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EFFECT OF ENZYME DEGLYCOSYLATION ON THE AMPEROMETRIC DETECTION OF GLUCOSE AT PDH-MODIFIED ELECTRODE

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ABSTRACT. The effect of deglycosylation of pyranose dehydrogenase (PDH), obtained from *Agaricus meleagris* and recombinantly expressed in *Pichia pastoris*, on the amperometric detection of glucose was investigated. Glycosylated (gPDH) and deglycosylated (dgPDH) PDH were immobilized on spectrographic graphite (G) simultaneously with an Os redox polymer (Os-RP). The amperometric response of G/Os-RP/gPDH and G/Os-RP/dgPDH to glucose was recorded using flow injection measurements and cyclic voltammetry. A significant increase in the maximum catalytic current density was observed for G/Os-RP/dgPDH [(148.7 ± 0.14) μA/cm^2) compared with G/Os-RP/gPDH [(81.4 ± 1.4) μA/cm^2]. Additionally, the deglycosylation of the enzyme resulted in a higher substrate-enzyme affinity (K_M^{app} = 2.44 ± 0.10 mM), compared with glycosylated PDH (K_M^{app} = 7.52 ± 0.34 mM).

Keywords: *pyranose dehydrogenase, enzyme deglycosylation, glucose amperometric detection, Os-redox polymer*

INTRODUCTION

PDH (EC 1.1.99.29) is a monomeric sugar oxidoreductase and is produced in a narrow group of fungi, classified based on ecophysiological characteristics as litter-decomposing fungi. PDH contains a covalently bound flavin adenine dinucleotide (FAD) [1] as a prosthetic group and was originally isolated from the edible mushroom *Agaricus bisporus* [2-4]. Beside *Agaricus bisporus* it was also purified form *Agaricus xanthoderma* [5]*, Agaricus rhacodes [6]* and *Agaricus meleagris* (*Am*) [1, 7].

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Structurally and catalytically PDH is related to two other enzymes: pyranose oxidase (POx) and cellobiose dehydrogenase (CDH). However, in contrast to POx, PDH displays broader substrate specificity and variable regioselectivity. Moreover, PDH is inactive with molecular oxygen as electron acceptor.

Depending on the substrate and enzyme source PDH is able to catalyze mono- as well as dioxidation reactions. Monoxidation occurs at C-1, C-2 or C-3 position and dioxidation at C-2,3 or C-3,4 positions [8]. All these characteristics made PDH very attractive for utilization in membraneless enzymatic biofuel cells (EBFCs). Another feature of PDH is its ability to work under physiological conditions (pH ~7), which is a significant advantage in the construction of implantable EBFCs.

Due to these unique properties, PDH was found to be very attractive for a number of technological applications and, consequently, its simple and effective production was considered. Thus, the *Am*PDH gens were expressed in various heterologous expression hosts such as *Aspergillus spp*. [9]*, Escherichia coli (E. coli)* and *Pichia pastoris (P. pastoris)* [10].

The expression in *Aspergillus spp.* was shown to be time consuming and involving a rather complicated genetic manipulation process. At the same time, PDH expression in *E. coli* resulted in no soluble or active enzyme [10]. A successful expression of *Am*PDH using *P. pastoris* as host organism was recently reported [10], and this approach allowed a large-scale production of PDH based on a simplified purification scheme. The MW of *Am*PDH expressed in *P. pastoris* was determined by SDS-PAGE to be ~93 kDa with an overglycosylation of ~30%, whereas the native *Am*PDH has a MW of ~66.5 kDa with 7 % glycosylation [1].

The removal of the glycan shell from the enzyme molecule decreases the distance between the enzyme redox center and the electrode surface [11-14] and, according to the Marcus equation [15], direct electron transfer should be facilitated. Therefore, *Am*PDH was deglycosylated with endoglycosidase H_f (Endo H_f) and the effect was studied in both direct and mediated electron transfer mode [16]. It was found that by removing the glycan shell from the *Am*PDH one can achieve direct electron transfer between the bound FAD in the enzyme active site and the electrode [16].

