Biosensors and Bioelectronics 24 (2009) 1382–1388

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Construction, assembling and application of a trehalase–GOD enzyme electrode system

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ARTICLE INFO

Article history:
Received 14 April 2008
Received in revised form 12 July 2008
Accepted 31 July 2008
Available online 20 August 2008

Keywords:
Trehalose
Screen-printed electrode
Trehalase reactor
GOD biosensor
Prussian Blue
Boletus edulis mushroom

ABSTRACT

Trehalose is a disaccharide important in foods, serving as a glucose source in many and also as an additive in the food preparation. Because of its peculiar physico-chemical properties it plays an important role as preservative in drying and deep-freezing treatments.

A new biosensor for trehalose determination has been realized by means of a flow system, based on a reactor in which the trehalase enzyme catalyses its hydrolysis into two α,D-glucose molecules, and a GOD (glucose oxidase) amperometric biosensor is employed for the glucose determination.

The optimum operative conditions have been laid out and a particular attention has been paid to the immobilization procedure of the two enzymes. The electrode used is of the SPE (screen-printed electrode) type and has been activated with the Prussian Blue (PB) and then assembled using GOD immobilized with Nafion. The reactor has been prepared with the trehalase enzyme chemically immobilized on an Immunodyne ABC membrane.

As demonstration of its utility, the biosensor has been tested on a real sample of *Boletus edulis* mushroom.

1. Introduction

Trehalose is a non-reducing disaccharide and the isomer we considered is the α,α-trehalose (1-*O*-(α-D-glucopyranosyl)-α-D-glucopyranoside), formed by two α,D-glucose molecules, linked with a 1,1 bond ($C_{12}H_{22}O_{11}$; M.W. 342). It is present in a large variety of microorganisms and plants (Richards, 2002) and it can serve as a reserve of carbohydrates and also to preserve the cells in response to stress conditions. In fact under stress conditions the trehalose metabolizing enzyme TPS (trehalose-6-phosphate synthase) increases its activity determining the accumulation of trehalose (El-Bashiti et al., 2005).

In humans trehalose is metabolized by the trehalase enzyme, producing two α,D-glucose molecules. Thus, a deficit of this enzyme in humans could determine a reaction similar to that caused by poisonous mushrooms, given that trehalose is particularly abundant in mushrooms (Terashita et al., 2003).

This disaccharide is also used as a glucose source and as an additive in food. Owing to its peculiar behaviour under dry and low temperature conditions, the trehalose can act as a preservative in food submitted to the deep-freeze procedure. The presence of trehalose prevents proteins denaturation and also, because of its particular amorphous glass forms under dry conditions, it can stabilize dry macromolecules; i.e. trehalose substitutes water molecules by forming hydrogen bonds with polar phospholipid heads groups and/or with aminoacids (Sampedro and Uribe, 2004).

In order to determine the trehalose concentration many methods are reported in the literature: those based on HPLC (Murray et al., 1997) or spectrophotometric measurements (Ferreira et al., 1997) and a FIA method using the spectrophotometric detection (Meyer zu Duttingdorf et al., 1997). All the reported methods are enzymatic assays and in some cases immobilized enzymes are used. In those methods preliminary extraction is always necessary and sometimes they show some matrix interferences.

In this work, we report a new flow system useful for the sensitive determination of trehalose, based on an immobilized trehalase reactor combined with a screen-printed GOD (glucose oxidase) biosensor (Tudorache and Bala, 2007; Ricci et al., 2003).

The best operative conditions have been optimised and, by means of the standard calibration curve, some mushrooms samples for the trehalose content have been analyzed as an example.

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doi:10.1016/j.bios.2008.07.084

2. Experimental

2.1. Reagents

All chemicals were commercial products of analytical grade.

Glutaraldehyde 25% (v/v), Nafion 5% (v/v), D-glucose and D-trehalose dihydrate, glucose oxidase (GOD) from *Aspergillus niger* VII S (195 IU mg^{-1}) (E.C. 1.1.3.4), trehalase from porcine kidney (1.35 IU mg^{-1}) (E.C.3.2.1.28) were from Sigma–Aldrich.

The Immunodyne ABC membrane was from Pall Corporation, Immobilon-AV pre-activated was from Millipore and the nitrocellulose membrane (0.01 μm pores) from Sartorius.

Unless otherwise stated, all solutions were prepared with phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}, pH 6.5. Standard solutions were daily prepared in the same buffer. Membranes with immobilized enzymes were stored at +4 °C for maximum 3 days, the only Immunodyne ABC membrane with trehalase enzyme was preserved in glycerol 50% (v/v).

2.2. Apparatus

Amperometric measurements were carried out using a VA 641 amperometric detector (Metrohm, Herisau, Switzerland), connected with a X-t recorder (L250E, Linseis, Selb, Germany). Cyclic voltammetry experiments were performed with an Autolab electrochemical system (Eco-Chemie, Utrecht, Netherlands) equipped with PG Stat12 and GPES software. The orbital shaker was from MPM Instruments (Bernareggio, Milan, Italy). Peristaltic pumps were Minipuls3 (Gilson, France), Rotavapor (Büchi, Labortechnik AG, SW).

