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Glucose Oxidation Using Oxygen Resistant Pyranose-2-Oxidase for Biofuel Cell Applications

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In this study, the effect of oxygen on glucose oxidation using Glucose Oxidase (GOx) and oxygen resistant Pyranose-2-Oxidase (P2O) has been studied. Enzyme solutions with ferrocene carboxylic acid (FcCOOH) as electron mediator were tested with glassy carbon electrode (GCE) under air and nitrogen saturated conditions in a three electrode electrochemical cell system. Electrochemical characterization of enzymes has been achieved in solution by using cyclic voltammetry (CV), linear sweep voltammetry (LSV) and chronoamperometry (CA). In the presence of glucose, CV and LSV results show increasing anodic peak current and decreasing cathodic peak current with increasing glucose concentrations, which reflects the ferrocene-mediated bioelectrocatalysis of glucose oxidation. The experiments with CA show enhanced stability with oxygen resistant P2O where GOx loses 30 % of its current density in the presence of oxygen after 3 hours. These results indicate that P2O, a promising enzyme with no oxygen reactivity and long stability, which can be used in enzymatic biofuel cell applications as an alternative to GOx.

1. Introduction

Enzyme-based biosensors and biofuel cells have been the focus of interest in recent years because of their various applications (Barton et al. (2004)). Their potential for in vivo applications could be used to provide a long-term or even permanent power supply for devices such as pacemakers, micro pumps, continuous blood sugar monitoring systems for diabetics (Bullen et al. (2006)). The key parameters for developing enzymatic biofuel cells for such purposes are the enhanced power density, high stability and the good cell performance in real systems (Heller (2004)).
Glucose Oxidase (GOx) has been commonly used for glucose oxidation in biofuel cells because of its well-known structure and range selectivity for glucose (Wilson et al. (1992)). GOx catalyses the oxidation of β-D-glucose into D-glucono-1,5-lactone, which then hydrolyses to gluconic acid and flavin adenine dinucleotide (FAD) which is the co-factor of the GOx for the catalytic reaction (Ivanov et al. (2010)). GOx has been very popular in the field of glucose oxidation, but has important drawbacks such as having restricted turnover rate for glucose at the same time showing high turnover rate for O_2, which is always risk for production of hydrogen peroxide (H_2O_2) (Zafar et al. (2010)). In enzymatic biofuel cell applications where the fuel and oxidant are in the same solution; enzyme activity towards oxygen becomes essential. Since GOx shows decent activity towards oxygen, there has been an undergoing search for alternative glucose-oxidizing enzymes.
Pyranose 2-Oxidase (P2O) is a wood degrading enzyme which has excellent reactivity with alternative electron acceptors for a range of sugar substrates. It has been characterised and purified from several different fungal sources (Leitner et al. (2001)), in which *Trametes multicolor* was found to be the best studied (Hallberg et al. (2004)). The first crystal structure of P2O from *Trametes multicolor* was reported using single anomalous dispersion by Hallberg et al. (2004). P2O can oxidase sugars at position C-2 by the Ping Pong Bi Bi mechanism as similar to other oxidoreductases (Wongnate et al. (2011)), as well as it can also oxidase some substrates at position C-3, such as 2-deoxy-D-glucose, 2-keto-D-glucose and

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methyl β-D-glucosides (Hallberg et al. (2004)). Therefore, this enzyme has recently become very popular as an anodic enzyme because of its wide range of substrate selectivity, and ability to be used without showing any anomeric selectivity (Spadiut et al. (2010)). The reaction mechanism of P2O consists of oxidative and reductive half reactions. During the reductive half reaction, the sugar is oxidized to the corresponding sugar derivative and the FAD is reduced to $FADH_2$ shown in Eq(1) and the oxidative half reaction consists of the reduction of O_2 to H_2O_2 and the re-oxidation of $FADH_2$ to FAD shown in Eq(2) (Kujawa et al. (2006)).

