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Monitoring of PQQ-Dependent Glucose Dehydrogenase Substrate Specificity for Its Potential Use in Biocatalysis and Bioanalysis

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Abstract Substrate specificity of 2,7,9-tricarboxypyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase was investigated in biosensor arrangement for understanding the suitability and the limitations of its use in bioanalysis and bioproduction of chemicals. The study demonstrated a very broad substrate specificity of biosensor utilising soluble form of PQQ-dependent glucose dehydrogenase. Nineteen saccharides out of 31 were oxidised by the sensor. Investigation confirmed strong importance of hydroxyl configuration in the positions 2 and 5 of oxidised saccharides. The broad specificity suggests that the PQQ-dependent glucose dehydrogenase could be utilised for analysis of other sugars than glucose in food samples for various production processes and for biofuel cells. In addition, the results showed that the substrate specificity of enzymes can be effectively and generally studied by biosensor arrangement for research purposes. This layout utilising immobilised enzyme allowed performing comprehensive study using a small amount of enzymes and thus saving the costs and time.

Keywords PQQ glucose dehydrogenase · Biosensor · Specificity · Saccharides · Bioanalysis

Introduction

Biocatalytic processes benefit from selectivity and substrate specificity of reactions catalysed by enzymes. In general, reaction specificity means that the enzyme catalyses only one type of reaction and substrate specificity stands for preference of the biocatalyst for only one type of substrate [1]. Requirements for the properties of the biocatalyst may however vary depending

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on its intended use. While high substrate specificity is usually required for bioanalytical purposes, e.g. for development of selective enzymatic assays or biosensors, enzymatic synthesis prefers more universal biocatalysts, operating on wider scale of substrates or reactions. As an example, the natural role of lipases is hydrolysis of triacylglycerols. Due to their broad substrate tolerance, lipases have a very limited use in bioassays of specific substances (with exception of e.g. triacylglycerols assay), but their reaction flexibility was employed in thousands of syntheses comprising ester hydrolyses and alcoholyses, direct esterifications, transesterifications, etc. Target substances prepared with the aid of lipases cover biofuels, polymer materials, detergents, pharmaceuticals, pheromones, etc. 2,7,9-Tricarboxypyrroloquinoline quinone (PQQ)-dependent dehydrogenases are known for possessing broad activity towards different substrates, e.g. the oxidation activity towards glycerol and some other linear polyhydroxylic alcohols [2]. PQQ-dependent glucose dehydrogenase (GDH-PQQ) is a typical example of enzyme with broad substrate specificity. Although originally commercialised for glucose assays due to its efficient and robust performance, its use for glucose monitoring decreased when highly specific FAD-dependent glucose dehydrogenase was introduced into test strips containing ferricyanide as a mediator [3]. GDH-PQQ belongs to quinoproteins, a class of dye-dependent dehydrogenases distinct from the flavin- and nicotinamide-dependent oxidoreductases [4]. These enzymes use PQQ as a prosthetic group and their natural electron acceptors are ubiquinones and cytochromes, while oxygen does not work as the acceptor [5]. GDH-PQQs occur in the nature as two different types: a membrane-bound enzyme (mGDH-PQQ) insoluble in water and a water-soluble sGDH-PQQ present in the cell interior [6–8]. Membrane-bound GDH-PQQs are widely distributed among many bacterial species including Gram-negative facultative anaerobes such as enteric bacteria and *Zymomonas* sp., as well as aerobic bacteria such as *Pseudomonas* sp. and acetic acid bacteria. All mGDH-PQQs are single peptides with molecular weights of about 87 kDa. Their primary structure, the enzymatic characteristics, such as cofactor binding stability, thermal stability and substrate specificity, are dependent upon the particular bacterial sources [9, 10]. sGDH-PQQ is a homodimeric enzyme consisting of two identical subunits of approximately 50 kDa. The enzyme oxidises a variety of mono- and disaccharides to the corresponding lactones [11, 12] and donates electrons to artificial electron acceptors, e.g. *N*-methylphenazonium methyl sulphate [13].

