Full Paper

Amperometric Biosensors for Detection of Sugars Based on the Electrical Wiring of Different Pyranose Oxidases and Pyranose Dehydrogenases with Osmium Redox Polymer on Graphite Electrodes

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Abstract
Electrical wiring of different types of pyranose oxidase (P2O) (fungal wild type, recombinant wild type with a hexa-histidine tag, mutant form E542K with a hexa-histidine tag) from *Trametes multicolor,* and recombinant P2O from *Coriolus* sp. overexpressed in *Escherichia coli* as well as of pyranose dehydrogenase (PDH) from *Agaricus meleagris* and *Agaricus xanthoderma* with an osmium redox polymer (poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-dipyridyl)$_2$-Cl$_2$]$^{2+/+}$) on graphite electrodes was carried out. After optimization studies using glucose as substrate, the biosensors, which showed the best characteristics in terms of linear range, detection limit and sensitivity were selected, viz. wild type P2O from *T. multicolor* and PDH from *A. meleagris.* These two enzymes were used and investigated for their selectivity for a number of different sugars.

Keywords: Nonselective sugar oxidizing enzyme, Flow injection analysis, Biofuel cell anode

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1. Introduction

The recent great interest in biofuel cells [1 – 3] has caused a renewed interest in sugar oxidizing enzymes other than glucose oxidase (GOx). Even though "wiring" of GOx with Os-redox polymers [4] has reached very efficient levels, GOx still suffers from some drawbacks. GOx oxidizes glucose at the C-1 position, from which follows that only one of the possible anomeric forms is the substrate for this enzyme, i.e., the β-form, which in aqueous solutions constitutes around 64% of the total glucose content. Additionally it is still a hydrogen peroxide producing oxidase meaning that molecular oxygen will always be competing with any mediator for being the electron acceptor to the reduced form of the enzyme and there is always a risk that some hydrogen peroxide will be formed. A compartment free biofuel cell that relies on using an oxygen reducing enzyme, e.g., laccase [5] or bilirubin oxidase [6], will of course rely on molecular oxygen being present. These multicopper blue oxidases are sensitive to even trace levels of hydrogen peroxide, its presence should therefore be avoided as much as possible. There are a number of other sugar oxidizing enzymes with bound cofactors. Some of them have been previously used for biosensor constructions, e.g., hexose oxidase [7], oligosaccharide dehydrogenase [8 – 10], aldose dehydrogenase [11], cellobiose dehydrogenase [12, 13], carbohydrate oxidase [14] and pyranose oxidase [15, 16]. In the present work, electrical wiring of various forms of two different sugar oxidizing enzymes, i.e., pyranose oxidase (P2O), both wild type and a mutant, as well as one commercial P2O, and additionally pyranose dehydrogenase (PDH) from two different origins, with an osmium redox polymer, (poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-dipyridyl)$_2$Cl$_2$]$^{2+/+}$), is reported.

Both P2O and PDH are not anomer specific as they do not oxidize the sugar at the C-1. Both enzymes are able to oxidize a whole range of different sugars, which may be beneficial for future biofuel cell studies. Additionally, PDH has no activity at all with molecular oxygen and therefore could be a very good candidate for a membrane free biofuel cell with a cathode relying on laccase or bilirubin oxidase.

Pyranose 2-oxidase (P2O; synonym, glucose 2-oxidase; pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) catalyzes the oxidation of several aldopyranoses at C-2 in the presence

of molecular oxygen to yield the corresponding 2-ketoaldoses and hydrogen peroxide. The enzyme, which is typically an intracellular tetrameric nonglycosylated flavoprotein, is widespread among wood-degrading basidiomycetes and it is hypothesized that P2O participates in the process of lignin degradation by providing hydrogen peroxide, a cosubstrate for lignin peroxidases [17]. Besides this principal activity, P2O can catalyze to a lesser extent the oxidation of certain substrates at C-3 [18]. P2O may also have another biological function [19], the enzyme may be involved in the reduction of quinones in the periplasm or in the extracellular environment.

Among the various P2Os, the most detailed studies were carried out with the enzyme from *Trametes multicolor* (*T. ochracea*). This enzyme was purified and characterized, cloned and efficiently expressed in *Escherichia coli*, and its crystal structure was determined [19, 20]. A variety of P2Os from different fungal sources have also been purified and characterized and the genes encoding for different P2Os have been sequenced. So far, the nucleotide sequence of P2O from *Coriolus versicolor* (accession number E11766), *Trametes ochracea* (AY291124), *Trametes hirsuta* (AR141573), *Peniophora gigantea* (AY370876), *Peniophora* sp. *SG* (AF535193), *Tricholoma matsutake* (AB043883), *Lyophyllum shimeji* (AB119106) and *Phanerochaete chrysosporium* (AY522922) are known. The data available at present reveal some general similarities among P2O from these different fungi. Typically, the enzyme has a molecular mass of approximately 270 kDa and is composed of four identical 68 kDa subunits. Each subunit contains one covalently bound flavin adenine dinucleotide as its prosthetic group [19, 21]. P2O is an enzyme with a high potential in biotransformations of carbohydrates. The application of P2O in bioprocesses, clinical chemistry analytics, and in synthetic carbohydrate chemistry was reviewed a few years ago [21]. In the present investigation, both different types of *T. multicolor* P2O described above (fungal wild type, recombinant wild type with a hexa-histidine tag, mutant form E542K with a hexa-histidine tag) and a commercially available P2O (recombinant P2O from *Coriolus* sp. overexpressed in *Escherichia coli*) were compared as the latter was recently investigated and reported from this laboratory [16]. The replacement of Glu542 with lysine was reported to improve thermal stability and to have a beneficial effect on the kinetic properties of P2O [22].