At the same time, it was shown that deglycosylation of *Am*PDH has a favorable effect on the biocatalytic oxidation of glucose.

Furthermore, aiming at investigating the use of PDH in the development of EBFCs, PDH was immobilized together with different Os redox polymers [17, 18]. Additionally, in order to increase the catalytic current output as well as the coulombic efficiency, PDH was co-immobilized with CDH [19].

In this work a comparative study of the behavior of glycosylated (gPDH) and deglycosylated PDH (dgPDH) was carried out by the separate immobilization of the above mentioned enzymes, coupled with an Os redox polymer (Os-RP), onto the surface of spectrographic graphite electrodes.

The bioelectrocatalytic activity for glucose oxidation of the so obtained modified electrodes was investigated by using flow injection (FI) and cyclic voltammetry (CV) measurements under different experimental conditions.

RESULTS AND DISCUSSION

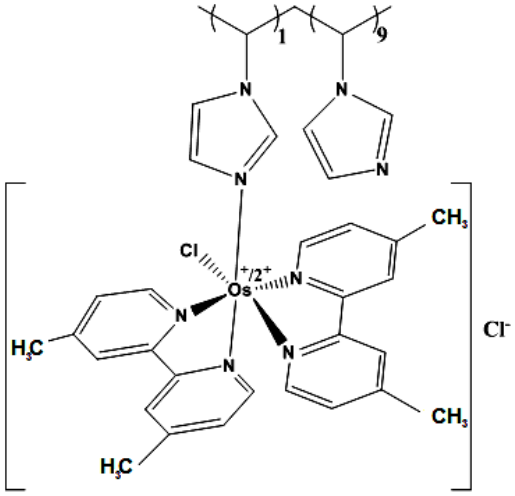
Optimization of bioelectrodes

The effect of deglycosylation on the bioelectrocatalytic activity of PDH was investigated by carrying out the immobilization of gPDH or dgPDH on the surface of graphite electrode. The enzyme immobilization was done by simple adsorption of the dissolved enzymes from a mixture containing the Os-RP and a cross-linking agent [poly(ethylene glycol) diglycidyl ether 400] (PEGDGE). In order to perform a reliable comparison, equal amounts (in terms of activity) of gPDH and dgPDH were deposited on the electrode surface (for more details see the Experimental section).

It is well known that an efficient electron transfer between the enzyme redox center, the redox mediator and the electrode surface requires a careful choice of the value of the applied potential. Thus, taking into account that the value of the formal standard potential (E°′) of the mediator must be ~50 mV higher than the corresponding value of the enzyme redox center [20], an Os-RP (Scheme 1) having a E°′ value of +32 mV *vs.* Ag|AgCl, 0.1M KCl was chosen to connect the enzyme redox center to the electrode. At the same time, the E°′ value of the selected Os-RP was more positive with ~170 mV than that of the bound FAD cofactor of AmPDH (−140 mV *vs.* Ag|AgCl, 0.1 M KCl (pH 7.4); [19]).

In order to find the optimum value for the applied potential, the amperometric response of the G/Os-RP/gPDH and G/Os-RP/dgPDH modified electrodes was recorded operated under flow conditions (a constant flow rate of 0.45 mL/min) at different applied potentials, gradually varied from -200 to +300 mV *vs.* Ag|AgCl, 0.1 M KCl in steps of 50 mV. For each value of the applied potential, the current response of the investigated modified electrodes was monitored during the injection of 50 µL of 5 mM glucose solution (Figure 1). Glucose was chosen as substrate for all measurements performed with the modified bioelectrodes because it was shown that glycosylated PDH exhibits a high activity towards this substrate [18].