In the past, these two forms had been considered to be the same enzyme or its interconvertible forms. However, further evidence has shown that the soluble form of enzyme is not a typical mGDH-PQQ. The membranes from several strains such as *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Gluconobacter suboxydans*, *Acetobacter aceti* and *Acinetobacter calcoaceticus* contain antigens cross-reactive with an antibody of mGDH-PQQ purified from *Pseudomonas fluorescens* [14], while sGDH-PQQ from the soluble fraction of *A. calcoaceticus* does not cross-react with the antibody [15]. Thereafter, sGDH and mGDH were purified separately from *A. calcoaceticus* and shown to be dissimilar in all aspects, including pH optima, kinetics, substrate specificity, ubiquinone reactivity, molecular size and immunoreactivity [11]. Properties such as the high catalytic activity, oxygen insensitivity and unnecessity of cofactor addition in contrast to NAD-dependent enzymes make GDH-PQQ suitable for in vivo blood D-glucose monitoring in the management of diabetes. These properties attracted electrochemists developing biosensors to utilise this enzyme not only for glucose monitoring [16–19] but also for its application in detection of heavy metals was found [20]. Moreover, direct electron transport from active centre of PQQ dehydrogenase to the modified carbon electrode surface was also demonstrated [21]. sGDH-PQQ was the first quinoprotein applied to glucose biosensor. Also, electrochemical test strip utilising sGDH-PQQ containing ferricyanide as a mediator was on the market. Previously, D-glucose oxidase and NAD-dependent GDH had been used as the enzyme for D-glucose biosensors. However, biosensors based on D-glucose oxidase are oxygen dependent

and thus sensitive to oxygen fluctuations in blood samples. Those based on NAD-GDH had poor stability due to the loss of the cofactor NAD. These problems were overcome by using PQQ-dependent GDH, which is oxygen insensitive and has a tightly bound PQQ. Moreover, the electron transfer rate of sGDH is significantly higher than that of D-glucose oxidase [16, 22]. Several biosensors based on GDH-PQQ were described previously [23, 24]. Although lower selectivity disqualified PQQ-GDH from glucose assays in favour of FAD-GDH, the beneficial properties mentioned above give it potential either for use in construction of biosensors for assays of other accepted substrates (if occur solely in the sample) or for biocatalytic transformations of mono- and oligosaccharides to valuable substances.

The purpose of this paper was to explore substrate specificity of sGDH-PQQ for understanding the limitations of its use in various biological fluids which may comprise interfering saccharides as well as for identification of potential synthetic applications of PQQ-GDH. Such approach is in the intentions of our previous efforts to use side activities or unusual substrate reactivity of enzymes for new applications [25–28]. The study was performed in biosensor arrangement as a simple, fast and cheap testing system with minimal consumption of the enzyme.

Materials and Methods

Materials

Soluble glucose dehydrogenase-PQQ dependent (sGDH-PQQ) from *A. calcoacetics* (1,010 U mg^{-1} solid) was obtained from Sorachim (Lausanne, Switzerland). *N*-methylphenazonium methyl sulphate, *N*-eicosane, D-glucose, D-fructose, anhydrous ethanol and chitosan from shrimp shells (85 % deacetylated) were supplied by Sigma-Aldrich (St. Louis, USA). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Riedel-de Haen (Seelze, Germany). Water deionised by Millipore Milli-Q purification system was used. All chemicals used were of analytical grade. A glucose stock solution was made with Milli-Q water and stored at least 24 h to achieve mutarotation equilibrium. Multi-walled carbon nanotubes (MWCNT, d=60–100 nm, L=5–15 μm, 95+% purity) were obtained from NanoAmor (Houston, USA). Planar basic sensors equipped with Ag/AgCl reference electrode (diameter 2 mm, screen-printed) deposited on the planar glass–epoxy laminate substrate were obtained from Biorealis (Bratislava, Slovakia). Samples of investigated saccharides were provided as a gift from Dr. Magdolen (Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia).

Apparatus

Electrochemical studies were performed with potentiostat model 559 from Amel (Milan, Italy), potentiostat Heka PG-284 (Lambrecht, Germany) and with electrochemical analyzer Omnilab from Biorealis (Bratislava, Slovakia).