Two different pyranose dehydrogenases (PDH, pyranose:acceptor oxidoreductase, EC 1.1.99.29) from *Agaricus meleagris* and *Agaricus xanthoderma* were additionally used as the biological component as a complementary redox enzyme to P2O. PDH also shows a nonselective substrate oxidizing profile but in contrast to P2O (and GOx) has no activity for molecular oxygen. PDH is synthesized as a highly glycosylated, extracellular, monomeric flavoglycoprotein (*A. meleagris*: MW, 66 kDa; pI, 4.2 – 4.4 (isoforms); glycosylation, 8%, *A. xanthoderma*: MW, 65 kDa; pI, 4.14 – 4.55 (isoforms); glycosylation, 5%) that does not accept oxygen as electron acceptor but instead reduces predominantly various quinones, natural and toxic products of lignin degradation, which catalyzes the quinone-dependent (di) oxidation of free (nonphosphorylated) sugars in their pyranose form to di- or tricarbonyl derivatives, aldoketoses or aldodiketoses [23]. This enzyme exhibits an extremely broad substrate tolerance and variable regioselectivity for the oxidation of both mono- and oligosaccharides. The selectivity of PDH for secondary alcoholic group(s) at the C-2, C-3, or C-2 + C-3 of the sugar is sugar substrate-dependent and changes also with the source of PDH. Thus, D-galactose was exclusively oxidized at C-2 (*A. bisporus*) [24], glucose (*M. rhacodes*) [25] and the D-glucosyl moiety of nonreducing oligosaccharides (*A. meleagris*) [26] at the C-3, whereas final C-2, C-3 double oxidation to the corresponding aldodiketose was demonstrated for D-glucose conversion by PDH from *A. bisporus* [24], and D-xylose by PDH from *A. meleagris* [27, 28].

Despite the large-scale industrial use of GOx, there are two main potential advantages in using P2O instead of GOx. The most important advantages are its high affinity for D-glucose and the lack of any anomeric preference [29 – 31]. Its ability to efficiently oxidize several sugars other than glucose may compromise its use for selective glucose monitoring. However, its use in biotechnology and in biofuel cells is envisaged as a better alternative than GOx, as many other sugars from, e.g., a lignocellulose hydrolysate can be oxidized by P2O and thus used for small scale energy production. This advantage of a broad substrate specificity is even more extended for PDH, which is amazingly promiscuous with respect to its sugar substrates, oxidizing efficiently various pentoses, hexoses, mono- and oligosaccharides, as well as various glycosides. Apart from the ability to act on monosaccharides with axial hydroxyl at C-2 (e.g., D/L-arabinose, D-mannose) and 1,4-glucooligosaccharides, other features distinguishing PDH from P2O are its preferred C-3 attack of D-glucose, its high degree of glycosylation and single polypeptide structure, unlike the homotetrameric P2O. This could make PDH a very interesting alternative to P2O for the proposed applications, e.g., in biotechnology or biofuel cells.

Graphite rod electrodes modified with the osmium redox polymer bound together with either of these two types of enzymes were placed in a flow through electrochemical cell and investigated in the flow injection mode for their efficiency to oxidize a number of different sugars. Optimization with respect to pH and buffer type was performed. Moreover, the analytical characteristics of the developed biosensors were investigated. In a previous work, the wiring of a commercial P2O was described by means of two different types of osmium polymers [16].

2. Experimental

2.1. Reagents and Materials

2.1.1. Enzymes

The three types of *Trametes multicolor* pyranose oxidase (P2O), used in this work were the following: fungal wild type

P2O (WT P2O; specific activity, 8 U/mg); recombinant wild type P2O with a hexa-histidine tag (WT HHT P2O; specific activity, 8 U/mg); mutant form E542K of P2O with a hexa-histidine tag (MT HHT P2O; specific activity, 5 U/mg). Recombinant *Coriolus* sp. P2O overexpressed in *Escherichia coli* (specific activity, 3 U/mg) was purchased from Sigma (St. Louis, MO, USA). The two types of PDH were from *Agaricus meleagris* (PDH AM; specific activity, 4 U/mg) and from *Agaricus xanthoderma* (PDH AX; specific activity, 5 U/mg). One U is defined as the amount of enzyme that converts 1 μmole of glucose per min under optimal electron acceptor conditions.

