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Thermostable Variants of Pyranose 2-Oxidase Showing Altered Substrate Selectivity for Glucose and Galactose

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The homotetrameric flavoprotein pyranose 2-oxidase (P2Ox) has several proposed biotechnological applications, among others as a biocatalyst for carbohydrate transformations toward higher-value products. To improve some of the catalytic properties of P2Ox from *Trametes multicolor*, we selected a semirational enzyme engineering approach, namely, saturation mutagenesis of the amino acid His450 located at a pivotal point of the active site loop and subsequent screening of the libraries thus obtained for improved activity with the sugar substrate D-galactose. A variant with improved catalytic characteristics identified was H450G, which showed a significant, 3.6-fold decrease in K_M together with a 1.4-fold increase in k_{cat} for its substrate D-galactose and an overall improvement in the catalytic efficiency by a factor of 5. By combining H450G with other amino acid replacements, we obtained the P2Ox variants H450G/V546C and H450G/E542K/V546C, which can be of interest for applications in food industry due to their increased activity with D-galactose, high activity with D-glucose, and considerably increased stability for the latter variant. While the His-tagged recombinant wild-type enzyme strongly prefers D-glucose to D-galactose as its substrate, H450G/E542K/V546C converts both sugars, which are found in lactose hydrolysates, concomitantly, as was shown by laboratory-scale biotransformation experiments. The 2-keto sugars thus obtained can conveniently be reduced to the corresponding ketoses D-fructose and D-tagatose.

KEYWORDS: Pyranose oxidase; hydrolyzed lactose; enzyme engineering; saturation mutagenesis; D-tagatose; D-fructose; substrate specificity; biocatalytic conversion

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 (*1*−*3*). P2Ox catalyzes the oxidation of various aldopyranoses at C2 to yield the corresponding 2-keto-sugars using oxygen as well as several other compounds (quinones, metal ions) as its electron acceptors. A search in the US Patent data bank (1976−2009) resulted in 438 patents found under the key word "pyranose oxidase", showing the applied interest in this enzyme. Actual applications of P2Ox can be found in food technology (e.g., the baking industry), bioelectrochemistry (e.g., enzymatic biofuel cells and biosensors (*4*)) and analytics, where the enzyme is used for the determination of 1,5-anhydro-D-glucitol, a marker for glycemic control in diabetes (*5*). The first proposed industrial application of P2Ox was the conversion of D-glucose to pure D-fructose via the intermediate 2-keto-D-glucose by the company Cetus in the early 1980s (*6*). In latter studies, various other mono- and disaccharides were converted to the corresponding 2-keto-sugars by P2Ox (*7*,*8*). Since then, P2Ox has become an attractive biocatalyst for biotransformations of carbohydrates, as it can be used for the synthesis of various carbohydrate derivates and rare sugars (*9*). Among others, the oxidation of D-glucose and D-galactose to 2-keto-D-glucose and 2-keto-D-galactose is of applied interest as these oxidized intermediates can subsequently be reduced at position C-1 to obtain the ketoses D-fructose and D-tagatose (*10*), both of which are of interest for the food industry. Lactose, a byproduct in the dairy industry, can be hydrolyzed conveniently by β-galactosidases resulting in equal amounts of D-glucose and D-galactose (*11*), which can subsequently be used as substrates for P2Ox-catalyzed conversions. Lactose represents about 4.5−5% (w/v) of whey, accumulating to approximately 6 million tons worldwide per year from 145 million tons of liquid whey (*12*), which has commonly been considered a waste product. About half of the world's whey production is disposed of as effluent, causing considerable environmental problems due to the high organic content and large volumes. Direct utilization of lactose in food and pharmaceuticals is limited, among others because of the reduced lactase activity encountered in adults (approximately 70−75% worldwide). Therefore, alternative ways to exploit the renewable resource lactose, including enzymatic sugar transformations, are of considerable commercial interest. For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose, however, is too low (5.7% relative activity compared to

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D-glucose (*8*)), leading to either very long conversion times or disproportionate amounts of the required enzyme when converting this sugar. In addition, conversions at elevated temperatures are desirable for applications in industry, as catalytic activities increase with higher temperatures and undesired microbiological growth is avoided as well. A thermostable variant of P2Ox with increased catalytic activity with D-galactose would therefore be of applied interest for food industry.

In this study, we examined various active-site variants of P2Ox isolated from the white rot fungus *Trametes multicolor* (*Tm*P2Ox) for increased activity with D-galactose. Furthermore, we combined various mutations, both at the active site and the subunit interface and known from former studies on P2Ox (*13*−*15*), to create a tailor-made, stable biocatalyst for the conversion of hydrolyzed lactose.

MATERIALS AND METHODS

Plasmids, Microorganisms and Media. The construction of the pET21d$^+$/P2Ox vector (pHL2), which expresses the C-terminally His-tagged P2Ox gene from *Trametes multicolor* (GenBank Accession No. AY291124) under control of the T7 promoter, has previously been described (*16*). The plasmids carrying the V546C as well as the E542K mutations have also been described elsewhere (*13*, *14*). Active, recombinant wild-type *Tm*P2Ox and mutational variants of P2Ox were expressed in *Escherichia coli* strain BL21 Star DE3 (Invitrogen; Carlsbad, CA). TB_{amp}-medium was used to cultivate *E. coli* cells for protein expression under appropriate selective conditions (ampicillin was added to 0.1 g/L). All chemicals used were purchased from Sigma (Vienna, Austria) and were of the highest grade available.