As can be seen in Figure 1, the bioelectrocatalytic current recorded for both modified electrodes becomes significant at potential values higher than -50 mV *vs*. Ag|AgCl, 0.1M KCl. Furthermore, an increase in the applied potential results in an increase in the amperometric response until values higher than +200 mV *vs.* Ag|AgCl, 0.1M KCl when a plateau is reached. Consequently, in order to achieve the highest sensitivity for all investigated bioelectrodes an applied potential of +200 mV *vs.* Ag|AgCl, 0.1M KCl was used for all further measurements.



Scheme 1. Structure of the Os redox polymer (Os-RP).

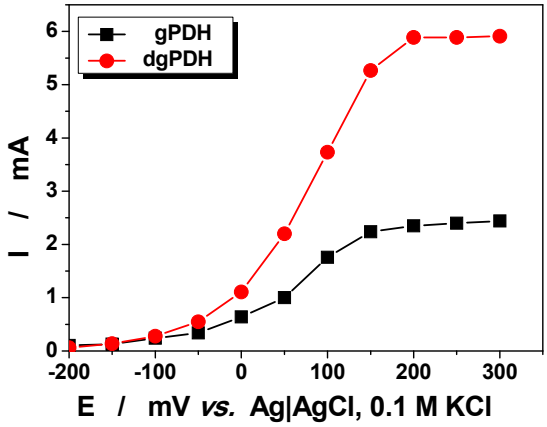


Figure 1. Dependence of the biocatalytic current on the applied potential observed at G/Os-RP/gPDH and G/Os-RP/dgPDH modified electrodes. *Experimental conditions: supporting electrolyte, 50 mM PB containing 137 mM NaCl (pH 7.4); flow rate, 0.45 mL/min; injected sample, 50 µL of 5 mM glucose.*

At the same time, the data from Figure 1 show that the maximum current response recorded at G/Os-RP/dgPDH electrode is approximately two-fold higher than that measured at G/Os-RP/gPDH. This behavior indicates that the deglycosylated PDH possesses a much higher bioelectrocatalytic activity than the glycosylated PDH.

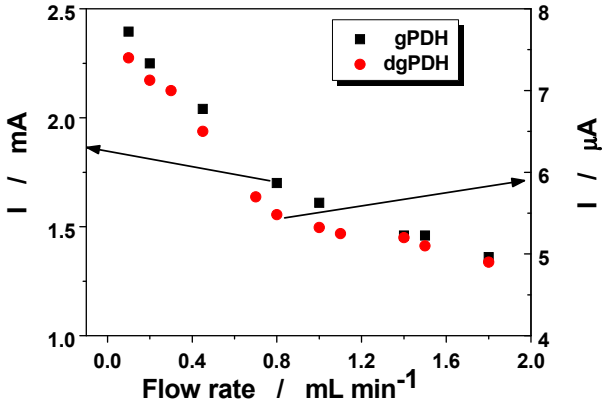


Figure 2. Influence of the flow rate on the amperometric response recorded at G/Os-RP/gPDH and G/Os-RP/dgPDH modified electrodes.
Experimental conditions: applied potential, 200 mV vs. Ag|AgCl, 0.1M KCl; supporting electrolyte, 50 mM PB containing 137 mM NaCl (pH 7.4); injected sample, 50 µL of 3 mM glucose.

PDH from *Agaricus meleagris* can oxidize D-glucose transiently to 2-dehydro-D-glucose or 3-dehydro-D-glucose and, further, to 2,3-didehydro-D-glucose [8]. Therefore, taking into account this complex behavior of PDH it was interesting to evaluate the influence of the flow rate on the current response of the modified bioelectrodes (Figure 2). The increase in the flow carrier rate leads, as expected for FI measurements, to a clear decrease into the current response as the time for the enzyme reaction to occur at the electrode surface decreases and also the enzyme product, which for glucose as substrate is also a substrate for PDH, is more rapidly removed from the electrode surface. Thus, for example at 1.8 mL/min for G/Os-PR-gPDH modified electrodes the decrease was ~50 % and for G/Os-PR-dgPDH modified electrodes was ~30 %. In order to choose an optimum value of the flow rate (high enough for shortening the time spent for a measurement, but not too high to significantly diminish the bioelectrode sensitivity), all further measurements were performed at a flow rate of 0.45 mL/min. For this flow rate value both types of biosensors showed a minor decrease in the current response (less than 10%).

