd	5`- ggtctgtccttnnncttggtgtacgca -3`
H548wobble_rev	5`- tgcgtaccaccaagnnnaaggacaagacc -3`
N593wobble_fwd	5`- cgtacggcgcgnnnccgacgctc -3`
N593wobble_rev	5`- gagcgtcggnnncgcccgtac -3`

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Table 2. Kinetic constants of wild-type P2Ox from *Trametes multicolor* and mutational variants for D-glucose and D-galactose as substrates and O₂ (air saturation) as electron acceptor.

variant	substrate	K _M [mM]	k _{cat} [s ⁻¹]	k _{cat} /K _M [mM ⁻¹ s ⁻¹]	k _{cat} /K _M relative to wt-P2O [%]
wild-type	D-glucose	0.698	35.4	50.8	100
	D-galactose	8.09	2.73	0.337	100
T169G	D-glucose	0.691	0.262	0.379	0.746
	D-galactose	2.48	0.273	0.110	32.6
T169N	D-glucose	0.327	2.41	7.30	14.4
	D-galactose	3.56	1.96	0.551	164
T169S	D-glucose	0.394	21.8	55.3	109
	D-galactose	12.9	5.53	0.429	127
Q448N	D-glucose	28.0	4.73	0.169	0.333
	D-galactose	940	0.433	0.000461	0.137
Q448S	D-glucose	30.0	5.42	0.181	0.356
	D-galactose	750	1.53	0.00204	0.605
Q448C	D-glucose	100	1.51	0.0151	0.0297
	D-galactose	1260	0.622	0.000494	0.147
R472L	D-glucose	0.886	30.9	34.9	68.7
	D-galactose	7.21	1.69	0.236	70.0
R472G	D-glucose	0.773	32.2	41.7	82.1
	D-galactose	6.38	2.07	0.324	96.1
V546G	D-glucose	5.70	23.6	4.13	8.13
	D-galactose	200	4.51	0.0226	6.71
V546P	D-glucose	2.20	15.8	7.19	14.2
	D-galactose	50.6	1.60	0.032	9.49
V546C	D-glucose	3.06	88.6	29.0	57.1
	D-galactose	46.2	6.57	0.142	42.1
H548I	D-glucose	0.811	31.6	39.5	77.8
	D-galactose	9.89	2.66	0.269	79.8
H548R	D-glucose	0.880	9.20	10.5	20.7
	D-galactose	9.27	0.356	0.0381	11.3
N593R	D-glucose	0.527	15.6	29.5	58.1
	D-galactose	7.39	1.57	0.213	63.2
V546G/T169G	D-glucose	0.984	0.119	0.12	0.24
	D-galactose	29.4	0.331	0.011	3.26
V546P/T169G	D-glucose	0.658	0.232	0.353	0.69

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	D-galactose	0.983	0.245	0.254	74.2
V546C/T169N	D-glucose	0.952	0.963	1.01	1.99
	D-galactose	10.4	0.654	0.0629	18.7