Generation of Mutants and Screening for Improved Variants. Based on the crystal structure (PDB 1TT0 (*17*)), position His450 of *Tm*P2Ox was targeted by site-saturation mutagenesis, which allowed the construction of a P2Ox variant library containing all possible codons at this position. The library size was determined by the mutagenic codon NNN (*18*). A total number of 360 colonies rather than the required 190 were screened to statistically cover more than 95% of all possible combinations with high probability. A convenient 96-well plate-screening assay, which allows a fast and reliable selection of improved P2Ox variants, has been described earlier (*13*). In brief, a total of six wells per plate were inoculated with *E. coli* carrying the wild-type P2Ox gene as a control. The average value of the specific activity of recombinant wild-type P2Ox (His_6-rP2Ox) with the substrate of interest was determined, and compared with activities for the P2Ox variants. The screening assay was performed with the two sugar substrates D-glucose and D-galactose using vigorous shaking to achieve saturating oxygen levels. Variants showing concomitantly high catalytic activity with D-galactose and reduced activity with D-glucose compared to the recombinant wild-type enzyme were selected, and their genes were sequenced to determine the introduced mutation at position His450.

For the introduction of site-directed mutations, the P2Ox gene was mutated by a two-step mutagenesis method using PCR and digestion with *Dpn*I (*19*). The plasmid carrying the P2Ox gene was used as a template for site-directed mutagenesis at position His450 with the primers H450G_fwd (5′-CACTCAGATCGGGCGCGATGCTTTCAGTTACGG-3′) and H450G_rev (5′-CCGTAACTGAAAGCATCGCGCCCGATCTGAG-TGT-3′). The additional mutations V546C and E542K were introduced as previously reported (*13*−*15*). 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 (Fermentas) 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; 58 °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 *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; Madison, WI). Five microliters of the PCR products were transformed into electrocompetent *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′-GCTAGT-TATTGCTCAGCGG-3′).

Protein Expression and Purification. P2Ox protein was expressed in *E. coli* BL21 Star DE3 cells and purified by immobilized metal affinity chromatography according to previously published protocols (*13*, *14*). The eluted, concentrated enzymes were washed 3 times using 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.

Enzyme Activity Assays. P2Ox activity was measured with the standard chromogenic ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay (*2*). 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 absorbance coefficient at 420 nm (ε_{420}) used was 42.3 $mM^{-1} \cdot cm^{-1}$. 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 using the BioRad Protein Assay Kit with bovine serum albumin as standard. All samples were measured at least in duplicates, and errors of these repeated measurements were always below 5%.

Steady-State Kinetic Measurements. Kinetic constants were calculated by nonlinear least-squares 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).

Thermal Stability. Kinetic stability of the *Tm*P2Ox variants was determined by incubating the enzymes in appropriate dilutions in 50 mM phosphate buffer (pH 6.5) at 60 and 70 °C and by subsequent measurements of the enzyme activity (A) at various time points (t) using the standard ABTS assay and glucose as the substrate (20 mM). A thermal cycler (thermocycler T3, Biometra; Göttingen, Germany) and thin-walled PCR tubes were used for all thermostability measurements. Residual activities (A_t/A_0, where A_t is the activity measured at time t and A_0 is the initial P2Ox activity) were plotted versus the incubation time. The inactivation constant k_{in} was obtained by linear regression of (ln activity) versus time. The half-life values of thermal inactivation $\tau_{1/2}$ were calculated using $\tau_{1/2} = \ln 2/k_{in}$ (*20*). At room temperature no inactivation of the enzyme in this buffered system was observed within the 48 h.

Electrophoresis. Electrophoresis was performed principally as described by Laemmli (*21*) to check the purity and the correct molecular mass of the purified mutational variants. Native PAGE was 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; Piscataway, NJ) was used as mass standard. Staining was performed with Coomassie blue.

Bioreactor Cultivations. To produce sufficient amounts of the recombinant wild-type enzyme and the mutational variants H450G/V546C and H450G/E542K/V546C, batch cultivations were carried out in a 5 L Biostat MD stirred tank reactor (Braun; Melsungen, Germany) with a working volume of 4 L. These cultivations were done in TB_{amp}-medium. A preculture grown in an Erlenmeyer flask containing 200 mL of 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%. The DO_2 level was maintained by automatically supplying filtered air (0−4 L/min) and adjusting the stirrer velocity. All parameters were controlled by a digital control unit (IMCS-2000, 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, and the optical density, total intracellular protein concentration and P2Ox activity were measured to monitor recombinant enzyme production. When the volumetric activity reached a maximum, cells were harvested by centrifugation and homogenized and enzymes were purified as described before (*13*).