Preparation of Nanocomposite

Nanocomposite working electrode was prepared according the procedure described in our previous work [29, 30]. Briefly, *N*-eicosane was melted at the temperature 45 °C and mixed with MWCNT. The mixture was stirred vigorously with a spatula until the homogenous composition was obtained. The suspension was subsequently transferred and spread on the conductive surface layer forming the working electrode on the planar basic sensor equipped with Ag/AgCl reference electrode. Finally, the layer of the nanocomposite was left to solidify and the surface was smoothed on a sheet of paper.

Preparation of Biosensors

The reference electrode was carefully cleaned with Milli-Q water and ethanol. Glucose dehydrogenase was dissolved in Milli-Q water before the procedure. The immobilisation of enzyme on the surface of the working electrodes was carried out by their sandwiching between chitosan layers (1 % *w*/*w*) (Fig. 1). The amounts of enzyme on the electrodes were optimised within the range of 1 to 12 units. Each layer was deposited after the previous one was dried. The prepared biosensors were stored at room temperature in a desiccator before use.

Measurements of Soluble PQQ-Dependent Glucose Dehydrogenase Substrate Specificity

Substrate specificity tests of sGDH-PQQ were performed by injecting of 10 μL saccharide standard solution (10 mM) into the 1 mL measuring media consisting of phosphate buffer solution (PBS) and *N*-methylphenazonium methyl sulphate. The response was measured as electric current (nanoampere), whereby the response for D-glucose was considered as 100 %. Each saccharide was measured 5×; results were averaged and expressed as percentage of D-glucose response.

Results and Discussion

Optimization of Working Conditions

The following principle using *N*-methylphenazonium methyl sulphate (PMS) as a mediator (Med) was utilised for the biosensor measurement of D-glucose or other saccharides based on soluble PQQ-dependent glucose dehydrogenase:

$$\mathrm{D-Glucose(other\ \ sugar)} + [\mathrm{Med}]_{\mathrm{ox}} \overset{\mathrm{sGDH-PQQ}}{\rightarrow} \mathrm{D-glucono-1,5-lactone(corresponding\ \ lactone)} + [\mathrm{Med}]_{\mathrm{red}} \quad (1)$$

$$[\mathrm{Med}]_{\mathrm{red}} \rightarrow [\mathrm{Med}]_{\mathrm{ox}} + \mathrm{e}^{-}\ \ (\mathrm{on\ \ the\ \ electrode\ \ surface}) \quad (2)$$

Working conditions of the developed biosensor had to be optimised before the specificity tests. Optimization was performed in 0.2 M PBS at pH 5.5. We decided to test PMS and its optimal amount was investigated in concentration range of 0.5–2 g L^{-1} in PBS. Based on the fact that no significant changes in current response after the addition of D-glucose standard

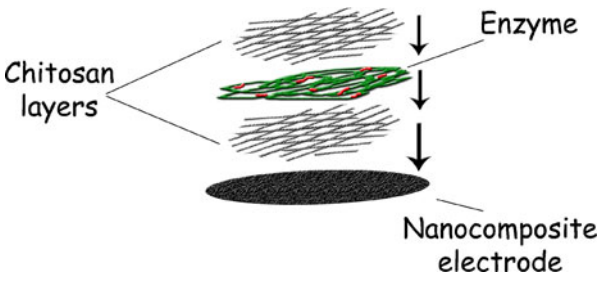

Fig. 1 Schematic illustration of enzyme immobilisation between chitosan layers onto nanocomposite electrode

(10 mM) were detected, we decided to choose for further work concentration 1 g L^{-1} of PMS in a working media.

To verify the optimal working potential, chronoamperometry measurements were performed by varying of values ranging from +25 to +200 mV. No significant differences in current response were noticed. Based on these results, we chose the working potential of + 50 mV for the next study.

Once the optimal working potential was established, the amounts of enzyme loadings on the working electrode were tested in the range from 1 to 12 units. As it can be seen in the Fig. 2, satisfactory current response started at loading of 6 units and peaked at 8 units. Higher enzyme loadings led to the current decrease, probably due to blockage of the surface of working electrode by immobilised protein. Hence, the amount of 8 units of GDH-PQQ was chosen for further work.