$$FAD + \text{D-glucose} \rightarrow FADH_2 + \text{2-keto-D-glucose} \quad (1)$$
$$FADH_2 + O_2 \rightarrow FAD + H_2O_2 \quad (2)$$

The use of P2O in the development of biosensors has been reported several times in literature, wherein the co-immobilisation of P2O with peroxidase on a carbon paste electrode has been one of earlier reports (Lidén et al. (1998)). Recently, there are number of studies have been reported using P2O in biosensor applications. Timur et al. (2006) reported the wiring of P2O with different flexible Osmium functionalised polymers on graphite electrodes and also showed that the wiring with Osmium polymers provided more efficient electron transfer from the reduced reaction centres of the enzyme. Tasca et al. (2007) used similar approach on P2O and supported the idea of enhanced electron transfer rate when wired with Osmium polymers. Although these studies showed promising results with Osmium polymers, there is a concern about their use in implantable applications. Osmium compounds are toxic and not biocompatible and can leach out therefore it can be risky for long term application (Yu et al. (2010)). Odaci et al. (2008) used carbon nanotube (CNT) modified carbon paste electrodes to fabricate P2O based biosensors to determine the glucose level in wine samples and showed that the results showed consistency with the standard methods. In another study, Ozdemir et al. (2010) used gold nanoparticles (AuNPs)-polyaniline(PANI)/AgCl/gelatin nanocomposite on glassy carbon electrode in which they tried to keep the enzyme with higher bioactivity and stability for glucose sensing applications. Although all the previous reports showed promising results, the wide sugar selectivity of P2O seems to have significant disadvantage on its use in biosensor applications. However, this could be an advantage if it is used in biofuel cells which yet not been studied to best of our knowledge.

Stability is one of the most important key elements for enzymatic biofuel cells for them to be utilized as implantable devices or other portable applications (Barton et al. (2004)). Since oxygen plays very important role in the enzymatic glucose oxidation reaction as an electron acceptor and is responsible for H_2O_2 production, the use of oxygen-resistant enzymes has a big advantage in biofuel cells. As P2O is an oxygen-dependent enzyme like GOx, H_2O_2 production during the sugar oxidation process would still be a problem in the application of biofuel cells. However, different mutant P2O enzymes developed by semi-rational protein design were reported to show good oxygen resistance, indicating that the enzyme could be promising for glucose oxidation in biofuel cell applications. Pitsawong et al. (2010) reported that the rapid reaction experiments on the *T. multicolor* P2O-wild type (P2O-WT) and mutant P20-T169G showed that the bio-molecular rate constant for the enzymes is 5.8±0.08x104 M^{-1} s^{-1} and 80±4 M^{-1} s^{-1} respectively. These results provide information about possible electrochemical applications of the oxygen resistant mutant. However, there have been no reports published on the electrochemical characterisation of P2O or its mutants showing the oxygen resistivity of the mutants in electrochemical systems for biofuel cell applications.

In this work, the use of oxygen resistant P2O mutant (P2O-T169G) in bio-electrochemical systems has been investigated by using electrochemical characterisation and compared with commercially available GOx. The redox behaviour of GOx and mutant P2O-T169G were characterised by cyclic voltammetry, linear sweep voltammetry and chronoamperometry under air and nitrogen saturated solutions conditions where ferrocene carboxylic acid (FcCOOH) was used as an electron mediator. The results showed that, P2O-169G could be promising alternative to GOx as it showed better stability and good oxygen resistance in electrochemical systems. This also supports previously reported values of rapid reaction experiments by Pitsawong et al. (2010) and provides a solid base for further development of biofuel cell anodes using P2O.

