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Analysis of *Agaricus meleagris* pyranose dehydrogenase N-glycosylation sites and performance of partially non-glycosylated enzymes

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ABSTRACT

Pyranose Dehydrogenase 1 from the basidiomycete *Agaricus meleagris* (*Am*PDH1) is an oxidoreductase capable of oxidizing a broad variety of sugars. Due to this and its ability of dioxidation of substrates and no side production of hydrogen peroxide, it is studied for use in enzymatic bio-fuel cells.

In-vitro deglycosylated *Am*PDH1 as well as knock-out mutants of the N-glycosylation sites N^{75} and N^{175}, near the active site entrance, were previously shown to improve achievable current densities of graphite electrodes modified with *Am*PDH1 and an osmium redox polymer acting as a redox mediator, up to 10-fold. For a better understanding of the role of N-glycosylation of *Am*PDH1, a systematic set of N-glycosylation site mutants was investigated in this work, regarding expression efficiency, enzyme activity and stability. Furthermore, the site specific extend of N-glycosylation was compared between native and recombinant *wild type Am*PDH1.

Knocking out the site N^{252} prevented the attachment of significantly extended N-glycan structures as detected on polyacrylamide gel electrophoresis, but did not significantly alter enzyme performance on modified electrodes. This suggests that not the molecule size but other factors like accessibility of the active site improved performance of deglycosylated *Am*PDH1/osmium redox polymer modified electrodes. A fourth N-glycosylation site of *Am*PDH1 could be confirmed by mass spectrometry at N^{319}, which appeared to be conserved in related fungal pyranose dehydrogenases but not in other members of the glucose-methanol-choline oxidoreductase structural family. This site was shown to be the only one that is essential for functional recombinant expression of the enzyme.

1. Introduction

The secreted fungal oxidoreductase pyranose dehydrogenase (PDH; EC 1.1.99.29) was first described by Volc et al. [1], and the main variant from *Agaricus meleagris* (*Am*PDH1) by Sygmund et al. [2]. PDH oxidizes a broad range of mono- and disaccharides at C1-C4, depending on the substrate, and is capable of dioxidation. Quinones and metallo-complexes serve as electron acceptors but not molecular oxygen [3,4]. Its biological function is not well understood but hypothesized to include a role in fungal response against quinones used by plants as defence and in lignin degradation by reducing quinones [4].

PDH has been studied for use in carbohydrate chemistry and bioconversion [2,3,5,6] but also successfully wired to and optimized on modified electrodes for use in biosensors or in enzymatic bio-fuel cells [7–13]. In the latter case, electrodes are modified with oxidoreductases to generate electric current by oxidizing substrates at the anode while usually reducing molecular oxygen to water at the cathode. The broad substrate range, lack of production of H_2O_2 and ability of dioxidation make PDH a promising can-

Abbreviations: *Am*PDH1, *Agaricus meleagris* pyranose dehydrogenase 1; AOX, alcohol oxidase; CDH, cellobiose dehydrogenase; GMC, glucose-methanol-choline; Fc^+, ferrocenium hexafluorophosphate; FIA, flow injection analysis; GlcNAc, *N*-acetylglucosamine; GOx, glucose 1-oxidases; HexNAc, *N*-acetylhexosamine; PDH, pyranose dehydrogenase; P2O, pyruvate 2-oxidase.

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didate for the anodic half reaction [4]. Shao et al. [14] reported a membraneless enzymatic bio-fuel cell prototype with a mixed *Am*PDH1/cellobiose dehydrogenase flavodomain anode. Recently, another prototype had been constructed, which operated in human physiological solution and powered transmission of sensing data (j_{max} = 0.275 mA cm^{-2}) [15].

*Am*PDH1 belongs to the glucose-methanol-choline structural family alongside glucose 1-oxidases (GOx; EC 1.1.3.4), cellobiose dehydrogenases (CDH; EC 1.1.99.18), pyranose 2-oxidases (P2O; EC 1.1.3.10) and alcohol oxidases (AOX; EC 1.1.3.13) [16,17]. It is to date the only pyranose dehydrogenase, which has been structurally resolved in X-ray crystallography (PDB-ID: 4H7U) [18] and features two tightly interwoven conserved domains, a Rossman-fold (PRK07364, GMC_oxred_N: pfam00732), which covalently binds the FAD co-factor *via* H103, and a substrate binding domain (GMC_oxred_C: pfam05199), which features H512, the only catalytic base of *Am*PDH1 [18–20]. As a secreted fungal enzyme, native *Am*PDH1 is N-glycosylated and was estimated to have a sugar content of 7% (m/m) [2].

Heterologous production of pyranose dehydrogenase was previously established in *Pichia pastoris* [21]. This host is known to be less prone to over-glycosylate heterologous proteins with extended *N*-glycan structures than the yeast model system *Saccharomyces cerevisiae*, but was observed to do so [22]. Indeed the purified recombinant product featured a comparable specific activity but also a more extensive and heterogeneous N-glycosylation pattern with a sugar content of approximately 30% [21].