Batch Conversion Experiments. His_6-rP2Ox and the variants H450G/V546C and H450G/E542K/V546C were compared in terms of

their ability to concomitantly oxidize D-glucose and D-galactose to the corresponding 2-ketoaldoses using oxygen as electron acceptor. Six batch conversion experiments (each with a volume of 300 mL) were performed in parallel in a multifermenter (Infors; Bottmingen, Switzerland). The specific activities of the enzyme samples used were 6.3 U/mg for His_6-rP2Ox, 2.7 U/mg for H450G/V546C, and 1.3 U/mg for H450G/E542K/V546C, employing standard assay conditions and D-glucose as the substrate. In order to compare these three variants directly in the discontinuous conversions, we used a total of 25 D-glucose units of each enzyme for the corresponding experiments. Catalase was used in excess (100,000 U) to decompose H_2O_2. The conversion experiments were conducted in 100 mM KH_2PO_4 buffer (pH 6.5) at 30 °C and at 50 °C, 300 rpm and a DO_2 concentration of 20%. Conversion reactions were performed with 100 mmol/L of each sugar substrate D-glucose and D-galactose. Samples (2 mL) were taken periodically, held at 95 °C for 3 min to inactivate the enzymes and clarified by centrifugation. 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) and a CarboPac PA-1 column (4 × 250 mm) at 30 °C (*22*).

RESULTS

Generation of Mutants. The strictly conserved active-site loop ^{450}HRDAFSYGA^{459} of pyranose oxidase is highly dynamic and has been proposed to play an important role in sugar substrate binding and discrimination (*16*, *23*). In order to improve the reactivity of *Tm*P2Ox with various substrates we chose the residues in the active-site loop as targets for saturation mutagenesis, aiming at improved reactivity with the substrate D-galactose. His450 is found at a pivotal position at the base of this loop (*23*), and was one of the targeted residues selected for mutagenesis studies (*24*). A screening assay based on microtiter plates (*13*) revealed several His450 variants with improved catalytic activity for D-galactose and concomitantly reduced activity with D-glucose, all of which were identified as His450Gly. In these selected variants, the amino acid glycine was encoded by either of the four possible codons (GGG, GGA, GGC, GGT), which proved the success of saturation mutagenesis and the reliability of the screening assay. In order to further improve this variant for the possible conversion of hydrolyzed lactose in food industry, we introduced additional mutations. Variant V546C is characterized by elevated k_{cat} values for both sugar substrates D-glucose and D-galactose, which is, however, accompanied by elevated K_M values (*14*). Variant E542K is characterized by reduced K_M values for both D-glucose and D-galactose and significantly increased stability (*13*). His-tagged recombinant wild-type P2Ox (His_6-rP2Ox) and the variants H450G, H450G/V546C and H450G/E542K/V546C were expressed and purified to apparent homogeneity (**Figure 1**) for further characterization.

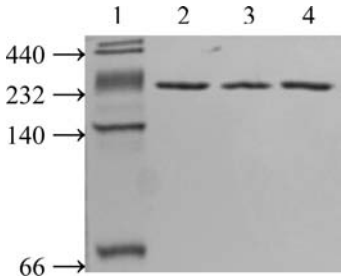

Figure 1. Native PAGE analysis of *Trametes multicolor* His-tagged recombinant wild-type P2Ox (His_6-rP2Ox) and mutational variants H450G/V546C and H450G/E542K/V546C. Lane 1, molecular mass marker proteins; lanes 2, 3 and 4, His_6-rP2Ox, H450G/V546C and H450G/E542K/V546C after purification by IMAC, respectively.

Kinetic Characterization of Mutational Variants. Initial rates of substrate turnover were recorded over a substrate range of 0.1 to 50 mM D-glucose and 0.1 to 200 mM D-galactose for His_6-rP2Ox and the mutational variants using the standard ABTS assay and air oxygen. Kinetic data are summarized in **Table 1**. Variant H450G showed similar binding (Michaelis constant K_M) of D-glucose as His_6-rP2Ox, but a 3.8-fold reduction in the turnover number k_{cat}, overall resulting in a 4-fold decreased catalytic efficiency k_{cat}/K_M. On the contrary, k_{cat}/K_M for D-galactose was increased 5-fold because of a significant reduction in K_M and slightly increased k_{cat} value for this substrate. Variant V546C of *Tm*P2Ox has been described earlier (*14*), and is characterized by dramatically increased K_M and k_{cat} values for both sugar substrates. The double mutant H450G/V546C showed elevated K_M values for both sugar substrates D-glucose and D-galactose compared to His_6-rP2Ox (2.6-fold and 1.4-fold, respectively). The turnover number for D-glucose decreased significantly (2.9-fold), whereas it increased 2.4-fold for D-galactose resulting in a 1.7-fold increase in catalytic efficiency for D-galactose, while k_{cat}/K_M for D-glucose was reduced 3.8-fold. The mutation E542K in *Tm*P2Ox results in a decrease of both K_M and k_{cat} value for the sugar substrates D-glucose and D-galactose, as has been shown before (*13*). This was confirmed when introducing this mutation into variant H450G/V546C. Overall, this triple mutant H450G/E542K/V546C showed a catalytic efficiency for its substrate D-galactose that was increased 1.2-fold compared to His_6-rP2Ox, while k_{cat}/K_M for D-glucose was decreased to 17% of its original value.

His_6-rP2Ox clearly prefers D-glucose to D-galactose as its sugar substrate; this is also expressed by the substrate selectivity value, i.e., the ratio of the catalytic efficiencies k_{cat}/K_M for the two substrates (*25*). This value is 177 for His_6-rP2Ox, while it is 8.9 for H450G, 27.4 for H450G/V546C and 24.4 for H450G/E542K/V546C (**Table 1**), indicating that these enzyme variants do not show such a strong preference for D-glucose as substrate anymore.

