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Improvement of the fungal enzyme pyranose 2-oxidase using protein engineering

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Abstract

Native pyranose 2-oxidase (P2Ox) was purified from *Peniophora* sp. and characterized. To improve its catalytic efficiencies and stabilities by protein engineering, we cloned and expressed the P2Ox gene in *Escherichia coli* and received active, fully flavinylated recombinant P2OxA. Selenomethionine-labeled P2OxA was used for X-ray analysis and the resulting crystal structure enabled the rational design using variant P2OxA1 with the substitution E542K as template. Besides increased thermal and pH stabilities this variant showed improved catalytic efficiencies (k_{cat}/K_m) for the main substrates. A new variant, P2OxA2H, with an additional substitution T158A and a C-terminal His_6-tag exhibited significantly decreased apparent K_m values for D-glucose (0.47 mM), L-sorbose (1.79 mM), and D-xylose (1.35 mM). Compared to native P2Ox, the catalytic efficiencies were substantially improved for D-glucose (230-fold), L-sorbose (874-fold), and D-xylose (1751-fold). This P2Ox variant was used for the bioconversion of L-sorbose under O_2-saturation in a molar scale. The structure–activity relationships of the amino acid substitutions were analyzed by modelling of the mutated P2Ox structures. Molecular docking calculations of various carbohydrates into the crystal structure of P2OxA and the analysis of the protein–ligand interactions in the docked complexes enabled us to explain the substrate specificity of the enzyme by a conserved hydrogen bond pattern which is formed between the protein and all substrates.

Keywords: Pyranose 2-oxidase gene; Rational protein design; Improved enzyme variants; Bioconversion; Structure–activity relationship; Molecular docking; Enzyme–ligand interactions

1. Introduction

The flavoenzyme pyranose 2-oxidase[1] (P2Ox, pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) is

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[1] Nucleotide sequence data is available at the GenBank accession no. AF535193 (pyranose 2-oxidase cDNA from *Peniophora* sp.).

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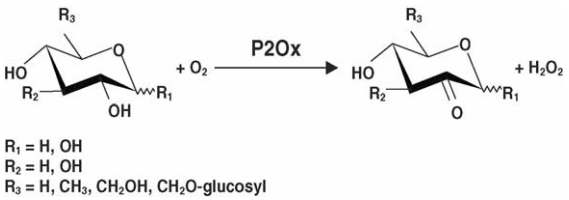

Fig. 1. General reaction scheme of pyranose 2-oxidase catalyzing the regioselective oxidation, e.g. of D-glucose to 2-keto-D-glucose (glucosone) concomitant with the reduction of oxygen to hydrogen peroxide via covalently bound FAD.

wide-spread among wood-degrading *Basidiomycetes* and localized in the hyphal periplasmic space. It is involved in the ligninolysis by oxidation of wood-specific sugars while supplying lignin peroxidases with H_2O_2 (Daniel et al., 1994). In some fungi P2Ox is also involved in a secondary metabolic pathway leading to the antibiotic cortalcerone (e.g. Baute et al., 1987; Volc et al., 1991). In *Tricholoma matsutake*, an unidentified P2Ox reaction product causes a strong antifungal activity (Takakura and Kuwata, 2003).

P2Ox catalyzes the regioselective C2-oxidation of D-glucopyranose and of structurally related mono- and disaccharides to the corresponding dicarbonyl sugars, while reducing molecular oxygen to hydrogen peroxide (Fig. 1, reviewed by Giffhorn, 2000; Volc et al., 2003). Certain substrates are oxidized to a smaller extent on carbon C3 (Freimund et al., 1998). The regeneration of reduced FAD can also be achieved by benzoquinones and related substances produced during lignin degradation (Leitner et al., 2001).

The high biotechnological potential of P2Ox in biotransformations of carbohydrates is documented by an increasing patent literature. The applications of P2Ox in clinical chemistry analytics, bioprocess monitoring, and synthetic carbohydrate chemistry has been reviewed (Giffhorn, 2000) and expanded by Bastian et al. (2005).

For a technical use of P2Ox, improvement of its catalytic efficiency and stability is needed (Freimund et al., 1998). Directed evolution (e.g. Kuchner and Arnold, 1997), rational protein design (Eijsink et al., 2004) and a combination of both methods are effective engineering tools for improving biocatalysts (Bornscheuer and Pohl, 2001). P2Ox engineering by a combination of directed evolution and rational protein design requires the availability and heterologous expression of the P2Ox gene. Currently, there are P2Ox gene sequences available from *Coriolus versicolor* (Nishimura et al., 1996), *Trametes hirsuta* (Christensen et al., 2000), *T. matsutake* (Takakura and Kuwata, 2003), *Peniophora* sp. (this work, AF535193), *Trametes ochracea* (Večerek et al., 2004), *Phanerochaete chrysosporium* (de Koker et al., 2004), *Peniophora gigantea* (Bastian et al., 2005), *Trametes pubescens* (Marešová et al., 2005), *Lyophyllum shimeji* (AB119106), and *Aspergillus nidulans* (AACD01000093). Most of them were cloned and functionally expressed. Recombinant *T. hirsuta* P2Ox is used in food industry for baking (Christensen et al., 2000). Recombinant *C. versicolor* P2Ox (Nishimura et al., 1998) was engineered by directed evolution, and the resulting enzyme variant with substitution E542K exhibited improved stabilities and K_m values for D-glucose and the diabetes marker 1,5-anhydro-D-glucitol and is used in diagnostics (Masuda-Nishimura et al., 1999).

Recently, we have described the engineering of recombinant *P. gigantea* P2OxB by a dual approach of site-directed mutagenesis and directed evolution (Bastian et al., 2005). For the latter, error prone PCR (Leung et al., 1989) was used and screening assays were established (Bungert et al., 2004; Bastian et al., 2005). The resulting variant (P2OxB2H) with amino acid substitutions E540K and K312E showed improved thermal and pH stabilities and catalytic efficiencies compared to native P2Ox, and was applied for model bioconversions (Bastian et al., 2005).

By structure based sequence comparisons (Albrecht and Lengauer, 2003), P2Ox was assigned to the glucose-methanol-choline (GMC) oxidoreductase protein family (Cavener, 1992). Meanwhile, the crystal structures of native *Trametes multicolor* P2Ox (Hallberg et al., 2004) and of recombinant *Peniophora* sp. P2Ox (Bannwarth et al., 2004) were solved. *Peniophora* sp. P2Ox forms a D_2 symmetric homotetramer, which is unique in the GMC oxidoreductase family. Each subunit consists of a substrate binding domain, an FAD binding domain with one covalently bound FAD, and a collar region. The four subunits are interconnected via four tetramerization loops. The tetramer contains a spacious central cavity from which the substrate enters one of the four reaction centers by penetrating a highly mobile peptide loop. This cavity can only be accessed by glucose-sized molecules through four pores (Bannwarth et al., 2004).