2. Experimental

2.1 Materials

Ferrocene carboxylic acid (FcCOOH), D-(+)-Glucose and Glucose oxidase (GOx, from Aspergillus niger lyophilized, powder, ~200U/mg) were purchased from Sigma-Aldrich. Mutant pyronase-2 oxidase (P2O-T169G) from *Trametes multicolor* were prepared as described previously (Pitsawong et al. (2010)). Stock

solutions of glucose were allowed to mutarotate for minimum 24h before use and were subsequently kept refrigerated at 4°C. Stock solution of 1mM FcCOOH were prepared by dissolving FcCOOH in 100mM phosphate buffer solution (PBS) at pH 7 and were subsequently kept refrigerated at 4°C. All the enzyme solutions were made by dissolving and/or diluting the enzyme stock solutions with 100mM PBS at pH 7 and were subsequently kept at -70°C.

2.2. Electrochemical characterisation of glucose oxidation with GOx and P2O in solution

The solutions for the electrochemical tests were prepared by mixing enzyme solutions with FcCOOH to give a final concentration of 1 mg/mL enzyme and 0.5 mM FcCOOH concentration in a total experimental volume of 2 mL. The electrochemical tests were performed in a three-electrode electrochemical cell system, in which the working electrode was GCE, a platinum coil was used as the counter electrode and the reference electrode was Ag/AgCl (~4M saturated KCl gel filled). The glassy carbon working electrode (GCE) has a diameter of 3 mm and a surface area of 0.071cm^2 was from the company IJ Cambria Scientific Ltd (UK). GCE was polished before each experiment with 1µm Diamond polish and 0.05 µm Alumina polish, rinsed thoroughly with distilled water between each polishing step, sonicated no more than 3 minutes in distilled water and dried under nitrogen. Prior to the electrochemical tests the solutions were saturated with either air or nitrogen between each consecutive glucose additions under constant stirring. The potentials described in this study are all against Ag/AgCl reference electrode unless otherwise specified. Cyclic voltammetry (CV), linear sweep voltammetry (LSV) and chronoamperometry (CA) were used to characterize the solution experiments. All the electrochemical measurements were carried out by an Autolab potentiostat-galvanostat (PGSTAT101) in an electrochemical cell containing 0.5 mM FcCOOH at pH 7 with different glucose concentrations. In order to investigate the effect of oxygen on the performance of GOx and P2O-T169G; CV experiments were performed at different scan rates from 500 mV/s to 10 mV/s, LSV experiments were performed at 1 mV/s and CA experiments were carried out as short CA (1 hour) and long CA (3 hours) at 350 mV.

3. Results and Discussion

3.1 Electrochemical characterization of GOx and P2O-169G in solution by using cyclic voltammetry (CV) and linear sweep voltammetry (LSV)

Figure 1 shows the cyclic voltammograms for GCE with GOx and P2O-T169G enzymes in solution under air and nitrogen at pH 7.

