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

M.L. Antonelli[a], F. Arduini[b,c,*], A. Laganà[a], D. Moscone[b,c], V. Siliprandi[a]

[a] Dipartimento di Chimica, SAPIENZA Università di Roma, Italy
[b] Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy
[c] Consorzio Interuniversitario Biostrutture e Biosistemi "INBB", Viale Medaglie d'Oro, 305 Roma, Italy

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.

* Corresponding author at: Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy.
Tel.: +39 06 72594404; fax: +39 06 72594328.
E-mail address: fabiana.arduini@uniroma2.it (F. Arduini).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
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

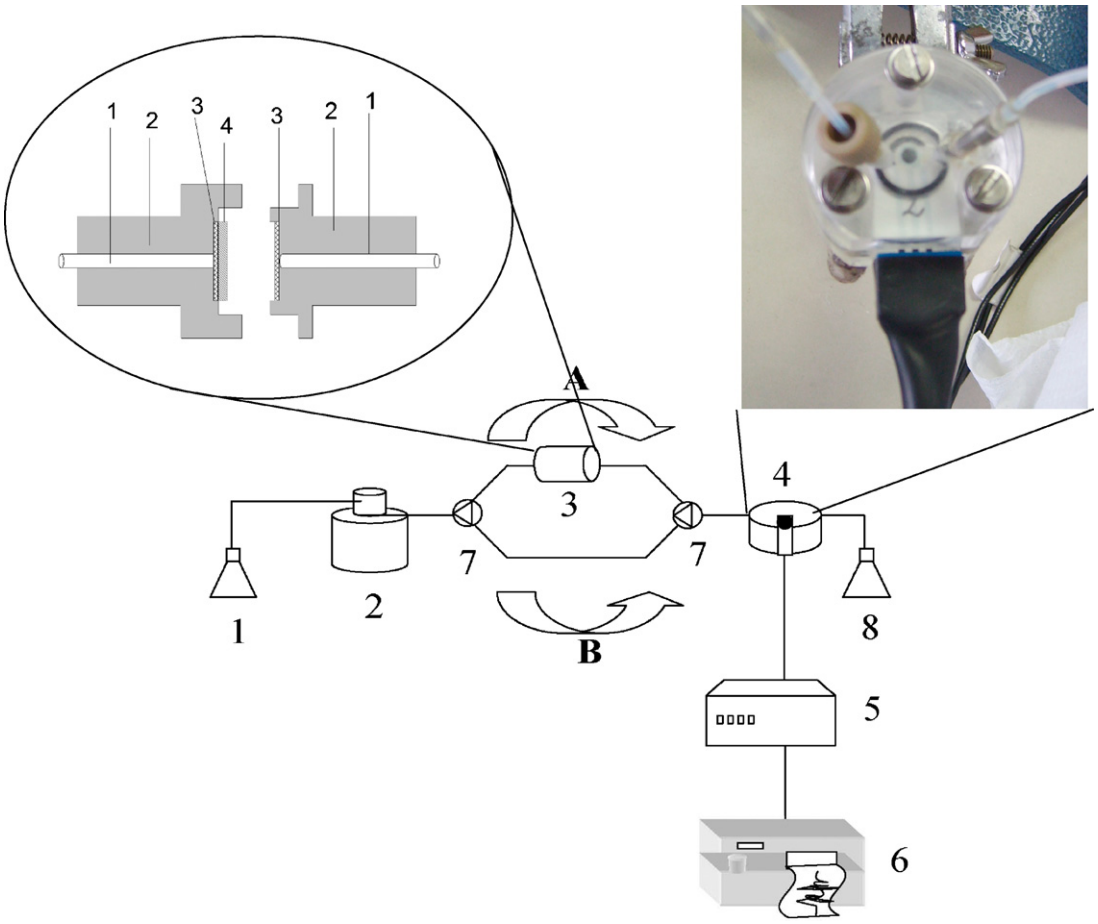

Fig. 1. Scheme of the flow system for the trehalose determination (1: sample solution; 2: peristaltic pump; 3: trehalase reactor; 4: GOD biosensor in the flow cell; 5: amperometer; 6: recorder; 7: switch (T valve); 8: waste. (A) Path to assay the total glucose in the sample (the intrinsic one *plus* the one produced by the trehalase); (B) path to assay the intrinsic glucose in the sample. Inset:
[-]
- Scheme of trehalase reactor: the fluid passes through the in- and out-connections (1) to the reactor that is enclosed by the Teflon adaptors (2). The enzyme is immobilized on membranes (4) between two stainless steel screens (3).
- Picture of GOD biosensor in the flow cell.