Table 1. Kinetic Properties and Substrate Selectivity of His-Tagged Recombinant 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[a]

	D-glucose			D-galactose			
enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\cdot s^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1}\cdot s^{-1}$)	selectivity: $(k_{cat}/K_M)_{Glc}/(k_{cat}/K_M)_{Gal}$
His_6-rP2Ox	0.939 ± 0.04	48.1 ± 0.50	51.2	8.79 ± 0.54	2.51 ± 0.05	0.286	177
H450G	0.987 ± 0.05	12.5 ± 0.15	12.7	2.45 ± 0.12	3.51 ± 0.04	1.43	8.88
V546C[b]	3.06 ± 0.14	88.6 ± 1.30	29.0	46.2 ± 3.21	6.57 ± 0.15	0.142	210
H450G/V546C	2.43 ± 0.14	16.8 ± 0.27	13.5	12.0 ± 0.38	5.92 ± 0.05	0.493	27.4
E542K[c]	0.521 ± 0.02	35.9 ± 0.33	68.9	3.87 ± 0.30	2.59 ± 0.04	0.669	103
H450G/E542K/V546C	0.770 ± 0.17	6.81 ± 0.35	8.83	7.80 ± 0.70	2.84 ± 0.06	0.364	24.4

[a] Substrate selectivity is given as the ratio of the catalytic efficiencies k_{cat}/K_M for the two substrates D-glucose and D-galactose. [b] Data from Spadiut et al. (*14*). [c] Data from Spadiut et al. (*13*)

Table 2. Kinetic Stability of His-Tagged Recombinant Wild-Type P2Ox from *T. multicolor* and Mutational Variants at 60 °C and 70 °C

	60 °C		70 °C	
variant	inactivation constant k_{in} (min^{-1})	half-life $\tau_{1/2}$ (min)	inactivation constant k_{in} (min^{-1})	half-life $\tau_{1/2}$ (min)
His_6-rP2Ox	687×10^{-4}	10	nd[a]	<1
H450G/V546C	345×10^{-4}	20	nd	<1
H450G/E542K/V546C	nd	>1200	112×10^{-4}	62

[a] Not determined.

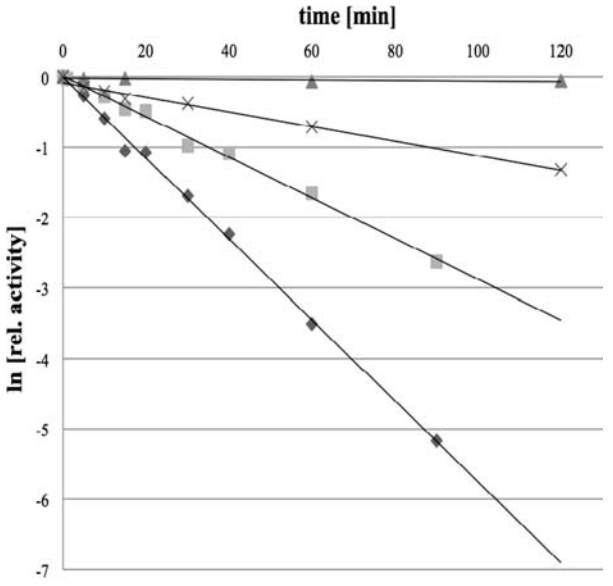

Figure 2. Inactivation kinetics of pyranose oxidase from *T. multicolor*, both recombinant His-tagged wild-type enzyme and variants, showing the residual activity after incubation at increased temperature for various time. Symbols: ◆, His_6-rP2Ox at 60 °C; ■, variant H450G/V546C at 60 °C; ×, variant H450G/E542K/V546C at 70 °C; ▲, variant H450G/E542K/V546C at 60 °C.

Thermal Stability. Kinetic stability, defined as the length of time in which an enzyme remains active before undergoing irreversible inactivation, of His_6-rP2Ox and of variants H450G/V546C and H450G/E542K/V546C was determined at 60 °C and at 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 2**). All enzymes showed first-order inactivation kinetics when analyzed in the ln(residual activity) versus time plot (**Figure 2**). The mutation E542K in combination with H450G and V546C stabilized P2Ox significantly. At 60 °C the half-life was increased by more than 120-fold compared to the rec wt enzyme and the double mutant. The effect of the mutation on stability was also obvious at 70 °C, where H450G/E542K/V546C was still active after 2 h with a $\tau_{1/2}$ of 62 min, whereas His_6-rP2Ox and H450G/V546C inactivated completely within 1 min.