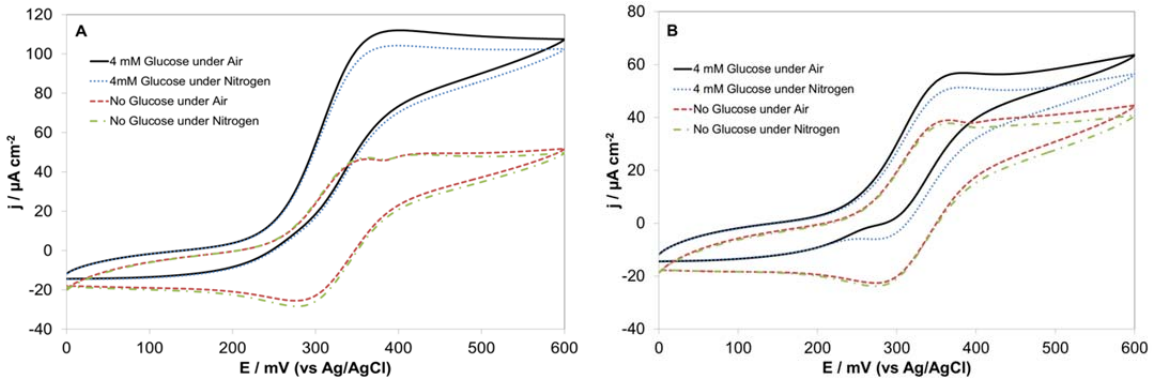

Figure 1: Cyclic voltammograms of air and nitrogen saturated solutions without glucose and 4 mM glucose concentrations added to 0.5 mM FcCOOH with (A) GOx (1 mg/mL) and (B) P2O-T169G (1 mg/mL), glassy carbon electrode, pH 7, scan rate 10mV/s.

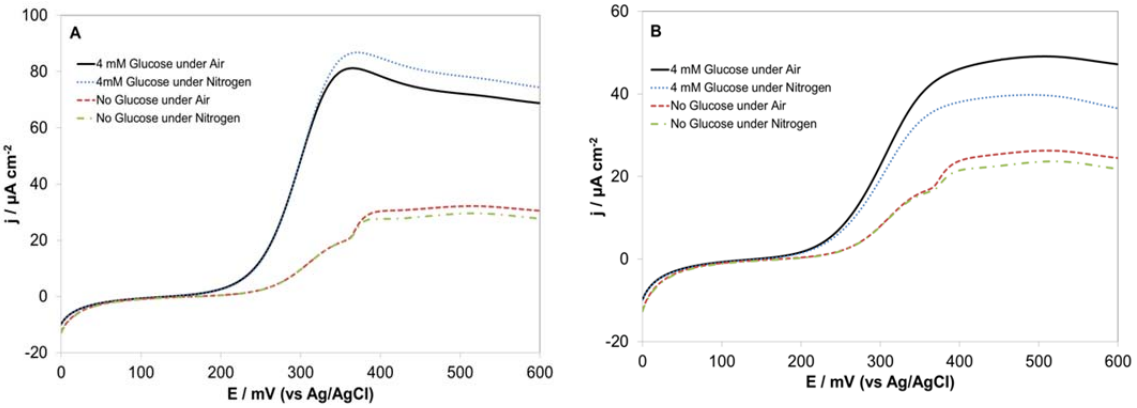

Figure 2: Linear sweep voltammograms of air and nitrogen saturated solutions without glucose and 4 mM glucose concentrations added to 0.5 mM FcCOOH with (A) GOx (1 mg/mL) and (B) P2O-T169 (1 mg/mL), glassy carbon electrode, pH 7, scan rate 1mV/s.

The CVs under air and nitrogen saturated solutions without glucose showed very similar features for GOx and P2O-T169G, where the anodic peaks at ~365 mV were observed. When 4 mM glucose was added to the solution, the anodic peak was increased and the cathodic peak were disappeared for GOx and significantly decreased for P2O-T169G. This showed the enzyme activity for catalysing glucose oxidation, and fast electron transfer from ferrocene mediator. The CV results of GOx and P2O-T169G show similar response for both nitrogen and air saturated conditions so that the effect of oxygen can't be seen clearly (Figure 1). This could be the fact that, ferrocene accepts electrons faster than oxygen; therefore faster electron transfer from ferrocene occurred, so that the effect of oxygen could not be seen.

Figure 2 shows the LSVs for GCE with GOx and P2O-T169G enzymes under air and nitrogen saturated solutions at pH 7. GOx showed similar anodic peaks at ~365 mV with CV experiments following by decreasing of current density with increasing potential which could be explained by mass transport limitations. However, P2O-T169G showed sharp increasing current density between ~230 mV and ~370 mV and kept increasing until ~400 mV, then reached plateau at ~420 mV. The LSV experiments show similar results with CV experiments except the current density increases when tested in air saturated solution for P2O-T169G (Figure 3). The decrease in current densities when tested under air saturated solutions was expected for GOx as it is sensitive to oxygen and the oxidizing nature of oxygen combined with the oxygen-resistant P2O-T169G could be reason of the increasing current density for P2O-T169G under air saturated conditions. The behaviour of P2O-T169G in LSV experiments (Figure 2) also shows that this enzyme might not be affected by mass transport limitations as GOx does. This could be because of the homotetrameric structure of P2O enzyme since it has different mechanism for substrates to enter the active side of the enzyme which was previously reported by Hallberg et al. (2004). The exchange current densities were calculated by using Tafel plot (data not shown) of LSV data at 0 mM and 4 mM glucose concentrations for GOx and P2O-T169G and presented in Table 1.