Bioelectrodes characterization

Cyclic voltammograms recorded at both modified electrodes, in the absence and presence of glucose (Figure 3), gave evidence for the electrocatalytic activity of the investigated bioelectrodes. In Table 1 are summarized the values for the catalytic efficiency [CE = 100*($I_{p,substrate}$ – $I_{p,0}$)/ $I_{p,o}$, where $I_{p,substrate}$ and $I_{p,0}$ stand for the peak current in presence and in absence

of the substrate, respectively, estimated at the same potential value]. It can be noticed that the catalytic efficiency corresponding to the G/Os-RP/dgPDH modified electrode is slightly higher than the value estimated for the G/Os-RP/gPDH modified electrode.

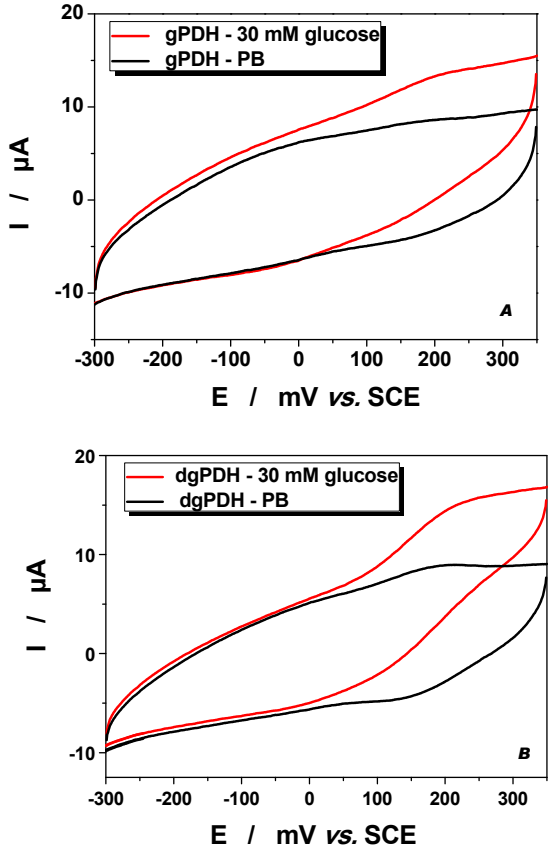


Figure 3. CV response recorded at G/Os-RP/gPDH (A) and G/Os-RP/dgPDH (B) modified electrodes in absence and in presence of 30 mM glucose. *Experimental conditions: supporting electrolyte, 50 mM PB containing 137 mM NaCl (pH 7.5); scan rate, 10 mV/s; starting potential, -0.300 V vs. SCE.*

Table 1. Bioelectrocatalytic efficiency of the *Am*PDH modified electrodes estimated for 30 mM glucose (for experimental conditions see Figure 3).

Bioelectrode	CE (%)
G/Os-RP/gPDH	56
G/O-RP/dgPDH	67

Moreover, the CV recorded at G/Os-RP/dgPDH in absence of the substrate shows a pair of better defined redox peaks, which can not be observed on the CV recorded at G/Os-RP/gPDH. This peculiar behavior of the G/Os-RP/dgPDH modified bioelectrode can be attributed to a better electrical connection existing in the electrochemical chain deglycosylated PDH – Os-RP - graphite electrode.