2.1.1.1. Organism and Culture Conditions

Pyranose 2-oxidase was produced either by fermentation of the wild type organism *Trametes multicolor* MB 49 from the culture collection of the Department of Applied Microbiology, University of Natural Resources and Applied Life Sciences Vienna, Austria, or recombinant *Escherichia coli* BL21 (DE 3). Recombinant *E. coli* contained either the plasmid pHL2 encoding the wild type enzyme and a C-terminal His_6-tag (WT HHT P2O) or the plasmid pCL22 encoding the E542K mutant and a C-terminal His_6-tag (MT HHT P2O) [32]. The fungus was cultivated in a 42 L laboratory bioreactor by using a medium based on whey powder and peptone from casein, to which lactose was fed as described by Leitner et al. [33]. The P2O activity routinely formed was 560 U/L, corresponding to 47 mg/L pure enzyme. The *E. coli* strain overproducing recombinant WT HHT P2O or MT HHT P2O was fermented in the same reactor using MHC-Gly medium. Before induction with 5 g/L lactose, the temperature was decreased to 25 °C to increase the production of native P2O over the formation of inclusion bodies. The obtained volumetric activities of 900 U/L equal 75 mg/L pure enzyme.

Pyranose dehydrogenase from *Agaricus meleagris* and *A. xanthoderma* was produced as previously described for another member of the genus *Agaricus* [34] by cultivating the fungi obtained from the Culture Collection of Basidiomycetes (CCBAS), Institute of Microbiology, Prague, Czech Republic, at 25 °C in static 500 mL Roux flasks. Mildly homogenized culture derived from malt-agar stock cultures was used to inoculate liquid glucose-corn steep medium. The average yield of PDH activity was 1750 U/L, corresponding to 30 mg/L pure enzyme for the *A. meleagris* culture, and 1500 U/L, equaling 28 mg/mL pure enzyme for the *A. xanthoderma* culture, respectively.

2.1.1.2. Enzyme Purification

WT P2O was purified with small deviations from the protocol given by Leitner et al. [35]. Shortly, the mycelium was separated from the culture medium by centrifugation, suspended in 50 mM phosphate buffer (pH 6.5) containing 10 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, and homogenized with an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany). Ammonium sulfate was slowly added to 30% saturation and solids were removed by centrifugation. The clear supernatant was applied to a Phenyl-Sepharose XK 50/30 column (GE Healthcare, Vienna, Austria), equilibrated with 50 mM phosphate buffer (pH 6.5) containing ammonium sulfate (30% saturation), and eluted with a linear (30 to 0%) $(NH_4)_2SO_4$ gradient. Fractions containing activity were pooled, dialyzed against 20 mM Bis-Tris buffer (pH 6.0) and loaded onto a Q-Source HR 16/10 column (GE Healthcare) equilibrated with the same buffer. P2O was eluted using a linear 0 to 500 mM KCl gradient. Active fractions were pooled, rigorously dialyzed against 10 mM Na-citrate buffer (pH 6.0), filter sterilized, and stored at −20 °C.

Recombinant *E. coli* cells containing His_6P2O were harvested by centrifugation, suspended in twice the volume of 50 mM phosphate buffer (pH 6.5) containing 10 mM imidazole and 1 M NaCl, and homogenized in a APV 2.000 homogenizer (APV Systems, Albertslund, Denmark) applying a pressure drop of 100 MPa. Cell debris was removed by ultracentrifugation (30,000 g for 30 min) and the sample applied to a Ni-Sepharose XK 50/30 column (GE Healthcare) column equilibrated with the same buffer. The His-tagged enzyme was eluted by a linear (10 mM to 500 mM) imidazole gradient. Fractions containing P2O were pooled, rigorously dialyzed against 50 mM phosphate buffer (pH 6.5), sterile filtered, and stored at −20 °C.

Pyranose dehydrogenase was purified by decanting the culture broth from mycelial pellets. The extracellular enzyme was then precipitated by ammonium sulfate (100% saturation), added slowly at 0 °C. After centrifugation (10000 g for 30 min) the pellet was resuspended in 20 mm Bis-Tris buffer (pH 6.0) and diafiltrated to remove low molecular weight phenolic compounds and to reduce the conductivity below 2 mS cm. The sample was applied to a EMD-DEAE-Fractogel 650 S column (5 × 30 cm) (Merck, Darmstadt, Germany), equilibrated with the same buffer and eluted by a linear (0 to 1 M NaCl) salt gradient. Fractions containing significant activity were pooled and foreign protein precipitated by slow addition of ammonium sulfate (50% saturation) at 0 °C. The solids were removed by centrifugation and the clear supernatant was loaded onto a PHE Source 15 (GE Healthcare) column, equilibrated with 50 mM phosphate buffer (pH 6.5) containing 295 g/L ammonium sulfate. The active enzyme was eluted by a linear (50 to 0%) $(NH_4)_2SO_4$ gradient. Fractions containing PDH activity were pooled, rigorously dialyzed against 50 mM phoshate buffer (pH 7.5), sterile filtered, and stored at −20 °C.

2.1.2. Other Chemicals

Poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-di'pyridyl)$_2$ Cl_2]$^{2+/+}$ (osmium redox polymer) was generously provided as a gift from TheraSense (Alameda, CA, USA). Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA, USA). L(+)-arabinose, D(+)-fucose, D(+)-fructose, D(+)-galactose, D(+)-glucose, glucosamine, D(+)-mannose, D(+)-cellobiose, D(+)-xylose, D-maltose, D-trehalose, β-lactose, 2-deoxy-D-glucose, 2-deoxy-D-galactose, D-maltotriose D-

maltoheptaose, D-maltopentaose were all of analytical grade and obtained from Sigma. D(+)-xylose and sucrose were purchased from ICN Biomedicals Inc. (Aurora, Ohio). Standard solutions of the various sugars were prepared by dissolving appropriate amounts in the carrier buffer, see below. The water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All solutions used for immobilization were prepared in Milli-Q water and the others used as substrate were in working buffer solutions and degassed before use to avoid air bubbles in the flow system.