Table 1: Exchange current densities for GOx and P2O-T160G

Enzymes	Exchange Current Density (x10^3 µA/cm^2)			
	Air Saturated Solution		Nitrogen Saturated Solution	
	0 mM Glucose	4 mM Glucose	0 mM Glucose	4 mM Glucose
GOx	1.43	5.60	1.06	5.73
P2O-T169G	1.40	5.72	0.95	4.15

The exchange current density values for both enzymes show similar values and behaviours between each other for different glucose concentrations. This shows that as GOx, P2O-T169G is also a FAD-dependent enzyme for glucose oxidation and using the similar glucose oxidation process.

3.2 Testing stability of GOx and P2O-T169G in solution by using chronoamperometry (CA)

To investigate the effect of oxygen on GOx and P2O-T169G for long term, chronoamperometry were performed for air and nitrogen saturated solutions with GCE and 0.5 mM FcCOOH at pH 7 for 1 hour (Figure 3) and 3 hours (Figure 4). In short (1 hour) CA experiments, when there is no glucose in the solution, the current density loses ~67 % of its initial value and stabilizes in a few minutes for GOx and presence of oxygen does not make any significant changes in current density.

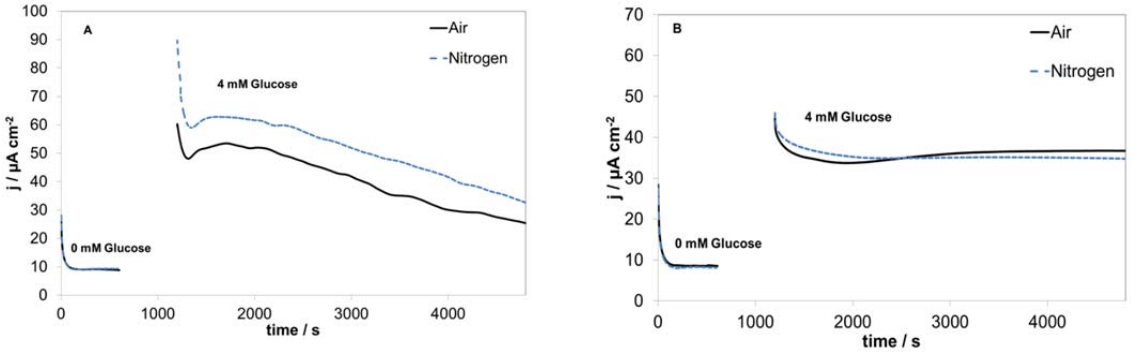

Figure 3: Chronoapmerograms of air and nitrogen saturated solutions without glucose and 4 mM glucose concentrations added to 0.5 mM FcCOOH with (A) GOx (1 mg/mL) and (B) P2O-T169 (1 mg/mL), glassy carbon electrode, pH 7, 10 minutes and 1 hour long at 350 mV for 0 mM glucose and 4 mM glucose concentrations respectively.

