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Indirect evidence of direct electron communication between the active site of galactose oxidase and a graphite electrode

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Abstract

Bi-enzymatic biosensor based on galactose oxidase (GalOD) and horseradish peroxidase (HRP) using ferrocene as an efficient mediator was constructed. When a dependence of a working potential on the sensor performance was examined, an unusual behaviour was observed. With increasing of an applied working potential a lower concentration of substrate to attain full linear range was needed. A fully linear dependence from the first substrate addition was observed at and above the working potential of 150 mV. This activation of the biosensor response by an applied working potential very well corresponds with a formal potential of GalOD (156 mV). When a membrane prevented GalOD access to the electrode surface was applied, no activation effect of a working potential on the sensor performance was observed. Thus, it can be assumed that direct electron communication between GalOD and the electrode occurred. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Biosensor; Galactose oxidase; Direct electron transfer; Copper oxidase

1. Introduction

Galactose oxidase is a 68-kDa enzyme belonging to the family of copper containing radical proteins produced especially by the fungi *Dactylium dendroides*. The enzyme is able to oxidise a wide range of analytes (galactose and primary alcohols including sugars/polysaccharides) to the corresponding aldehydes, coupled to the reduction of O_2 to H_2O_2 [1].

The enzyme contains two one-electron redox centers: a mononuclear copper center and a ligating tyrosyl radical (Tyr272) that is covalently cross-linked at a position ortho to the phenolic oxygen of cysteine residue (Cys228). Ligated Cu^{II} has square–pyramidal coordination (Fig. 1). The reaction mechanism is rather complicated [1] and is shown in Eq. (1).

$$\underset{(GalOD_{ox})}{Cu^{2+} - Tyr^{\bullet}} \xleftrightarrow{+e^-(-e^-)} \underset{(GalOD_{semi})}{Cu^{2+} - Tyr} \xleftrightarrow{+e^-(-e^-)} \underset{(GalOD_{red})}{Cu^{+} - Tyr} \quad (1)$$

Oxidised form of GalOD reacts with a substrate leading to the reduced form of GalOD, which is oxidised by oxygen. Oxidised form of GalOD contains a very stable radical, which persists at ambient temperature with a half-life of nearly a week [2]. Due to this process, the enzyme is present in a mixed redox state consisting of $GalOD_{semi}/GalOD_{ox}$ in a ratio of ~95:5 [1]. Copper in the active site of the enzyme plays a crucial role because it cannot be replaced by other metals such as zinc or cobalt, and copper atom is localised in the outer part of the enzyme [3].

Free radical chemistry of GalOD allows rapid and indiscriminant oxidation of variety of reducing substrates producing hydrogen peroxide. This enzyme can be either 'on' or 'off' depending on the redox state of the active site. In addition, there may be a regulatory significance to the redox activation step characteristic for the radical copper oxidases [2]. In 1985, Johnson et al. [4] observed that the active site of GalOD is strongly influenced by the solution potential, and standard redox potential of the active site was determined to be 400 mV vs. SHE (corresponds to 156 mV vs. SCE).

In our experiment for higher efficiency of galactose detection, horseradish peroxidase (HRP) was co-immobilised together with galactose oxidase [5]. However, HRP could communicate with an electrode via direct electron

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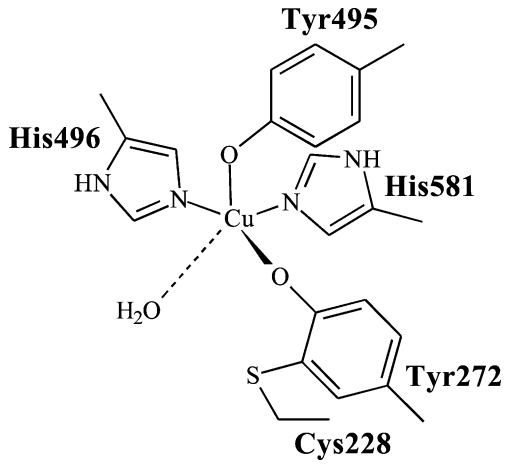

Fig. 1. The structure of the active site of GalOD with square–pyramidal coordination of Cu.

transfer, it is better to use mediators to overcome the slow heterogeneous electron transfer between HRP and the electrode [6]. For this purpose, ferrocene was successfully used [7]. As a result, a "reagentless" galactose biosensor was constructed.

2. Experimental

2.1. Reagents

Galactose oxidase (GalOD), horseradish peroxidase (HRP), dialysis membrane and galactose were supplied from Sigma. Ferrocene was purchased from Merck. All other reagents were supplied by Lachema (Brno, Czech Republic).

2.2. Apparatus

Biosensor measurements were carried out on Amperometric Detector ADLC2 (Laboratorní přístroje, Prague, Czech Republic) using a graphite electrode (Electrocarbon Topo'lčany, Slovak Republic) as a working electrode and saturated calomel electrode (SCE) as a reference electrode.