Enzyme Production and Substrate Conversion Experiments. Batch cultivations of *E. coli* BL21DE3 carrying the genes for either the His-tagged wild-type or the mutated enzymes H450G/V546C and H450G/E542K/V546C were performed 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 His_6-rP2Ox with D-glucose reached a maximum level of 230 $U \cdot L^{-1}$, corresponding to approximately 30 mg of recombinant protein per liter. The purified enzymes were used for batch conversion experiments with an equimolar mixture of D-glucose and D-galactose (100 mM each) as substrate and oxygen as electron acceptor. Twenty-five D-glucose units of each enzyme was used for the corresponding conversion experiments, which were conducted at both 30 and 50 °C (**Figure 3**, **Table 3**). At 30 °C His_6-rP2Ox clearly preferred D-glucose to D-galactose as substrate. Only when D-glucose was completely consumed, D-galactose was converted as evident from HPLC analysis, albeit at a very low rate of 0.018 $g \cdot L^{-1} \cdot h^{-1}$ during the batch conversion (and hence is not evident in **Figure 3A** because of the scale used). This conversion rate was 130-fold lower than the value of 2.34 $g \cdot L^{-1} \cdot h^{-1}$ calculated for D-glucose, again stressing the prominent discrimination of D-galactose oxidation versus that of D-glucose. At 50 °C the clear preference of the rec wt enzyme for D-glucose was again obvious. Because of thermal inactivation over time, the conversion rate rapidly dropped within approximately 10 h, and the enzyme was completely inactivated after 15 h as was evident from residual D-glucose in the reaction mixture and the complete lack of 2-keto-D-galactose. This sequential oxidation of D-glucose and D-galactose when using His_6-rP2Ox was shown before, both when using oxygen and benzoquinone as electron acceptor during the bioconversions (*14*).

Variant H450G/V546C converted both sugar substrates concomitantly and showed a 4-fold improved conversion of D-galactose compared to His_6-rP2Ox at 30 °C. Similar as for His_6-rP2Ox, the conversion stopped after approximately 15 h at 50 °C, presumably again because of thermal inactivation of the enzyme. Because of the reduced conversion rate of D-glucose, ~25% of the initial amount of D-glucose was still detected in the reaction mixture at that time.

Variant H450G/E542K/V546C showed the highest conversion rate of D-galactose in the batch conversion studies. This triple mutant converted both sugar substrates concomitantly at high rates of 1.78 $g \cdot L^{-1} \cdot h^{-1}$ for D-glucose and 0.23 $g \cdot L^{-1} \cdot h^{-1}$ for D-galactose. A further increase in the conversion rate for D-galactose was observed for this thermostable variant at 50 °C, reaching a value of 0.29 $g \cdot L^{-1} \cdot h^{-1}$; this corresponds to a 16-fold increase compared to His_6-rP2Ox employed at 30 °C.

DISCUSSION

Lactose is the main constituent of milk solids, accounting for 40% of the solids in cow's milk. β-D-Galactosidase (EC 3.2.1.23) efficiently hydrolyses lactose into D-glucose and D-galactose (*11*), and hence these sugars can be obtained in huge amounts as lactose hydrolysates. They have significant potential as a fermentation substrate, but can also be used as substrates for further enzymatic transformations to more valuable food ingredients. A conceivable transformation is the isomerization of these D-glucose/D-galactose mixtures to the corresponding ketoses D-fructose and D-tagatose. D-Tagatose is almost as sweet as sucrose (92% relative sweetness in 10% solutions) yet its caloric value is estimated to be almost negligible (*26*), and therefore its use allows a significant reduction in caloric value of food at a comparable level of sweetness. Both D-fructose and D-tagatose can be obtained from the respective aldoses by employing isomerases, glucose (xylose) isomerase and arabinose isomerase, respectively (*26*). However, only the equilibrium concentrations can be attained when using this approach. Another biocatalytic