2.2. Equipment

For the flow injection measurements the electrodes were mounted into a flow-through amperometric cell of the wall-jet type [36] containing a platinum wire counter electrode and an Ag | AgCl (0.1 M KCl) reference electrode. The potential of the working electrode against the reference electrode was kept at the required value (+ 300 mV vs. Ag | AgCl) using a three electrode potentiostat (Zäta Elektronik, Höör, Sweden). The electrode response was registered with a recorder (BD 112, Kipp & Zonen, Delft, The Netherlands). Samples were injected with an injector (Rheodyne, type 7125 LabPRO, Cotati, CA, USA) supplied with an injection loop of 25 μL.

A 0.1 M ethanolamine, Tris-HCl or PBS buffer at various pHs was used as the carrier in the flow system and pumped at a flow rate of 1.0 mL min^{-1} (Minipuls 2, Gilson, Villier-le Bel, France). Before use the carrier buffer was thoroughly degassed under vacuum to prevent microbubbles to appear in the flow system. Alternatively, the ethanolamine carrier buffer at pH 9.2 was saturated with oxygen at 35 °C and then cooled to room temperature and used to compare the influence of oxygen on the response to glucose for the WT P2O modified electrode. Connections between the various parts were made with Teflon tubings, i.d. 0.5 mm, and Altex screw couplings. Amperometric measurements were performed at an applied potential of + 300 mV vs. Ag | AgCl [16].

2.3. Preparation of the Biosensors

Graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were polished on wet emery paper (Tufback Durite, P1200) [37, 38] washed thoroughly with Milli-Q water, sonicated for 2 min, and then rinsed with Milli-Q water and dried in an oven at 105 °C. Poly(1-vinylimidazole)$_{12}$-[Os(4,4-dimethyl-2,2-di'pyridyl)$_2$Cl$_2$]$^{2+/+}$ (osmium redox polymer) was dissolved in Milli-Q water at a concentration of 10 mg/mL. A premixed solution including 2.0 μL of redox polymer (2 pg), 1.0 μL of a freshly prepared poly(ethylene glycol) diglycidyl (PEGDGE) solution (2.5 mg/mL in water) was placed on top of the polished end of the electrode and spread evenly using the microsyringe tip. After a waiting time of 10 min, 5 μL of the enzyme solution (equal to 0.4 U), unless stated otherwise, was evenly spread on top of the first layer of the electrode and allowed to stand overnight at 4 °C and constant humidity for complete cross-linking reaction before mounting the electrode into the electrochemical cell. Each enzyme was dissolved by diluting the proper amount of enzyme with phosphate buffer to a final concentration of 80 U/mL and this solution was used as stock enzyme solution. Electrode preparations for later use were kept at 4 °C [16].

3. Results and Discussion

3.1. Cyclic Voltammetry of the Osmium Redox Polymer

The osmium redox polymer [39] was used for electrical wiring of the four different types of P2O and the two different types of PDH to the graphite electrodes. Polymeric mediators have been successfully used to wire a series of different redox enzymes and applied for construction of a variety of biosensors [1, 4 – 6, 39 – 41]. Recently, they were shown also to wire whole living *Gluconobacter oxydans* cells [42]. The possibility to wire P2O to graphite electrodes was anticipated [16] and the experimental results obtained showed that a fairly sensitive biosensor based on P2O and the redox polymer can be fabricated for multianalyte detection. The formal redox potential ($E^{\circ\prime}$) of the polymer was determined to be + 140 mV (vs. Ag | AgCl). This value agrees well with the literature value [39] and with our previous results [16, 42]. In an easily electrically "wired" enzyme connected to the electrode, the current measured as the analytical response signal represents the actual turnover rate of the enzyme. Unless the maximum turnover rate is reached, the turnover and the current increase linearly with the diffusional flux of substrate and with substrate concentration. In other words, the concentration of the substrate is transduced to the measured electrical current. The proper working potential to use in the flow system was chosen according to the CV results shown in Figure 1. In the absence of any enzyme the anodic peak has a potential of about + 200 mV (Fig. 1a). When coimmobilized with PDH AM (Fig. 1b) the whole wave is slightly moved with around 50 mV into a more negative region most certainly reflecting strong interactions between the redox polymer and the enzyme. The less amount of electrochemically active $Os^{2+/3+}$-groups seen is due to the shielding effect when cross-linked to the enzyme. An even further decrease in electroactivity of the same taken amount of redox polymer (Fig. 1c), when WT P2O was used, is possibly the result of that the enzyme molecules are bigger than those of PDH AM. However, the shift in $E^{\circ\prime}$ is less than when PDH AM was used. For both enzyme systems the amperometric measurements were performed at + 300 mV vs. Ag | AgCl in further experiments, so that the osmium polymer is completely present in its oxidized form using either of the enzymes.