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For Peer Review

Legends to Figures

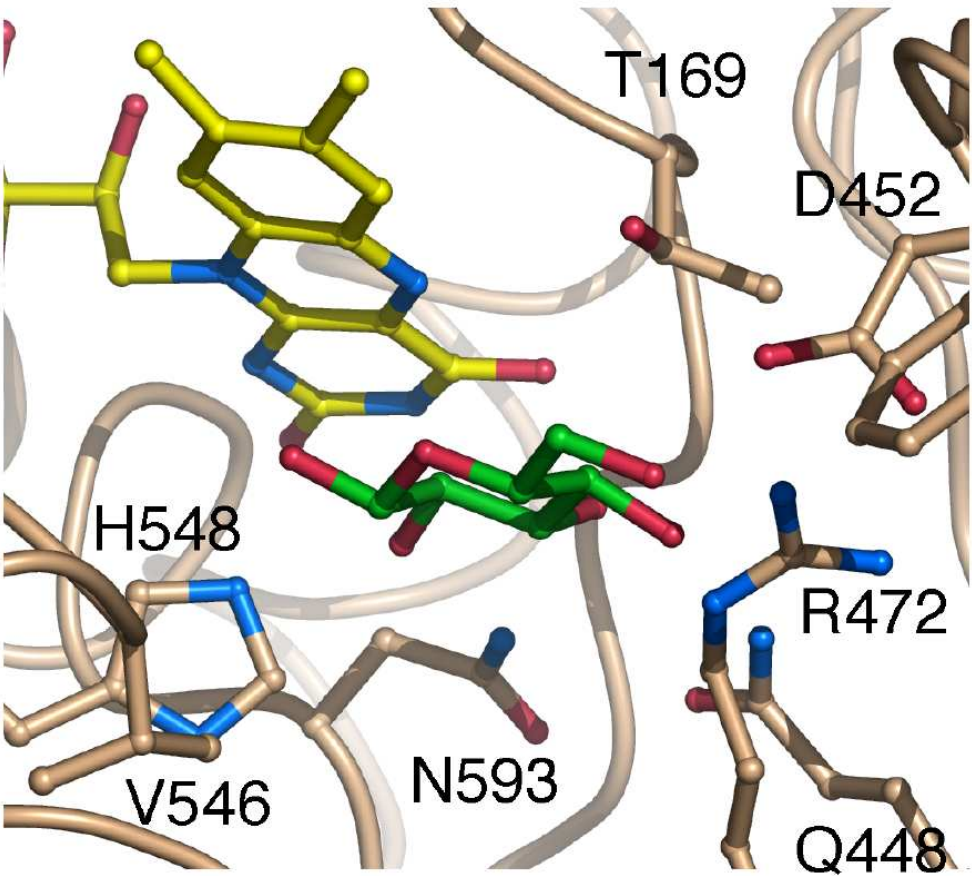
Figure 1. Theoretical models showing the presumed binding of β -D-glucose in the active site of *T. multicolor* pyranose 2-oxidase. The protein chosen for modeling was the crystal structure of pyranose 2-oxidase H167A in complex with 2-fluoro-2-deoxy-D-glucose (PDB code 2IGO; [16]). The FAD co-factor is colored yellow, and the monosaccharide is shown in green. For clarity, protein backbone atoms have been omitted. The monosaccharide is oriented for oxidation at C2. Modeling was performed using the program O [23], and the picture was made using MacPyMOL [22].

Figure 2. SDS-PAGE analysis of mutational variants of pyranose 2-oxidase from *T. multicolor*. Lane 1, molecular mass marker proteins (Precision Plus Protein Dual Color, Biorad); samples from *E.coli* BL21 Star DE3 expressing variants H548I lanes 2 – 4, H548R lanes 5 – 7 and N593R lanes 8 – 10. Lanes 2, 5 and 8 crude extract; lanes 3, 6 and 9, samples purified by IMAC; lanes 4, 7, 10, flow through from IMAC columns.

Figure 3. Sequence alignment of pyranose oxidases from different organisms.

Abbreviations used and accession numbers: T. mats., *Tricholoma matsutake* (BAC24805); L.s., *Lyophyllum shimeji* (Q75ZP8); P.c., *Phanerochaete chrysosporium* (AAS93628); T.m., *Trametes multicolor* (= *Trametes ochracea*; AAX09279); P. sp., *Peniophora* sp. (AAO13382); T.v., *Trametes versicolor* (P79076); T.p., *Trametes pubescens* (AAW57304); P.g., *Phlebiopsis gigantea* (AAQ72486); E.n., *Emericella nidulans* (Q5B2E9).

Figure 4. Conversion of D-galactose by wild-type pyranose oxidase from *T. multicolor* and several of its mutational variants. Conversions were performed in a total volume of 10 ml using equal amounts of P2Ox protein (2.7 mg/mL) at 30 °C using 50 mM phosphate buffer pH 6.5. Pure oxygen was supplied continuously to the reaction mixture through a sparger; catalase was added (350 U/mL) to remove hydrogen peroxide. Symbols: λ , wild-type P2Ox; τ , V546C; υ , T169S; σ , T169N; ν , V546C/T169N.



76x68mm (300 x 300 DPI)