2.3. Procedures

Working electrodes were prepared from graphite rods with a diameter of 5 mm. Ferrocene was adsorbed on the electrode surface by dipping of the electrode into paraffin solution of ferrocene overnight [8]. Suspension of the enzymes in phosphate buffer was dropped on the electrode surface and then dried and subsequently covered with a dialysis membrane. Each electrode contained 8–10 U of GalOD and 12–15 U of HRP, both dissolved in a phosphate buffer.

To prevent direct contact between an electrode and GalOD, a cellulose acetate (CA) membrane was used. The membrane preparation is well described in our recent study [9]. On a ferrocene adsorbed graphite electrode, a solution of HRP was spread. After water evaporation, a CA membrane was applied on HRP layer. Subsequently, a solution of GalOD was spread on a CA membrane and water evaporation was allowed. The electrode was subsequently covered by a dialysis membrane and rubbed by an O-ring.

3. Results and discussion

The effect of working potential was examined from 0 to +250 mV. For better clarity of presented results, the influence of the working potential on the sensor performance is shown from 0 to +150 mV (Fig. 2.) The first addition of galactose did not cause a current flow through the electrode at a working potential of 0 mV. The second addition of galactose caused only a low current flow, after which the current was increased further by the addition of galactose up to a total of five additions. Subsequently, a linear dependence was observed between the current flow and galactose added to the reaction vessel. At a potential of 50 mV, similar behaviour was observed, but linear dependence was established after the third addition of galactose. A fully linear dependence after the first galactose addition was observed at and above a working potential of 150 mV.

As was mentioned above, the redox potential of Cu-coordinated tyrosine-272 present in the enzyme centre is 156 mV vs. SCE. Thus, at potential below 156 mV, the enzyme is in its intermediate state—$GalOD_{semi}$. With lowering working potential from 156 mV down to 0 mV, the equilibrium is shifted more to the intermediate state of the enzyme, but even at a potential of 0 mV, there are some enzyme molecules present in its oxidised state able to oxidise galactose in the presence of oxygen producing H_2O_2. The hydrogen peroxide involved in the reaction

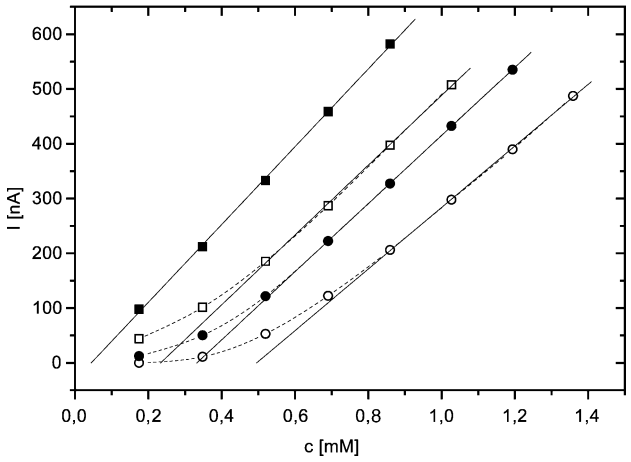

Fig. 2. Calibration curves for galactose detection with immobilised GalOD and HRP. Measurement was carried out in 0.1 M phosphate buffer pH 8.0 determined at ○ 0 mV, ● 50 mV, □ 100 mV and ■ 150 mV and a temperature of 28 °C.

probably oxidises the redox centre of the enzyme and an increase in enzyme activity by this autocatalytic process was observed. This fact can explain the enhancement of the sensitivity of sensor response with increased concentration of galactose at a working potential below 150 mV. An applied potential could affect the redox state of the enzyme and thus galactose oxidase activity, only when some form of direct communication between the active site of GalOD and the electrode occurs.

To prove that GalOD activity is influenced by an applied working potential, additional experiment was carried out, when GalOD was spread on the electrode covered by a CA membrane. It was confirmed that when GalOD had no access to the graphite electrode, no effect of an applied working potential on the sensor performance was observed. The output signal was linear from the first galactose addition, when a working potential from -100 to $+250$ mV was applied (data not shown).

4. Conclusion

It was confirmed that the redox state of GalOD was influenced by an applied working potential and an activation of the enzyme occurred. When GalOD was spread on a CA membrane, this phenomenon was not observed. Galactose oxidase is a small molecule (68 kDa) with an active site localised close to the surface of the enzyme. Based on these facts, it can be concluded that GalOD is able to communicate directly with a graphite electrode. This could be supported by the fact that laccase, ascorbate oxidase and superoxide dismutase (copper containing oxidoreductases) are able to communicate with the electrode via direct electron transfer [10].

However, more direct evidence of direct electron transfer between galactose oxidase and a graphite electrode is necessary.

Acknowledgements

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