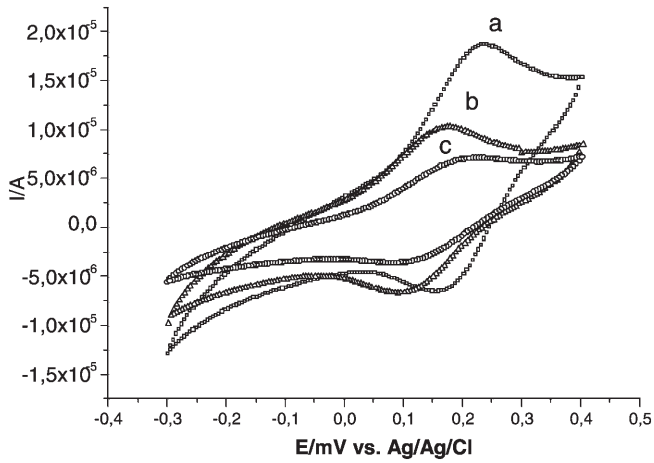

Fig. 1. Cyclic voltammogram of poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-dipyridyl)$_2$Cl$_2$]$^{2+/+}$ (osmium redox polymer) on graphite electrodes: a) with no enzyme, b) crosslinked with 0.4 U of PDH AM, and c) crosslinked with 0.4 U of WT P2O. Experimental conditions: 10 mV/s, 100 mM ethanolamine buffer, pH 9.4.

3.2. Optimization of the Biosensor Systems

3.2.1. Effect of pH

The optimum pH-buffer combination for the P2O systems from *T. multicolor* was found to be at about 10 – 10.5 in ethanolamine buffer and at about 8 in PBS buffer for P2O from Sigma, as already reported in a previous work [16]. For the PDH systems the optimum pH-buffer combination was found to be at about 9 – 9.5 in Tris-HCl buffer and ethanolamine buffer for PDH from *A. xanthoderma* and from *A. meleagris*, respectively. Figures 2 – 5 show the results obtained from the pH optimization studies of the biosensors. It is important to note that the activity and pH profile of all the investigated forms of both P2O and PDH are very much

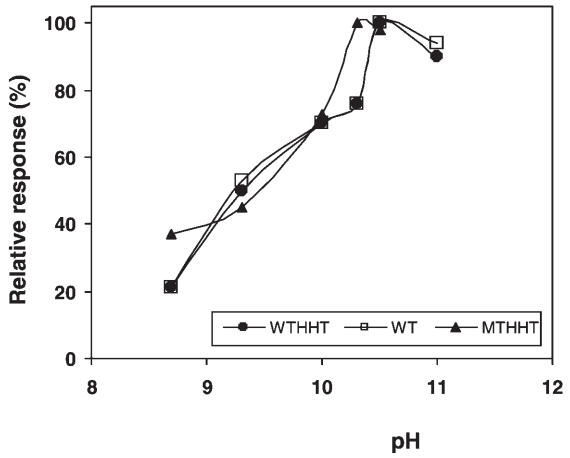

Fig. 2. Effect of pH on the response of P2O based biosensors. Experimental conditions: pH 7.5 – 9 Tris-HCl, pH 8.5 – 11 ethanolamine buffer; applied potential +300 mV vs. Ag | AgCl; 1 mM glucose for WTHHT and 0.1 mM for WT and MTHHT; flow rate 1.0 mL min^{-1}.

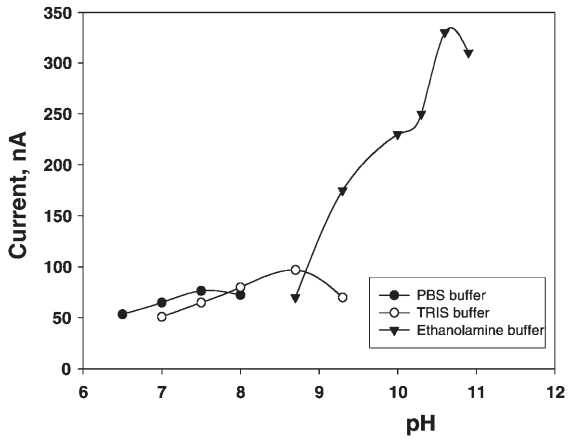

Fig. 3. Effect of different buffers and pHs on the response of a WT P2O based biosensor. Experimental conditions: pH 6.5 – 8 phosphate buffer, pH 7 – 9.3 Tris-HCl buffer, pH 8.5 – 11 ethanolamine buffer; applied potential 300 mV vs. Ag | AgCl; glucose concentration 0.1 mM, flow rate 1.0 mL min^{-1}.

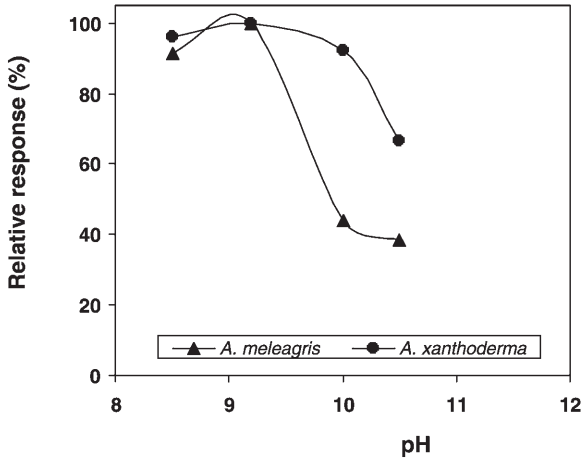

Fig. 4. Effect of pH on the response of PDH based biosensors. Experimental conditions: in ethanolamine buffer (0.1 M), applied potential +300 mV vs. Ag | AgCl; glucose 1 mM; flow rate 1.0 mL min^{-1}.

influenced by the buffer components used. In ethanolamine buffer the pH optima are all found in the more alkaline region and with responses several times higher than at the pH optima found for the other two buffer systems used.

