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Effect of deglycosylation on the mediated electrocatalytic activity of recombinantly expressed *Agaricus meleagris* pyranose dehydrogenase wired by osmium redox polymer

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ABSTRACT

The effect of deglycosylation of pyranose dehydrogenase (PDH) obtained from *Agaricus meleagris* (*Am*) and recombinantly expressed in *Pichia pastoris* on its electrocatalytic activity was investigated. Glycosylated (g*Am*PDH) and deglycosylated PDH (dg*Am*PDH) were immobilised on spectrographic graphite (G) simultaneously with an osmium redox polymer (Os-RP) using poly(ethylene glycol)(400) diglycidyl ether (PEGDGE) as cross-linking agent. The amperometric response to glucose, recorded at G/(Os-RP)-g*Am*PDH and G/(Os-RP)-dg*Am*PDH bioelectrodes, was optimised under flow injection conditions concerning the applied potential, enzyme loading, working pH and flow rate. The G/(Os-RP)-dg*Am*PDH bioelectrode is characterised by better kinetic and electroanalytical parameters compared with the G/(Os-RP)-g*Am*PDH bioelectrode: (i) a higher value of the maximum catalytic current density, $j_{max} = (146.6 \pm 2.6)\,\mu A\,cm^{-2}$ *vs.* $j_{max} = (80.9 \pm 1.9)\,\mu A\,cm^{-2}$; (ii) a lower value of the apparent Michaelis–Menten constant, $K_M^{app} = (2.4 \pm 0.1)\,mM$ *vs.* $K_M^{app} = (7.5 \pm 0.3)\,mM$; (iii) a higher slope of the linear domain, $(43.6 \pm 1.1)\,\mu A\,cm^{-2}\,mM^{-1}$ *vs.* $(9.74 \pm 0.16)\,\mu A\,cm^{-2}\,mM^{-1}$. Additionally, the time dependent decay of the amperometric response to glucose shows a slightly better operational stability for the G/(Os-RP)-dg*Am*PDH bioelectrode than that for the G/(Os-RP)-g*Am*PDH. The enzyme deglycosylation induces significant changes in the order of substrate selectivity for g*Am*PDH and dg*Am*PDH.
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1. Introduction

Pyranose dehydrogenase (PDH; EC 1.1.99.29) is an extracellular glycosylated enzyme (7% glycosylation) with a molecular weight of about 75,000 Da, which is produced by a small family of wood-degrading basidiomycetes [1]. It was first isolated from *Agaricus bisporus* [2] and originally mistaken for cellobiose dehydrogenase (CDH), which has similar structural and catalytical properties [3]. PDH carries one covalently bound FAD in the active site and belongs to the class of GMC [glucose-methanol-choline] oxidoreductases [4]. However, it has a flavinylation type, VAO [6-*S*-cysteinyl-8α-(N1-histidyl)-FAD] [5] different from that reported for other members of flavoprotein family [6]. The unique bicovalent flavinylation in PDH is responsible for the broader substrate specificity and variable regioselectivity of the enzyme compared to that of pyranose oxidase (POx) from the same GMC class [7].

PDH can mono- and dioxidise a variety of sugars at C-2, C-3 and C-2,3 positions to their corresponding aldonolactones or (di)dehydrosugars (aldos(di)uloses) [8–10] with equal catalytic efficiency making the enzyme attractive for applications in analytical chemistry, industrial production of rare sugars, and construction of enzymatic biofuel cells (EBFC). In comparison POx does not show such a broad substrate specificity [10].

PDH has no anomeric specificity and does not show any catalytic activity towards molecular oxygen [11]. This inability to utilise molecular oxygen as electron acceptor is advantageous for construction of EBFCs, where oxygen-active enzymes such as POx will transfer part of the subtracted electrons to oxygen rather than to the artificial mediator, resulting in the formation of hydrogen peroxide, which can damage both the enzyme and the mediator.

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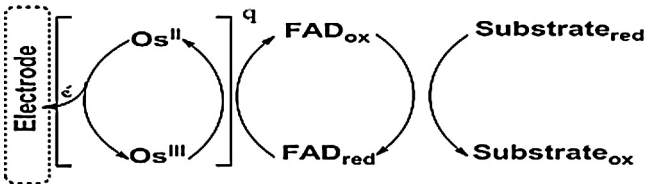


Scheme 1. Mediated electron transfer between substrate and electrode *via* (Os-RP)-PDH. *(in 1. Introduction).*

The potential application of PDH for fabrication of amperometric biosensors [12,13] and EBFCs [14] has been recently demonstrated. In these studies PDH isolated from the natural source *Agaricus meleagris* (*Am*PDH) has been used in combination with osmium redox polymer (Os-RP) as mediator. In the mediated electrocatalysis scheme (Scheme 1) Os-RP was used to shuttle electrons from the enzyme active site FAD/$FADH_2$ to the electrode surface. At the same time, it was observed that Os-RP stabilises the enzymes by forming a protective net around the protein molecules beneficial for the operational stability of the electrodes [13].

Recently, it was shown that *Am*PDH recombinantly expressed in *Pichia pastoris* [15] can successfully be applied for biotechnological applications [16] as a cheap alternative to natural *Am*PDH. However, when expressed in *P. pastoris* PDH becomes substantially more glycosylated (30%) [15], which does not promote electrochemical communication with electrodes. Thus, taking into account the beneficial effect of enzyme deglycosylation observed for both direct [17–22] and mediated electron transfer [23–25], it was demonstrated that deglycosylation of *Am*PDH increases significantly the current output of graphite electrodes modified with deglycosylated *Am*PDH and Os-RP [16].