The trehalase reactor was a home-made Teflon apparatus, and a drawing is shown in Fig. 1. The electrochemical cell of the "thin-layer" type was a plexiglass home-made apparatus, formed by a flow-through cell with two tubes (in and out) and the appropriate holder for the GOD electrode (Fig. 1).

2.3. Electrodes

Screen-printed electrodes (SPEs) were home-produced with a 245 DEK (Weymouth, England) screen-printing machine. Graphite-based ink (Elettrodag 421) from Acheson (Milan, Italy) was used to print the working and counter electrode. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia (Milan, Italy).

The electrodes were produced in foils of 48 strips. Each sensor consists of three screen-printed elements: two carbon electrodes, working and counter, and a silver electrode acting as pseudoreference, respectively. The diameter of the working electrode was 0.3 cm, which resulted in an apparent geometric area of 0.07 cm^2. After the printing step, the foils were stored dry, at room temperature, in the dark.

2.4. Preparation of Prussian Blue-modified screen-printed electrodes

Prior to Prussian Blue (PB) modification, screen-printed electrodes were pre-treated in a 0.05 M phosphate buffer + 0.1 M KCl, pH 7.4, by applying a positive potential of 1.7 V during 3 min.

Prussian Blue modification of SPEs was then accomplished by placing a drop (10 μL total volume) of precursor solutions onto the working electrode area. This solution is obtained by mixing directly on the working electrode surface 5 μL of 0.1 M potassium ferricyanide ($K_3Fe(CN)_6$) in 10 mmol L^{-1} HCl to 5 μL of 0.1 mol L^{-1} Fe(III) chloride in 10 mM HCl. The drop was carefully placed exclusively on the working electrode area, in order to avoid the formation of PB on the reference and counter electrodes, an event that could notably increase the internal resistance of the system. The solution was left on the electrode for 10 min and then rinsed with a few millilitres of 10 mmol L^{-1} HCl. The electrodes were then left 90 min in the oven at 100 °C to obtain a more stable and active layer of PB. To control the correct deposition of PB on the electrode, cyclic voltammograms have been performed as reported before (Ricci et al., 2003). The PB-modified electrodes were stored dry at room temperature in the dark and remain stable for almost 1 year.

2.5. GOD enzyme immobilization

PB-modified SPEs were used as support for enzyme immobilization. To produce the biosensor, a cross-linking method was adopted (Ricci et al., 2005). 4 μL of a 2.5% (v/v) glutaraldehyde solution were applied with a syringe exclusively on the PB-modified working electrode. The solution was then left to evaporate. Then, 4 μL of a GOD and Nafion® mixture were applied on the working electrode. The mixture was obtained by mixing 2 μL of Nafion (0.1% (v/v) diluted in water) and 2 μL of a stock enzyme solution (25 mg mL^{-1} corresponding to 4875 IU mL^{-1}).

The above mixture (4 μL) was placed onto the working electrode area and allowed to dry for 45 min at room temperature.

The biosensors were then kept in phosphate buffer solution 0.05 M + 0.1 M KCl, pH 6.5 at 4 °C until use.

2.6. Trehalase enzyme immobilization

2.6.1. Physical immobilization on nitrocellulose membrane

8 μL of trehalase solution (3.7 IU mL^{-1}) were entrapped between two membranes of nitrocellulose (disk shaped) and then inserted into the reactor (Elekes et al., 1995).

2.6.2. Chemical immobilization on Immobilon-AV membrane

10 μL of trehalase solution (3.7 IU mL^{-1}) were adsorbed on the pre-activated (disk shaped) Immobilon-AV membrane (Campanella et al., 1999). After 1 h the treated membrane was washed for 10 min with phosphate buffer (0.1 mol L^{-1}), pH 8.

2.6.3. Chemical immobilization on Immunodyne ABC membrane

A disk shaped Immunodyne ABC membrane (Kelly et al., 1998; Sibanda et al., 1999) was treated with a 2% (p/v) PEI (polyethyleneimmine) solution and maintained under solution stirring for 1 h, washed with distilled water under mixing for 10 min and then treated with a (2.5%, v/v) glutaraldehyde solution under stirring for 40 min. On each side of the so treated membrane, rewashed with distilled water, 13 μL of trehalase solution (3.7 IU mL^{-1}) were deposed and left to dry for 1 h. After rewashing, the membrane was dipped in a glycine solution (0.5 mol L^{-1}) for 30 min with stirring. For preservation, the so prepared membrane was maintained in a glycerol solution (50% (v/v) in buffer pH 6.5) at +4 °C.

2.7. Assembling of the flow system

The system devised out for the trehalose assay (Fig. 1) consists of a peristaltic pump, in order to flow the solutions into the measuring system, a reactor containing the immobilized trehalase enzyme, a GOD amperometric biosensor for the glucose assay, an amperometer and a recorder. A switch (T-valve) allows the solution to flow or into the reactor and then through the GOD biosensor (path A), or directly into the GOD biosensor (path B). By using path B, it is possible to measure the current response due to the glucose concentration initially present in the sample solution, while through