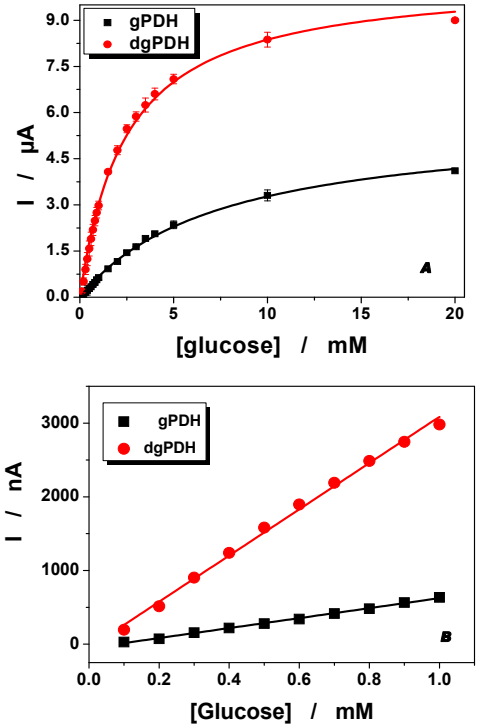


Figure 4. Calibration curves (A), and the linear range (B) of G/Os-RP/gPDH and G/Os-RP/dgPDH using glucose as substrate. *Experimental conditions: applied potential, 200 mV vs. Ag|AgCl, 0.1M KCl; supporting electrolyte, 50 mM PB containing 137 mM NaCl (pH 7.4); flow rate, 0.45 mL/min.*

The kinetic parameters corresponding to the modified bioelectrodes were estimated by using nonlinear fitting of the calibration curves to glucose exhibited in Figure 4A. The results are summarized in Table 2. It can be noticed that the biosensor based on dgPDH showed a much higher catalytic current density [I_{max} = (148.7 ± 0.14) µA/cm^2] than that measured for gPDH [I_{max} = (81.4 ± 1.4) µA/cm^2]. This finding proves once again the higher bioelectrocatalytic activity of the deglycosylated PDH.

Table 2. The kinetic parameters estimated for the modified bioelectrodes (for experimental conditions see Figure 4).

Biosensor	K_M^{app} (mM)	J_{max} ($\mu A/cm^2$)	Sensitivity ($\mu A/mMcm^{-2}$)	R^2/N
G/Os-RP/dgPDH	2.4 ± 0.1	148.7 ± 0.14	61.4 ± 2.9	0.996 / 19
G/Os-RP/gPDH	7.5 ± 0.3	81.4 ± 1.4	10.9 ± 0.3	0.996 / 19

At the same time, deglycosylation of PDH resulted in a lower value of the apparent Michaelis-Menten constant (K_M^{app}= 2.4 ±0.1 mM) compared with that estimated for gPDH (K_M^{app} = 7.5 ± 0.3 mM). This behavior suggests either a higher enzyme-substrate affinity for dgPDH, or a higher permeability to glucose of the deglycosylated enzyme matrix associated with a higher accessibility of the Os-RP for the deglycosylated enzyme.

For both modified bioelectrodes the linear range was practical the same, being placed between 0.1 up to 1 mM (Figure 4B).

The increase in the maximum current response and the substrate affinity for the G/Os-RP/dgPDH bioelectrode could be due to: (i) a faster electron transfer between the Os-RP and the covalently bound FAD cofactor of dgPDH; (ii) an increase in the substrate accessibility at the dgPDH active center, due to a higher permeability of the Os-RP – dgPDH network [14].

dgPDH selectivity

*Am*PDH showed a noticeable bioelectrocatalytic activity toward various sugars such as: 2-deoxy-D-galactose, 2-deoxy-D-glucose, cellobiose, fucose, lactose, maltose, mannose, sucrose, trehalose, galactose, xylose [18]. Aiming to investigate the selectivity of deglycosylated PDH, the sensitivities of the G/Os-RP/dgPDH bioelectrode towards galactose, xlyose, mannose, and lactose were estimated as the I_{max}/K_M^{app} ratios, calculated with the values obtained by the fitting of the experimental calibration curves to the Michaelis-Menten equation. The obtained results are shown in Figure 5.