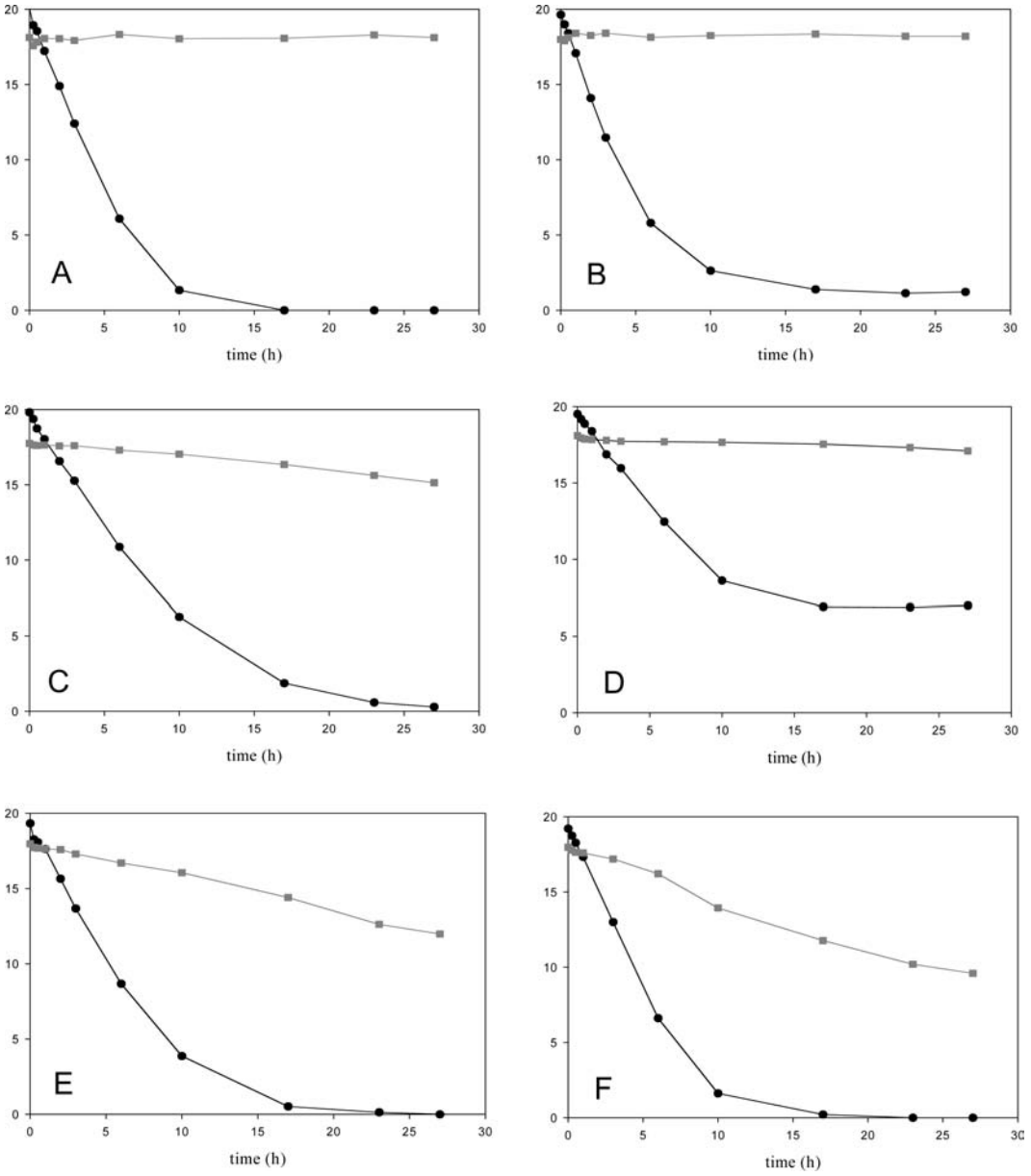

Figure 3. Batch conversion experiments of an equimolar mixture of D-glucose and D-galactose (100 mM each) and oxygen as electron acceptor in saturating concentrations, using His-tagged recombinant wild-type *Tm*P2Ox, the variant H450G/V546C or H450G/E542K/V546C as biocatalysts. **A**, His_6-rP2Ox and oxygen at 30 °C; **B**, His_6-rP2Ox and oxygen at 50 °C; **C**, H450G/V546C and oxygen at 30 °C; **D**, H450G/V546C and oxygen at 50 °C; **E**, H450G/E542K/V546C and oxygen at 30 °C; **F**, H450G/E542K/V546C and oxygen at 50 °C. Symbols: ●, D-glucose; ■, D-galactose.

Table 3. Batch Conversion Experiments of Equimolar Mixtures of D-Glucose and D-Galactose (100 mM Each) Performed at 30 °C and at 50 °C and Using His-Tagged Recombinant Wild-Type Pyranose Oxidase from *T. multicolor* and the Variants H450G/V546C and H450G/E542K/V546C[a]

	batch A	batch B	batch C	batch D	batch E	batch F
enzyme	His_6-rP2Ox	His_6-rP2Ox	H450G/V546C	H450G/V546C	H450G/E542K/V546C	H450G/E542K/V546C
temp (°C)	30	50	30	50	30	50
D-Glc conversion ($g \cdot L^{-1} \cdot h^{-1}$)	2.34	2.30	1.49	1.18	1.78	2.10
D-Gal conversion ($g \cdot L^{-1} \cdot h^{-1}$)	0.018[b]	0.00	0.068	0.023	0.23	0.29

[a] These biocatalysts were added based on equal P2Ox activities with D-glucose under standard assay conditions. [b] D-Galactose was not converted until D-glucose was completely oxidized as was evident from HPAEC analysis.

approach is via the corresponding 2-ketosugars 2-keto-D-glucose and 2-keto-D-galactose, which subsequently can be easily and efficiently reduced by catalytic hydrogenation to yield the corresponding ketoses (*10*, *27*). As the P2Ox-catalyzed reaction is quasi-irreversible (*28*), close to theoretical yields can be obtained. For the simultaneous conversion of D-glucose and D-galactose in biotechnological processes, however, the catalytic activity of P2Ox with D-galactose is too low relative to that of D-glucose (5.7% relative activity). This simultaneous conversion of D-glucose and D-galactose is important when lactose hydrolysates are used as a starting material for the envisaged bioconversion, since P2Ox is known to overoxidize its primary reaction product, 2-keto-D-glucose, thus forming 2,3-diketo-D-glucose (*9*), once the primary oxidation at C-2 is completed.

In several previous studies the improvement of *Tm*P2Ox both in terms of stability and reactivity was discussed (*13*, *14*). Recently, we reported on the stable P2Ox variant T169G/V546C, which converts D-glucose and D-galactose concomitantly at identical rates (*15*). However, the conversion rates were rather low (0.05 $g \cdot L^{-1} \cdot h^{-1}$ for D-glucose and 0.06 $g \cdot L^{-1} \cdot h^{-1}$ for D-galactose, respectively), making this variant less interesting for industry because of very long process times.