3.2.2. Effect of Amount of Enzyme

The effect of the amount of enzyme on the electrode response was tested using WT P2O (Fig. 6). The properties of the biosensors depend on the enzyme activity used, and the optimum amount was found to be 5 μL, corresponding to 0.4 U of enzyme, prepared as described in Experimental. When lower and higher amounts of the enzyme were employed the current response of the biosensor decreased. A lower amount of enzyme leads to less enzyme molecules entrapped in the hydrogel and therefore to a lower current response. A lower response was also observed when an

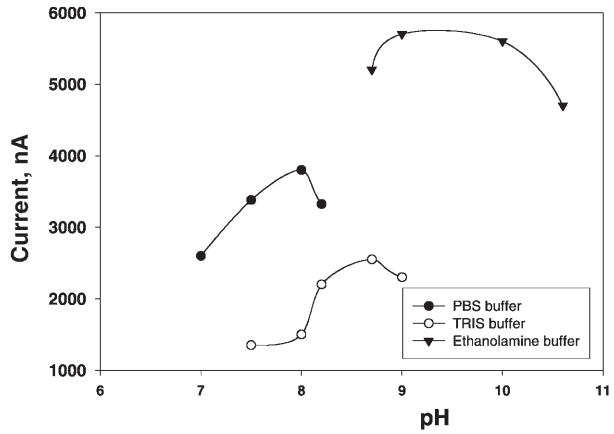

Fig. 5. Effect of different buffers and pHs on PDH from *Agaricus meleagris* based biosensors response. Experimental conditions: pH 7–8.5 phosphate buffer, pH 7.5–9 Tris-HCl, pH 8.5–10.5 ethanolamine buffer; applied potential +300 mV vs. Ag | AgCl; glucose concentration 1 mM; flow rate 1.0 mL min^{-1}.

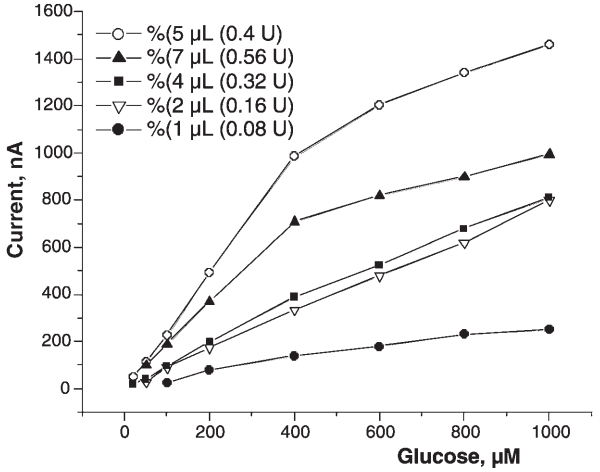

Fig. 6. Effect of amount of WT P2O on the electrode response. Experimental conditions: in ethanolamine buffer (0.1 M, pH 10.5), +300 mV vs. Ag | AgCl.

amount higher than 5 µL was employed. This could be due to the fact that the presence of a larger amount of enzyme causes a decrease of mediator molecules involved due to diffusion problems and therefore a reduction of the electron transfer capability, resulting in a lower current response. Similar observations were found for all the other enzymes studied (data not shown). Further investigations were therefore pursued with an amount of 0.4 U of enzyme per electrode.

3.3. Analytical Characteristics

The analytical characteristics for glucose of the biosensors based on the various forms of P2O and PDH are summarized in Table 1. The biosensor based on PDH AM showed the best results with the largest linearity (5–1000 µM), the lowest LOD (5 µM) and the highest sensitivity (6.01 nA/µM). On the other hand, among the P2O-based biosensors, the WT P2O gave the better analytical characteristics when considering the linear range (10–400 µM), LOD (8 µM) and higher sensitivity (2.48 nA/µM) in comparison with the other P2O-based biosensors. The commercial P2O preparation was also tested in the operational conditions, but performed poorer than WT P2O in terms of LOD and sensitivity. The values of the apparent Michaelis–Menten constant, K_M^{app}, were also evaluated using the direct fitting approach (Table 1). It is interesting to note that the sensors based on WT P2O and its His-tagged equivalent have virtually the same value, whereas the sensor based on MTHHP P2O is higher, i.e., has less affinity for glucose. Sensors based on the two PDHs have values in the same range even though the one based on PDH AM has a sensitivity ten times higher.