The pH of working media is a very important factor which can affect the biosensor performance. The pH dependence of the sGDH-PQQ-based biosensor was investigated over the range from 5.0 to 7.5 in 0.2 M PBS, as shown in Fig. 3. The highest relative response occurred as early as at pH 5.5 and decreased as pH increased further. Following experiments were performed at optimised working conditions mentioned above.

Substrate Specificity

Broad substrate specificity of enzymes used for construction of biosensor may complicate its real application due to interferences and thus decrease the accuracy of assays. Study of biosensor selectivity belongs therefore to obvious testing procedures for prediction of their accuracy and reliability in real measurements. The enzyme used for the construction of biosensor is sGDH from *A. calcoaceticus*. Its substrate specificity was briefly studied by Matsushita et al. [15] and compared to the specificity of the counterpart dehydrogenase obtained from the membrane fraction of the same strain. More particular study of structural aspects of sGDH specificity was done by Olsthoorn et al. [31, 32]. Our study focused on the

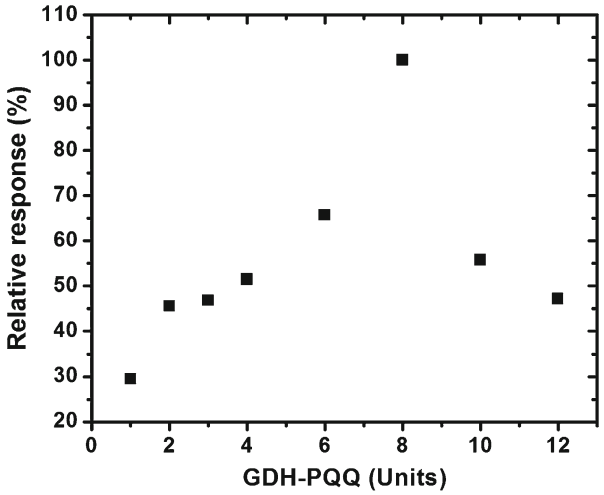

Fig. 2 Effect of enzyme loadings on the response of biosensor based on soluble PQQ-dependent glucose dehydrogenase from *A. calcoaceticus* (the response at 8 units=100 %). Experimental conditions: 1 g L^{-1} *N*-methylphenazonium methyl sulphate, 100 μM D-glucose in 0.2 M phosphate buffer at pH 5.5, applied potential +50 mV vs. Ag/AgCl

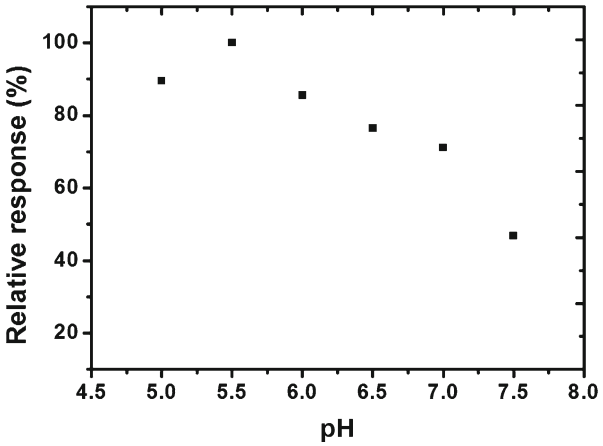

Fig. 3 Effect of pH on the response of biosensor based on soluble PQQ-dependent glucose dehydrogenase from *A. calcoaceticus* (the response at pH 5.5=100 %). Experimental conditions: 1 g L^{-1} *N*-methylphenazonium methyl sulphate, 100 μM D-glucose in 0.2 M phosphate buffer, applied potential +50 mV vs. Ag/AgCl

selectivity of sGDH-PQQ to assess its usability for analytical or synthetic purposes in presence of other saccharides and to widen the knowledge on sGDH substrate specificity. Saccharide specificity was investigated in a biosensor arrangement as a current response to the consecutive additions of saccharide solutions. More than 30 saccharides were therefore tested to explore the biosensor response to varying sugar structures, including aldoses, ketoses, alditols, glucosamine and oligosaccharides.