In order to improve P2Ox with respect to its substrate promiscuity and selectivity (i.e., increased activity with its poor substrate D-galactose), we used a semirational approach, namely, saturation mutagenesis of residue His450, which is located at the active site loop involved in substrate recognition (*23*). When screening the libraries of P2Ox variants mutated at position 450, we found several mutants that reacted faster with D-galactose and concomitantly slower with D-glucose than His_6-rP2Ox. All of these variants were identified as His450Gly. The replacement of the aromatic, polar amino acid histidine by the small, apolar glycine apparently influenced the positioning and/or flexibility of the active site loop, which drastically affected the catalytic characteristics of this enzyme variant. Variant H450G has been crystallized, is currently analyzed in more detail in our laboratory and will be discussed elsewhere (unpublished data).

A mutation in *Tm*P2Ox that was recently identified by us is V546C, which is characterized by significant increases in k_{cat} for both D-galactose and D-glucose, albeit at the cost of elevated Michaelis constants (*14*). Since increased k_{cat} values are desirable for technological processes, where substrates are converted at high concentrations well above K_M, we combined this mutation with H450G, resulting in the double mutant H450G/V546C. This double mutant indeed showed increased k_{cat} values, but also higher K_M values than H450G. In order to create a thermostable variant of P2Ox, which converts D-glucose and D-galactose concomitantly at high rates, we combined H450G/V546C with E542K, which had shown positive effects on the catalytic properties and stability of *Tm*P2Ox before (*13*, *14*). The resulting triple mutant H450G/E542K/V546C shows properties that make it interesting for applications in food industry. Recombinant wild-type P2Ox clearly prefers D-glucose to D-galactose resulting in a substrate selectivity, the ratio of k_{cat}/K_M for these two substrates, of 177. This ratio changes to 24.4 for H450G/E542K/V546C, due to an increase in k_{cat}/K_M for D-galactose and a decrease in k_{cat}/K_M for D-glucose. In contrast to the previously reported variant T169G/V546C (*15*), the k_{cat} value for D-galactose was increased and also was reasonably high for D-glucose compared to His_6-rP2Ox. While His_6-rP2Ox converted D-galactose only when D-glucose was depleted from the reaction mixture, H450G/E542K/V546C catalyzes the concomitant oxidation of both sugars, as was confirmed in small-scale bioconversion experiments. Introducing the E542K mutation into the variant enabled conversions at higher temperatures, which is preferable because of higher reaction rates and a decreased possibility of microbial contamination. The triple mutant showed considerably increased thermostability as is evident from a remarkable increase in half-life times, both at 60 and 70 °C. Thus, bioconversions based on this thermostable variant will be feasible at temperatures of up to 60 °C.

In conclusion, the semirational approach selected for the engineering of P2Ox with respect to its substrate specificity and promiscuity proved successful. By combining the newly identified H450G variant with other mutations we obtained a thermostable biocatalyst that shows significantly improved catalytic properties compared to His_6-rP2Ox. This variant holds promise for applications in food industry for the conversion of hydrolyzed lactose toward a sugar mixture of considerably increased sweetness. In addition, this work shows that individual mutations identified in *Tm*P2Ox can be combined beneficially, enabling the design of biocatalyts with desired and tailored properties as our knowledge on structure/function relationship on P2Ox increases.

ABBREVIATIONS USED

P2Ox, pyranose 2-oxidase; FAD, flavin adenine dinucleotide; *Tm*P2Ox, pyranose 2-oxidase from *Trametes multicolor*; His6-rP2Ox, His-tagged recombinant wild-type pyranose 2-oxidase; ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); PAGE, polyacrylamide gel electrophoresis; DO_2, dissolved oxygen concentration;

LITERATURE CITED

(1) Volc, J.; Denisova, N. P.; Nerud, F.; Musílek, V. Glucose-2-oxidase activity in mycelia cultures of basidiomycetes. *Folia Microbiol.* **1985**, *30*, 141–147.

(2) Danneel, H.-J.; Ullrich, M.; Giffhorn, F. Goal-oriented screening method for carbohydrate oxidases produced by filamentous fungi. *Enzyme Microb. Technol.* **1992**, *14*, 898–903.

(3) Leitner, C.; Haltrich, D.; Nidetzky, B.; Prillinger, H.; Kulbe, K. D. Production of a novel pyranose 2-oxidase by basidiomycete *Trametes multicolor*. *Appl. Biochem. Biotechnol.* **1998**, *70*−*72*, 237–248.

(4) Tasca, F.; Timur, S.; Ludwig, R.; Haltrich, D.; Volc, J.; Antiochia, R.; Gorton, L. Amperometric biosensors for detection of sugars based on the electrical wiring of different pyranose oxidases and pyranose dehydrogenases with osmium redox polymers on graphite electrodes. *Electroanalysis* **2007**, *19*, 294–302.

(5) Yamanouchi, T.; Ogata, N.; Tagaya, T. Clinical usefulness of serum 1,5-anhydroglucitol in monitoring glycaemic control. *Lancet* **1996**, *347*, 1514–1518.

(6) Neidleman, S. L., Amon, W. F., Geigert, J. Process for the production of fructose. US Patent 4,246,347 to Cetus Corporation, 1981.

(7) Freimund, S.; Huwig, A.; Giffhorn, F.; Köpper, S. Rare keto-aldoses from enzymatic oxidation: substrates and oxidation products of pyranose 2-oxidase. *Chem.—Eur. J.* **1998**, *4*, 2442–2455.