Since the WT P2O- and PDH-AM based biosensors showed the best characteristics in terms of linearity, detection limit and sensitivity, these systems were selected for screening the selectivity for some other carbohydrates. The results obtained with these two systems are shown in Tables 2 and 3, respectively. Only sugars, which exhibited a response, are included in the tables. The PDH AM-based biosensor can detect cellobiose, maltotriose, trehalose and xylose with lower LOD values as well as better linearity in comparison with the P2O-based systems. Moreover, mannose, glucosamine, lactose, sucrose and maltose did not cause any response signal in the P2O-based system in contrast to the PDH-based biosensor. It is interesting to note that even if though maltose is not a substrate for the WT P2O-based sensor, higher maltooligosaccharides are rather good substrates. As the degree of polymerization (DP) increases the sensitivity increases and the K_M^{app} value decreases reflecting that a higher DP has a higher affinity for the enzyme. For the PDH AM-based sensor the picture looks very much different for the maltosaccharides. Maltose gives a moderate response. When increasing the DP to 3 the response increases around 5 times. However, when further increasing the DP to 4 the response decreases and continues to decrease for DP 5. In all 13 sugars could be efficiently oxidized by WT P2O and 17 sugars could be oxidized by PDH AM. Fructose was the only sugar investigated, which was not detected by either of these two biosensors, which is expected as it is not a pyranose. Further investigations will reveal more clearly trends in turnover rate (sensitivity) and affinity (K_M^{app}) with structural features and DP values of the sugar.

To test the influence of oxygen on the response to glucose for the WT P2O modified electrode ethanolamine buffer pH 9.2 accurately degassed was pumped through the FIA system and the outcoming current was registered as baseline. Then the same ethanolamine buffer but saturated with oxygen at 35^0C and cooled to room temperature was used. In this case the outcoming current was about 5 nA lower than the one registered with the degassed buffer. The same experiment with the same buffers was carried out also after

Table 1. Comparison of results with the different biosensors based on P2O and PDH enzymes using glucose as substrate. A 0.1 M ethanolamine buffer at pH 10.5 was used for WT P2O, WT HHT P2O, MT HHT P2O, 0.1 M PBS buffer at pH 8 for P2O (Sigma), and 0.1 M ethanolamine buffer at pH 9.4 for the two PDHs.

Enzyme	Linear range (μM)	Sensitivity (nA/μM)	LOD [a] (μM)	Equation	R^2	K_M^{app} (mM)	No. of tested electrodes
WT P2O	10 – 400	2.48 ± 0.03	8	$y = 2.4823x - 7.8767$	0.9997	0.32 ± 0.01	5
WT HHT P2O	10 – 400	1.50 ± 0.01	5	$y = 1.5013x - 2.6747$	0.9998	0.35 ± 0.02	5
MT HHT P2O	100 – 1000	0.05 ± 0.01	72	$y = 0.0505x + 5.1637$	0.9987	1.51 ± 0.29	5
P2O (Sigma)	100 – 4000	0.06 ± 0.02	311	$y = 0.056x + 7.5$	0.9986	2.72 ± 0.19	5
PDH (*A. xanthoderma*)	10 – 400	0.64 ± 0.01	11	$y = 0.6396x + 5.7688$	0.9995	0.44 ± 0.03	5
PDH (*A. meleagris*)	5 – 1000	6.01 ± 0.22	5	$y = 6.0143x + 26.599$	0.9998	0.65 ± 0.01	5

[a] The limit of detection (LOD) was calculated using S/N.

Table 2. Carbohydrate analyses using the WT P2O based biosensor. Experimental conditions: + 300 mV vs. Ag | AgCl; 100 mM ethanolamine buffer, pH 10.5, flow rate 1 mL/min.

Sugar	Linear range (mM)	Sensitivity (nA/mM)	LOD (mM)	Equation	R^2	K_M^{app} (mM)	No. of tested electrodes
2-Deoxy-D-galactose	1 – 20	17.57 ± 0.27	0.53	$y = 17.574x + 5.581$	0.9997	10.32 ± 1.14	3
2-Deoxy- D-glucose	1 – 20	31.50 ± 1.61	1.76	$y = 31.509x - 17.339$	0.9973	11.85 ± 1.79	3
Arabinose	1 – 10	7.23 ± 0.43	1.01	$y = 7.2346x + 6.693$	0.9965	1.55 ± 0.10	3
Cellobiose	0.01 – 1	59.12 ± 0.20	0.0052	$y = 59.124x - 11.102$	0.9999	0.71 ± 0.13	3
Fucose	1 – 20	6.48 ± 0.15	0.8	$y = 6.485x + 21.884$	0.9994	6.25 ± 0.77	3
Galactose	0.5 – 10	1.61 ± 0.05	0.5	$y = 1.617x + 4.946$	0.9999	11.81 ± 1.07	3
Maltoheptaose	0.5 – 10	18.36 ± 0.17	0.16	$y = 18.356x + 7.029$	0.9999	6.02 ± 0.88	3
Maltopentaose	0.1 – 10	9.64 ± 0.19	0.26	$y = 9.645x + 15.369$	0.9999	6.14 ± 0.80	3
Maltotriose	1 – 10	6.84 ± 0.13	0.32	$y = 6.847x - 6.124$	0.9999	6.61 ± 0.77	3
Trehalose	1 – 20	9.31 ± 0.21	0.77	$y = 9.307x + 5.487$	0.9994	7.51 ± 0.85	3
Xylose	0.5 – 10	11.63 ± 0.29	0.42	$y = 11.62x + 3.821$	0.9996	6.21 ± 1.05	3

Table 3. Carbohydrate analyses using *A. meleagris* PDH based biosensor. Experimental conditions: + 300 mV vs. Ag | AgCl; 100 mM ethanolamine buffer, pH 9.4; flow rate 1 mL/min.