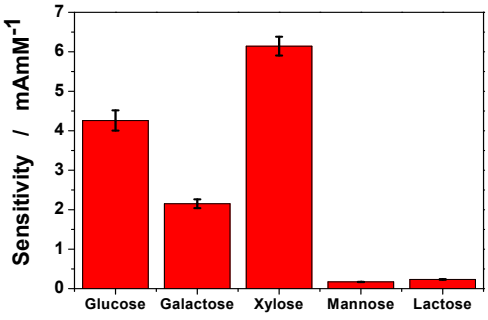


Figure 5. Sensitivity of G/Os-RP/dgPDH modified electrodes towards different substrates. The error bars correspond to the standard deviation for 3 successive measurements. *Experimental conditions: see Figure 4.*

The dgPDH modified electrodes show a noticeable sensitivity for all investigated substrates. It is interesting to notice that dgPDH exhibits the highest sensitivity for xylose, contrarily to gPDH which showed the highest sensitivity for glucose [18]. This unexpected change in the enzyme selectivity after its deglycosylation is not explained until now.

Short term stability of bioelectrodes

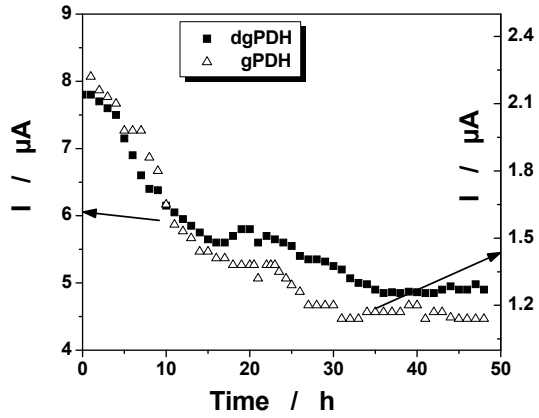


Figure 6. Time evolution of the amperometric response of G/Os-RP/gPDH and G/Os-RP/dgPDH modified electrodes for glucose.
Experimental conditions: applied potential 200 mV vs. Ag|AgCl, 0.1M KCl; 50 mM PB, 137 mM NaCl, 5 mM glucose, pH 7.4; flow rate 0.450 mL/min

The operational stability of the bioelectrode is one of its key parameters and, consequently, it was evaluated further. Figure 6 shows the results of the test of the stability performed for both bioelectrodes based on *Am*PDHs, under a constant flow of 5 mM glucose.

For both modified electrodes, a pronounced decrease in the current response was observed during the first 20 h. Thus, for G/Os-RP/gPDH electrode the current decreases to ~37% of the initial response, while for G/Os-RP/dgPDH electrode the decrease was slightly smaller (~25%). Furthermore, after ~35 h spent under a constant flow of glucose, the current response of both bioelectrodes reached a plateau placed at ~63% for G/Os-RP/dgPDH and at ~56% for G/Os-RP/gPDH.

CONCLUSIONS

In the present study the effect of deglycosylation of PDH on the amperometric response to glucose of G/Os-RP/PDH modified electrodes was investigated. The obtained results confirmed the beneficial effect of the removal of the glycan shell on the behavior of the bioelectrode. This effect

materialized as: (i) an increase in the sensitivity of the bioelectrode for amperometric detection of glucose; (ii) an increase in the enzyme affinity towards its main substrate (glucose); (iii) an unchanged short term operational stability; (iv) a change of the enzyme selectivity towards its substrates.

In conclusion, it was established that, by simple immobilization of the deglycosylated PDH simultaneously with a convenient Os redox polymer and a cross-linker (PEGDGE) on the surface of a graphite electrode, a modified bioelectrode with a good sensitivity, selectivity and short term operational stability can be obtained. The bioelectrochemical characteristics of this bioelectrode recommend it as a suitable transducer for biofuel cells.