(8) Leitner, C.; Volc, J.; Haltrich, D. Purification and characterization of pyranose oxidase from the white-rot fungus *Trametes multicolor*. *Appl. Environ. Microbiol.* **2001**, *67*, 3636–3644.

(9) Giffhorn, F. Fungal pyranose oxidases: occurrence, properties and biotechnical applications in carbohydrate chemistry. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 727–740.

(10) Haltrich, D.; Leitner, C.; Neuhauser, W.; Nidetzky, B.; Kulbe, K. D.; Volc, J. A convenient enzymatic procedure for the production of aldose-free D-tagatose. *Anal. N.Y. Acad. Sci.* **1998**, *864*, 295–299.

(11) Nguyen, T.-H.; Splechtna, B.; Steinböck, M.; Kneifel, W.; Lettner, H. P.; Kulbe, K. D.; Haltrich, D. Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54*, 4989–4998.

(12) González-Siso, M. The biotechnological utilization of cheese whey: a review. *Bioresour. Technol.* **1996**, *57*, 1–11.

(13) Spadiut, O.; Leitner, C.; Salaheddin, C.; Varga, B.; Vertessy, B.; Tan, T.-C.; Divne, C.; Haltrich, D. Improving thermostability and catalytic activity of pyranose 2-oxidase from *Trametes multicolor* by rational and semi-rational design. *FEBS J.* **2009**, *276*, 776–792.

(14) Spadiut, O.; Pisanelli, I.; Maischberger, T.; Peterbauer, C.; Gorton, L.; Chaiyen, P.; Haltrich, D. Engineering of pyranose 2-oxidase: improvement for biofuel cell and food applications through semi-rational protein design. *J. Biotechnol.* **2009**, *5*, 250–257.

(15) Spadiut, O.; Radakovits, K.; Pisanelli, I.; Salaheddin, C.; Yamabhai, M.; Tan, T. C.; Divne, C.; Haltrich, D. A thermostable triple mutant of pyranose 2-oxidase from *Trametes multicolor* with improved properties for biotechnological applications. *Biotechnol. J.* **2009**, *4*, 525–534.

(16) Kujawa, M.; Ebner, H.; Leitner, C.; Hallberg, B. M.; Prongjit, M.; Sucharitakul, J.; Ludwig, R.; Rudsander, U.; Peterbauer, C.; Chaiyen, P.; et al. Structural basis for substrate binding and

regioselective oxidation of monosaccharides at C3 by pyranose 2-oxidase. *J. Biol. Chem.* **2006**, *281*, 35104–35115.
(17) Hallberg, B. M.; Leitner, C.; Haltrich, D.; Divne, C. Crystal structure of the 270 kDa homotetrameric lignin-degrading enzyme pyranose 2-oxidase. *J. Mol. Biol.* **2004**, *3*, 781–796.
(18) Georgescu, R.; Bandara, G.; Sun, L. Saturation Mutagenesis. In *Directed Evolution Library Creation: Methods and Protocols*; Arnold, F. H., Georgiou, G., Eds.; Springer; New York; 2003; pp 75−83.
(19) Li, S.; Wilkinson, M. F. Site-directed mutagenesis: a two-step method using PCR and *Dpn*I. *Biotechniques* **1997**, *4*, 588–590.
(20) Polizzi, K. M.; Bommarius, A. S.; Broering, J. M.; Chaparro-Riggers, J. F. Stability of biocatalysts. *Curr. Opin. Chem. Biol.* **2007**, *11*, 220–225.
(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(22) Splechtna, B.; Nguyen, T.-H.; Steinböck, M.; Kulbe, K. D.; Lorenz, W.; Haltrich, D. Production of prebiotic galacto-oligosaccharides from lactose using β-galactosidases from *Lactobacillus reuteri*. *J. Agric. Food Chem.* **2006**, *54*, 4999–5006.
(23) Hallberg, B. M.; Leitner, C.; Haltrich, D.; Divne, C. Crystal structure of the 270 kDa homotetrameric lignin-degrading enzyme pyranose 2-oxidase. *J. Mol. Biol.* **2004**, *341*, 781–796.
(24) Salaheddin, C.; Spadiut, O.; Ludwig, R.; Tan, T.-C.; Divne, C.; Haltrich, D.; Peterbauer, C. Probing active-site residues of pyranose 2-oxidase from Trametes multicolor by semi-rational protein design. *Biotechnol. J.* **2009**, *4*, 535–543.
(25) Morley, K. L.; Kazlauskas, R. J. Improving enzyme properties: when are closer mutations better? *Trends Biotechnol.* **2005**, *23*, 231–237.
(26) Oh, D. K. Tagatose: properties, applications, and biotechnological processes. *Appl. Microbiol. Biotechnol.* **2007**, *76*, 1–8.
(27) Leitner, C.; Neuhauser, W.; Volc, J.; Kulbe, K. D.; Nidetzky, B.; Haltrich, D. The Cetus process revisited: A novel enzymatic alternative for the production of aldose-free D-fructose. *Biocatal. Biotransform.* **1998**, *16*, 365–382.
(28) Prongjit, M.; Sucharitakul, J.; Wongnate, T.; Haltrich, D.; Chaiyen, P. Kinetic mechanism of pyranose 2-oxidase from *Trametes multicolor*. *Biochemistry* **2009**, *48*, 4170–4180.

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