Sugar	Linear range (mM)	Sensitivity (nA/mM)	LOD (mM)	Equation	R^2	K_M^{app} (mM)	No. of tested electrodes
2-Deoxy-D-galactose	1 – 20	26.721 ± 0.85	0.61	$y = 26.721x - 4.180$	0.9994	12.14 ± 1.54	3
2-Deoxy-D-glucose	0.05 – 1	23.254 ± 0.14	0.01	$y = 23.254x + 12.825$	0.9993	0.65 ± 0.07	3
Arabinose	1 – 20	6.713 ± 0.29	1.48	$y = 6.713x + 7.438$	0.9981	8.63 ± 0.76	3
Cellobiose	0.005 – 2	53.207 ± 2.08	0.0011	$y = 53.207x + 0.125$	0.9977	1.05 ± 0.02	3
Fucose	1 – 20	9.001 ± 0.43	1.66	$y = 9.009x + 16.410$	0.9976	12.31 ± 1.52	3
Galactose	0.5 – 10	3.069 ± 0.12	0.699	$y = 3.069x - 1.222$	0.9982	7.16 ± 0.85	3
Glucosamine	0.005 – 1	32.95 ± 0.51	0.0021	$y = 32.951x - 0.309$	0.9995	0.74 ± 0.08	3
Lactose	0.5 – 15	18.06 ± 0.49	0.683	$y = 18.057x - 0.156$	0.9989	10.84 ± 0.91	3
Maltoheptaose	0.05 – 1	21.46 ± 0.57	0.044	$y = 21.461x + 2.974$	0.9992	0.51 ± 0.14	3
Maltopentaose	0.1 – 1	94.59 ± 2.55	0.046	$y = 94.592x + 16.983$	0.9985	0.64 ± 0.13	3
Maltose	0.01 – 0.5	28.671 ± 1.09	0.019	$y = 28.671x - 41.823$	0.9987	0.34 ± 0.05	3
Maltotriose	0.005 – 1	133.29 ± 1.06	0.0012	$y = 133.286x - 0.199$	0.9999	0.64 ± 0.09	3
Mannose	0.1 – 1	96.31 ± 5.33	0.111	$y = 96.311x + 3.967$	0.9983	0.62 ± 0.09	3
Sucrose	0.01 – 0.5	44.35 ± 1.99	0.034	$y = 44.351x - 83.043$	0.9979	0.35 ± 0.09	3
Trehalose	0.005 – 0.05	44.43 ± 0.18	0.0035	$y = 44.435x - 0.5128$	0.9981	0.05 ± 0.01	3
Xylose	0.005 – 10	11.23 ± 0.22	0.0023	$y = 11.234x - 4.123$	0.9988	5.84 ± 0.94	3

the addiction of glucose to the solutions in order to obtain a 1 mM glucose buffer solution. With the degassed solution a current of 1560 nA was obtained, while with the oxygen saturated solution the current response was 1550 nA. This small loss of current shows that the Os-redox polymer competes very well with molecular oxygen in the electron collecting process.

Our results show that it is possible to construct fairly sensitive second generation biosensors by wiring all the investigated forms of P2O and PDH with the Os redox polymer for multianalyte analysis of common sugars. The present detection systems can suitably be used in a chromatographic analysis set-up as was previously reported with P2O [15]. Especially the use of PDH enhanced the electrode response for glucose compared with that when using P2O. The reproducibility of the WT P2O- and PDH AM-based biosensors was tested in flow injection mode by using 0.1 mM glucose as substrate and 25 subsequent injections. The standard deviation (*SD*) and variation coefficient (*cv*) were calculated to be ± 0.004 mM and 4.4% and ± 0.002 mM and 1.8% for the WT P2O- and PDH AM-based biosensors, respectively.

Storage stability experiments were performed with the WT P2O- and PDH AM-based biosensors under dry and wet storage conditions at 4^0C. Under dry storage conditions, both biosensors retained almost 100% of their original activity after 2 days and about 90% after one week. In contrast, when the enzyme electrodes were stored in their working buffer solutions, their activities were decreased by about 10% after only one day of storage and more than 50% after one week. The lower stability showed in the second case is likely due to the leakage of the enzyme or the mediator from the bioactive membrane layer. Alternatively, the rather high pH values of the buffers could result in inactivation.

4. Conclusions

The investigated P20 and PDH-based biosensors using an Os-redox polymer allowed carbohydrate analysis with high sensitivity due to the fast electron collection efficiencies of the Os-redox polymer that in the case of P2O compete very well with that of molecular oxygen. Comparison of results obtained with a carefully degassed carrier with those obtained with a carrier buffer in equilibrium with air did not show any significantly different results. Moreover, the nonselectivity of both enzymes suggests the application of these two systems for either the determination of various sugars or as the enzyme in biofuel cell anodes. For both the WT P2O- and PDH AM-based biosensors it was possible to convert several of the sugars much more efficiently than glucose, thus exhibiting higher electrode sensitivities. Another advantage of using these enzymes is that they do not exhibit any anomeric selectivity for the α- and β-forms of the sugars in contrast to the commonly used GOx. All these characteristics together with the high sensitivity, good stability and reproducibility of these biosensors, simplicity and low cost of manufacturing make them new interesting alternatives for biotechnological applications including biofuel cell anodes.

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