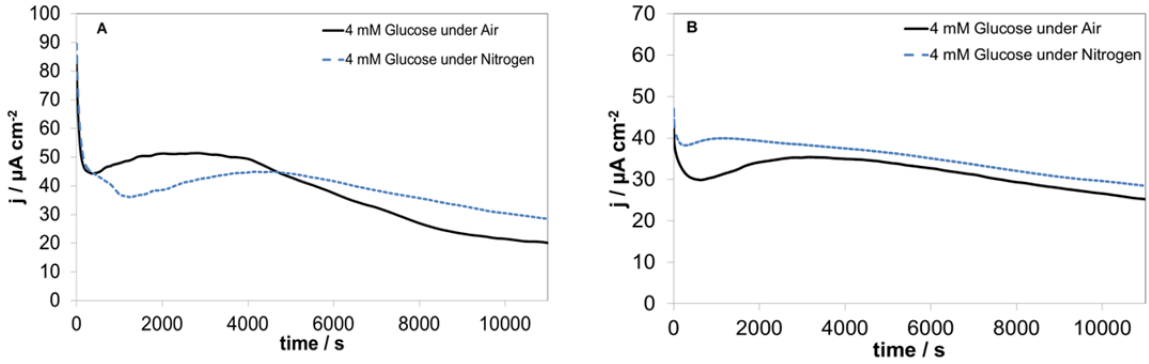

Figure 4: Chronoapmerograms of air and nitrogen saturated solutions without glucose and 4 mM glucose concentrations added to 0.5 mM FcCOOH with (A) GOx (1 mg/mL) and (B) P2O-T169 (1 mg/mL), glassy carbon electrode, pH 7, 3 hours long at 350 mV for 4 mM glucose concentration.

The same behaviour can be seen for P2O-T169G where the total current density lost is ~70 %. When 4 mM glucose added, the current density shows a sharp decrease from the initial value and reaches a plateau. Taking the difference between plateau and the end of 1 hour, the change in current density under nitrogen and air saturated conditions for GOx is 48 % and 53 % respectively. There is no remarkable change for P2O-T169G as the value slightly reaches plateau and stays constant. Although the current density values for P2O-T169G was initially much lower than GOx, the current density values for P2O-T169G stays higher than GOx after an hour. It is calculated that the net current density difference at the end of 1 hour between air and nitrogen saturated conditions for GOx and P2O-T169G is 22 % and 5.6 % respectively. This shows that the effect of oxygen presence on the performance of P2O-T169G is small, whereas it causes a big performance loss in the performance of GOx. For long CA experiments, Figure 4 shows the behaviour of enzymes for 4 mM glucose concentration over 3 hours. As similarly with short CA experiments, the differences in current density values were calculated from the plateau to the end of the 3 hours. The change in current density under nitrogen and air saturated conditions for GOx is 36% and 61%, respectively. It is ~28 % for P2O-T169G under both conditions. Also, the net current density difference at the end of 3 hours between air and nitrogen saturated conditions for GOx and P2O-T169G is 30 % and 11.4 % respectively. As clearly seen from the calculated values, similar to the short stability experiments P2O-T169Gdoes not show as much activity towards oxygen as GOx. Although ~28 % of its initial current density was lost in the long stability experiment, this loss can be seen for both air and nitrogen saturated conditions which suggests that oxygen is not the reason of the reduction. It is more likely because of being in solution as it has been known that enzymes are very instable when used in solution. The results from short or long stability experiments show that P2O-T169G could be a very good alternative to GOx because of its good stability even in the presence of oxygen.

4. Conclusions

The oxygen resistibility of P2O-T169G and feasibility of its application in electrochemical systems was shown using electrochemical characterisation methods. Stability study with chronoamperiometry showed that oxygen has no significant effect on the performance of P2O-T169G whereas it affected GOx remarkably. The current density of GOx was decreased by 30 % in the presence of oxygen while P2O-T169G almost showed no change in current density when tested in solution. If used in immobilized systems, even more enhanced result could be achieved. Further studies on enzyme electrode with immobilised enzymes for glucose oxidation are undertaking. In conclusion, this study suggests that when used in biofuel cells P2O-T169G could be an alternative to GOx with its good oxygen resistance and stability.

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