As expected, neither alditols such as D-mannitol and D-sorbitol, nor ketoses (D-fructose, D-sorbose, D-tagatose) were oxidised since the enzyme converts generally the hemiacetal group of saccharide to the corresponding lactone. It is however surprising that the relative rates of oxidation of aldoses resemble the reactivity pattern of mGDH rather than sGDH (Table 1). The set of monosaccharides tested in oxidations by mGDHs and sGDHs in Matsushita report [15] is however too limited to draw any reliable conclusion when comparing the published results with our observations. We can only speculate if the immobilisation procedure might contribute to changes in enzyme conformation.

The anomeric specificity of sGDH is strictly limited to β-anomer of glucopyranose in $^4C_1$ chair conformation (Fig. 4) which has all hydroxyl residues in equatorial positions [31]. Analogously, extent of oxidation of other aldoses depends on the level to which one of their equilibrium conformers mimics the β-glucopyranose in solution. Anyway, our results summarised in Table 1 confirm strong importance of hydroxyl configuration in the position 2 of saccharides as found by Olsthorn [31]. Virtually all tested aldoses (with exception of L-mannose) bearing hydroxyl with D-configuration in this position were oxidised with slight preference for the 2,3-L-*threo* configuration comparing to 2,3-D-*erythro* sugars. D-Glucosamine, bearing D-amino group in position 2, was not oxidised. On the other side, four saccharides with L-hydroxyl in position 2 (D-arabinose, D-mannose, D-altrose and D-talose) were also oxidised in moderate to low extent. Configuration in position 5 has similar important effect on sugar oxidation, the reactivity being D-hexose>pentose>L-hexose. The intermediate reactivity of pentoses probably results from the shape of their pyranose form, when neither potentional interaction of the end hydroxymethyl with active site nor steric hindrance limiting their access to the reaction centre occurs. Oxidation of oligosaccharides follows the pattern reported for sGDH. The disaccharides comprising glucose on the reducing

Table 1 Oxidation of aldoses by soluble PQQ-dependent glucose dehydrogenase from *A. calcoaceticus*. Comparison of biosensor's relative responses to glucose with data for free enzymes found in literature

Saccharide	Configuration in position			Relative response	Activity of free enzymes		
	2,3	4	5	Biosensor [%]	sGDH[a] k_{cat}/K_M [mM^{-1} s^{-1}] [32]	sGDH[a] [%] [15]	mGDH[b] [%] [15]
D-Glucose	L-*threo*	D-	D-	100	1,700	100	100
D-Glucosamine	L-threo (2-amino)	D-	D-	0	0	–	–
D-Galactose	L-*threo*	L-	D-	65.2	150	30	79
L-Arabinose	L-*threo*	L-	–	55.6	1,450		
D-Xylose	L-*threo*	D-	–	45.2	46	14	81
L-Altrose	L-*threo*	L-	L-	39.2	–	–	–
D-Allose	D-*erythro*	D-	D-	96.9	1,470	–	–
D-Ribose	D-*erythro*	D-	–	36.5	11	5	46
L-Lyxose	D-*erythro*	L-	–	21.0	–	–	–
L-Talose	D-*erythro*	D-	L-	18.6	–	–	–
L-Mannose	D-*erythro*	L-	L-	0	–	–	–
D-Altrose	D-*threo*	D-	D-	40.8	–	–	–
D-Arabinose	D-*threo*	D-	–	11.1	–	–	–
L-Xylose	D-*threo*	L-	–	0	–	–	–
L-Glucose	D-*threo*	L-	L-	0	–	–	–
L-Galactose	D-*threo*	D-	L-	0	–	–	–
D-Mannose	L-*erythro*	D-	D-	41.3	50	–	–
D-Talose	L-*erythro*	L-	D-	12.8	–	–	–
L-Allose	L-*erythro*	L-	L-	0	–	–	–
D-Lyxose	L-*erythro*	D-	–	0	0.24	–	–