EXPERIMENTAL SECTION

Materials

Pyranose dehydrogenase from *Agaricus meleagris* (*Am*PDH) was recombinantly expressed in *Pichia pastoris* and deglycosylated with Endo Hf (New England Biolabs, Bionordiska AB, Stockholm, Sweden) as described elsewhere [10]. Poly(ethylene glycol)(400) diglycidyl ether (PEGDGE), ferricenium hexafluorophosphate and D(+)-glucose were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The osmium redox polymer ([Os(dmbpy)2(PVI)10Cl]$^{+2/+}$, E°' = +76 mV *vs.* SCE, where dmbpy stands for 4,4'-dimethyl-2,2'-bipyridine and PVI is poly(N-vinylimidazole)) was synthesized as described in [21].

In all experiments the supporting electrolyte was 50 mM phosphate buffer at pH 7.4, containing 137 mM NaCl salt. The phosphate buffer solution was prepared using Na_2HPO_4 and NaH_2PO_4 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and its pH value was adjusted to 7.5 by using 4M NaOH (Fischer Scientific) or 5 mM HCl (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

The water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Equipment

Flow injection measurements were performed with a flow-through amperometric cell of the wall-jet type [22], containing a platinum wire as counter electrode and an Ag|AgCl, 0.1M KCl as a reference electrode. The applied potential to the working electrode was controlled by using a low currents potentiostat (Zäta Electronics, Höör, Sweden). The response of the working electrode, (spectrographic graphite, Ringsdorff Werke GmbH, Bonn, Germany, type RW001; 3.05 mm diameter and 13% porosity) was registered using a chart recorder (BD 112, Kipp & Zonen, Utrecht, The Netherlands). An injector (Rheodyne, type 7125 LabPR, Cotati, CA, USA) with a 50 µL sample loop was used to inject the samples.

CV measurements were performed with a standard three electrodes electrochemical cell connected to a computer controlled potentiostat (AutoLab PGSTAT30, Metrohm Nordic AB, Bromma, Sweden). The modified graphite electrode was used as working electrode, a SCE as reference electrode and a platinum foil as counter electrode. Before every measurement argon was purged through the solution for 10 min.

Enzyme assay

For the determination of the *Am*PDH activity the protocol described previously [16, 23] was used. It consists of spectrophotometric monitoring of ferricenium (Fc^+) reduction to ferrocene at 300 nm [molar absorptivity 4.3 $mM^{-1}cm^{-1}$], for 3 min at 20 °C in a standard cuvette [23]. One milliliter of the standard solution contained 100 µmol of phosphate buffer (pH 7.4), 50 µmol of D-glucose, 0.4 µmol of $Fc^+PF_6^-$ (prepared daily by dissolving 3.3 mg of salt in 5 ml of 5 mM HCl) and the appropriately diluted enzyme. One unit of enzyme activity was defined as the amount of the enzyme necessary for the reduction of 2 µmol of Fc^+ per 1 min at 20 °C.

Preparation of bioelectrodes

Graphite electrodes were prepared using the procedure described elsewhere [16, 18]. Spectrographic graphite rods (geometric area of 0.071 cm^2) were polished on wet emery paper (Tufbak, Durite, P1200). Afterwards, they were carefully rinsed with Milli-Q water and dried at room temperature. *Am*PDH was immobilized on graphite electrodes by adsorbing the enzyme solution prepared in the presence of Os redox polymer and the cross-linking agent (PEGDGE) by using the following procedure: 2 µl of an aqueous Os-RP solution (10 mg/ml) and 1 µl of freshly prepared PEGDGE (68% v/v, in water) were placed on the top of the electrode.

After 10 min of incubation 5 µl of enzyme solution (250 U/ml) was added. The modified electrodes were left overnight at 4^o C to complete the cross-linking.

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