Yakovleva et al. [9] found that recombinant *Am*PDH1/Os-polymer-modified graphite electrodes yielded higher currents when PDH was deglycosylated *in-vitro* prior to use. This created interest in producing PDH with no or reduced N-glycosylation. As expression in *Escherichia coli* yielded only non-functional enzyme in form of inclusion bodies [21], knock out mutants, with a focus on N-glycosylation sites around the active site access, were expressed in *Pichia pastoris*. The mutant *Am*PDH1 N75G/N175Q showed a higher j_{max} (290 μA cm^{-1}) compared to the glycosylated recombinant wild type enzyme [9,23]. Interestingly, however, this double mutant largely retained the over-glycosylation pattern of recombinant *Am*PDH1 wt. When all three glycosylation sites, which were at this point confirmed by mass spectrometry, were knocked out, the purified enzyme formed a well-defined band close to deglycosylated *Am*PDH1. However, the current output of electrodes modified with this triple mutant was not improved compared to the wild type (52 μA cm^{-1}) [23]. These results suggest a site specific heterogenous N-glycosylation of *Am*PDH1, with differential effects of individual N-glycosylation moieties on enzyme performance and stability. In this work this was investigated further by elucidating the N-glycosylation pattern of native and recombinant *Am*PDH1 and the impact of individual N-glycosylation sites on the recombinantly expressed enzyme. To this end a systematic set of N-glycosylation site knock-out mutants was expressed and characterized.

2. Material and methods

2.1. Chemicals

Chemicals were of the highest grade and purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. GE Healthcare (Chalfont-St. Giles, UK) was the supplier for the Phenyl-Sepharose FF chromatography resin and Thermo Fisher Scientific (Waltham, MA, USA) for restriction enzymes and Phusion polymerase. NEB-5α competent *Escherichia coli* from New England Biolabs (Ipswich, MA, USA) was used for cloning and *Pichia pastoris* X33 from Invitrogen (Carlsbad, CA, USA) for heterologous expression. Alfa Aesar & Co KG (Karlsruhe, Germany) graphite rods (AGKSP grade, Ultra "F" purity, ASTM C-6, 3.81 cm, 3.05 mm diameter) were used as working electrodes and the polymer [Os(4,4′-dimethyl-2,2′-bipyridine)$_2$(poly(1-vinylimidazole)$_{10}$)Cl$_2$]$^{+2/+}$([Os(dmbpy)$_2$(PVI)$_{10}$Cl]$^{+2/+}$, $E^{0'}$ = 320 mV *vs.* NHE) for electrode modification.

2.2. Mutagenesis and microtiter plate screening

Site directed mutagenesis was done by overlap extension PCR, with Phusion PCR Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), as reported elsewhere [23].

The site saturation library of transformed *Pichia pastoris* X33 was created according to previously described protocols [23] and screened by a modified microtiter plate expression protocol based on Weis et al. [21,24]. 300 μL BMD1 medium (100 mM potassium phosphate buffer pH 6, 1.34% yeast nitrogen base without amino acids, 0.4 μg mL^{-1} biotin, 1% D-glucose) per well were inoculated and after 64 h of incubation (25 °C, 360 rpm, 80% humidity) 300 μL BMM2 medium (100 mM potassium phosphate buffer pH 6, 1.34% yeast nitrogen base without amino acids, 0.4 μg mL^{-1} biotin, 1% methanol) were added. 70 μL BMM10 medium (100 mM potassium phosphate buffer pH 6, 1.34% yeast nitrogen base without amino acids, 0.4 μg mL^{-1} biotin, 5% methanol) were added at 73 h, 89 h and 112 h after inoculation. After 136 h the supernatant was harvested by plate centrifugation (3500 rpm, 4 °C, 10 min) and transferred to a new plate. Enzymatic activity was determined by measuring 50 μL of supernatant per well in a Fc$^+$-96-well-microtiter assay (100 mM sodium phosphate buffer pH 7.5, 0.2 mM ferrocenium hexafluorophosphate, 25 mM D-glucose, 30 °C, total volume: 200 μL).

2.3. Shake flask expression screening

Shake flask expression screening was done by modifying the microtiter plate protocol from Weis et al. [24] for 50–150 ml shake flasks. Per shake flask, 10 mL BMD1 medium was inoculated and incubated at 30 °C and 130 rpm. Induction was started by adding 2.2 mL BMM2 after 64 h. 2.2 ml, 2.4 mL and 2.4 mL BMM10 were added after 73 h, 88 h and 112 h after inoculation, respectively. The supernatant was harvested 136 h after inoculation (centrifuging cell suspension for 20 min at 4000 rpm) and measured for volumetric activity in a standard assay as described below, with a modified measuring time. After 100 s of incubation at 30 °C measurement was conducted for 900 s.

2.4. Shake flask expression re-screening

Chosen mutants were expressed at 1 L shake flask scale together with *Am*PDH1 wt as positive control and with pPICZ-B without gene insert as negative control. 100 mL BMGY (100 mM potassium phosphate buffer pH 6, 2% peptone, 1% yeast extract, 1.34% yeast nitrogen base without amino acids, 0.4 μg mL^{-1} biotin, 1% glycerol) were inoculated with pre-culture (10 mL BMGY incubated for 24 h at 30 °C and 130 rpm after inoculation) and induction was started after 27 h (1 mL 50% methanol 4 times a day). Incubation temperature was reduced to 25 °C after induction. Cell suspension samples were taken 24 h and 95 h after induction. 15 mL of the sample supernatant (4000 rpm, 20 min) were concentrated in 10 kDa Amicon ultrafiltration filter units (Millipore Corp., Billerica, MA, USA) at 4000 rpm to 0.24-0.45 mL. Volumetric activity of the concentrated supernatant was determined by standard ferrocenium assay.

2.5. Enzyme expression and purification

PDH was expressed in methanol induced *Pichia pastoris* in a stirred-tank bioreactor (7L, MBR, Wetzikon, Switzerland) and puri-