[a] Soluble

[b] Membrane bound

end were oxidised by high velocity despite the α- or β-glycosidic bond in the position 4 (Table 2). The reactivity decreased when end hydroxymethyl in position 6 was glycosylated (in case of melibiose). Non-reducing oligosaccharides were generally not oxidised. Slight

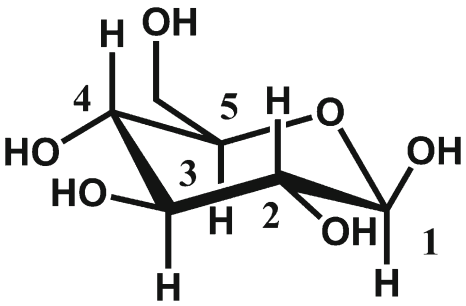

Fig. 4 Structure of β-D-glucopyranose

Table 2 Oxidation of disaccharides by soluble PQQ-dependent glucose dehydrogenase from *A. calcoaceticus*. Comparison of biosensor's relative responses to glucose with data for free enzymes found in literature

Saccharide	Relative response	Activity		
	Biosensor [%]	sGDH[a] k_{cat}/K_M [mM^{-1} s^{-1}] [32]	sGDH[a] [%] [15]	mGDH[b] [%] [15]
D-Glucose	100	1,700	100	100
Cellobiose	70.9	1,080	–	–
Lactose	75.4	790	4	74
Maltose	69.4	800	12	72
Melibiose	27.3	11	–	–
Sucrose	6.6	–	–	–
Trehalose	0	–	–	–
Raffinose	0	–	–	–

[a] Soluble

[b] Membrane bound

oxidation of sucrose, which was free of glucose, may be explained by invertase occurrence in the enzyme preparation. This was however not confirmed experimentally.

Our results demonstrated a very broad substrate specificity of sGDH-PQQ-based biosensor. This report provides therefore a useful basis for evaluation of results from glucose assays in various biological fluids according to potential biosensor interferences. On the other side, the biosensor may be used as an inexpensive device utilising small amount of enzyme in monitoring of saccharides other than glucose in processes comprising only one oxidisable sugar, e.g. in enzymatic hydrolysis of raffinose to melibiose [25] (raffinose and fructose are not oxidised), hydrolysis of cellulose to cellobiose or glucose, hydrolysis of sucrose, reduction of aldoses to alditols (alditols are not oxidised) or isomerase processes (ketoses are not oxidised). Biosensor may be also valuable in analysis of food samples with dominant single sugar (e.g. for lactose determination in milk). In addition, the results suggest a potential use of free sGDH-PQQ, as a unique stable and efficient dehydrogenase which does not require addition of costly coenzymes in preparative scale processes. The enzyme can be perspectively used either for elimination of undesired saccharides from reaction mixtures (for example removal of aldoses from invert mixtures) or production of valuable aldonic acid as are for example lactobionic and cellobionic acids. Last but not least, GDH-PQQ is a versatile enzyme particularly suitable for biofuel cells [33] because of the ability to oxidise various sugars demonstrated in this study and also in our previous work [34]. It was showed that GDH-PQQ was able to oxidise not only glucose and maltose but also maltotriose, maltotetraose and short chain dextrins, i.e. hydrolysis products of starch which could be interesting from economical point of view.

Conclusion

In this study, biosensor arrangement was used to explore substrate specificity of soluble PQQ-dependent glucose dehydrogenase from *A. calcoaceticus* and to understand the limitations of its use for glucose assays in various biological fluids comprising interfering saccharides. Our results demonstrated a very broad substrate specificity of biosensor based

on soluble form of PQQ-dependent glucose dehydrogenase and confirmed strong importance of hydroxyl configuration in positions 2 and 5 of oxidised saccharides. This report provides therefore a useful basis for evaluation of results from glucose assays in various matrix according to potential biosensor interferences. Further studies should be aimed to the monitoring of substrate specificity at different working conditions, such as pH, ionic strength, etc. Moreover, analysis of solutions or real samples containing more complex saccharide composition and comparison with reference analytical methods will be further investigated.

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