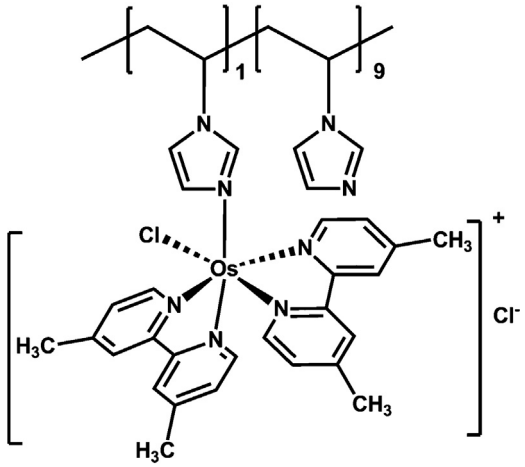
The present study aims at providing a detailed comparison between amperometric biosensors based on recombinantly expressed glycosylated (g*Am*PDH) and deglycosylated PDH (dg*Am*PDH) "wired" with Os-RP. The investigated biosensors consisted of graphite electrodes modified with the corresponding enzyme-redox polymer complexes. The enzyme modified electrodes were mounted into an electrochemical flow-through cell inserted in a single line flow-injection system and investigated in terms of operational conditions, kinetics, operational stability, and substrate selectivity.

2. Experimental

2.1. Reagents

All chemicals were of analytical grade. Poly(ethylene glycol)(400) diglycidyl ether (PEGDGE), ferricenium hexafluorophosphate ($Fc^+PF_6^-$), D-glucose, L-fucose, D-xylose, sucrose, D-cellobiose, D-mannose, D-maltose and 2-deoxy-D-glucose and D-galactose were purchased from Sigma-Aldrich (Steinheim, Germany), while lactose was obtained from Merck (Darmstadt, Germany) and silver nitrate was from Scharlau (Barcelona, Spain). The osmium redox polymer [Os(4,4′-dimethyl-2,2′-bipyridne)$_2$(poly(*N*-vinylimidazole)$_{10}$Cl]Cl (Os-RP; Scheme 2), with a formal standard potential of 320 mV *vs.* NHE, was synthesised as described elsewhere [26]. All other chemicals were obtained from Sigma-Aldrich (www.sigmaaldrich.com) if not stated otherwise.

A 50 mM phosphate buffer containing 137 mM NaCl (PB) was prepared from sodium phosphate dibasic and sodium phosphate monobasic salts; the ethanolamine-HCl buffer and Bis-Tris buffer were prepared from ethanolamine or bis-(2-hydroxy-ethyl)-amino-tris(hydroxymethyl)-methane. The pH value of the buffer solutions was adjusted with sodium hydroxide solution (Fischer Scientific Pittsburgh, Pennsylvania, USA) or hydrochloric acid. All solutions were prepared with deionised water (18.2 MΩ), purified with a Milli-Q system (Millipore, Bedford, MA, USA).



Scheme 2. Structure of the osmium redox polymer (Os-RP) [26] (in Section 2.1).

2.2. Equipment

Flow injection measurements were performed with a flow-through amperometric cell of the wall-jet type described elsewhere [27,28] containing a Pt wire as counter electrode; an Ag|AgCl (0.1 M KCl, +288 mV *vs.* NHE) as reference electrode; a spectrographic graphite rod (Ringsdorff Werke GmbH, Bonn, Germany, type RW001; 3.05 mm diameter and 13% porosity) as the working electrode. The potential applied to the working electrode was controlled by a low current potentiostat (Zäta Electronics, Höör, Sweden). The amperometric response of the working electrode was registered on a chart recorder (model BD 112, Kipp & Zonen, Utrecht, The Netherlands). The carrier flow was maintained at the desired flow rate with a peristaltic pump (Minipuls 3, Gilson, Villier-le-Bel, France). An automatic injector (Rheodyne, type 7125 LabPR, Cotati, CA, USA), equipped with a 50 μL sample loop, was used to introduce the samples.

2.3. Enzyme preparation

Pyranose dehydrogenase from *A. meleagris* (*Am*PDH) was recombinantly expressed in *P. pastoris* [15]. *Am*PDH was deglycosylated using Endo Hf (New England Biolabs, Bionordiska AB, Stockholm, Sweden) according to the manufacturer's recommendations. The volumetric activity of g*Am*PDH and dg*Am*PDH was the same (340 U mL^{-1}), while the protein concentrations were 23.8 mg mL^{-1} and 18.2 mg mL^{-1}, respectively.

The molecular weights of g*Am*PDH and dg*Am*PDH were estimated using vertical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), performed with a Bio-Rad system (Sundbyberg, Sweden) according to a previously reported procedure [29]. Briefly, the proteins were solubilised with sodium dodecyl sulphate buffer, heated for 10 min at 95 °C, loaded onto the gel and analysed under the following experimental conditions: 10% of acrylamide in resolving gel; 200 V of applied potential; MES running buffer and 1 μg of protein load per well. Finally, the gels were silver stained using the method described elsewhere [30]. The estimated molecular masses were ~90,000 Da and ~65,000 Da for g*Am*PDH and dg*Am*PDH, respectively (Fig. 1).

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