the path A, the additional amount of glucose, deriving from the enzymatic hydrolysis of the trehalose present in the sample solution, can be measured. By subtracting the response obtained during the flow-through path B from that obtained from path A, it is possible to quantify the only current due to the glucose produced from the trehalose hydrolysis ($I_A - I_B$).

By means of the calibration curve I (nA) versus [trehalose] (mol L^{-1}) the trehalose concentration is allowed to be determined.

2.8. Sample preparation

The real samples examined were exsiccated *Boletus edulis* mushrooms. In order to obtain a sample solution containing trehalose, an extraction procedure has been worked out by modifying the reported ones (Yang et al., 2001; Chuanbin et al., 1998).

A quantity of 80 mg of exsiccated *B. edulis* mushrooms were minced and dissolved in 50 mL of 80% ethanol solution. The suspension was shaken for 45 min at room temperature, then filtered on a 0.45 μm filter paper, and the residue washed 3 times with 25 mL of 80% ethanol solution. The filtered solution and the washing solutions were mixed and put into the rotavapor at 40 °C long enough for complete solvent evaporation. The insoluble residue was dissolved in 10 mL of buffer solution pH 6.5 and again filtered.

The so prepared sample solution was ready to be pumped into the flow system.

2.9. Final operative conditions

By using the flow system described, formed by the trehalase reactor and the GOD screen-printed electrode biosensor for glucose, all measurements on standards and also on real samples were performed under the best operative conditions, resulting from the optimisation process: $T = 25$ °C; flow rate = 50 μL min^{-1}; pH 6.5 (phosphate buffer 0.05 mol L^{-1} + KCl 0.1 mol L^{-1}); trehalase immobilized on Immunodyne ABC membrane (0.05 IU on each membrane side); GOD biosensor: SPE Prussian Blue pre-treated, GOD immobilized with Nafion and glutaraldehyde on the electrode surface.

3. Results and discussion

3.1. PB-modified electrode performances

PB films (potassium iron(III)hexacyanoferrate(II)) are usually prepared by electrochemical reduction of solutions containing Fe(III) and hexacyanoferrate(III) ions or chemically synthesized directly from solutions containing Fe(III) and hexacyanoferrate(II) ions (Ricci and Palleschi, 2005b).

In our case, PB has been chemically synthesized starting from both iron(III) precursor (potassium ferricyanide ($K_3Fe(CN)_6$) and iron(III) chloride) directly on the surface of the carbon working electrode previously activated by oxidation at a positive potential (+1.7 V).

We have already demonstrated that, in the presence of activated carbon particles, an autoreduction or a catalytic reduction of the highly reactive ferric-ferricyanide complex, which presumably is initially formed, seems to occur. In this way we obtain a layer of PB with improved characteristic of excellent stability in a wide range of pH (3–9), high sensitivity, long operational lifetime, and fast response time (Moscone et al., 2001).

The catalytic activity towards the reduction of H_2O_2 of PB-modified SPE was evaluated in batch by amperometric measurements. Then the response time, detection limit, linearity range, sensitivity and electrode reproducibility were studied. All the measurements were performed in 0.05 mol L^{-1} phosphate buffer + 0.1 mol L^{-1} KCl pH 7.4 (10 mL) and at an applied potential of −0.050 V versus screen-printed internal silver pseudoreference electrode.

The calibration curve of hydrogen peroxide obtained with three different electrodes (using each electrode for all the concentration values tested) showed a good linearity in a range between 1×10^{-6} and 5×10^{-4} mol L^{-1} with a detection limit of 5×10^{-7} mol L^{-1} (signal/noise = 3). The regression equation of the linear portion of the curve was $Y = 34.5X - 36.8$ where Y represents the current in nA and X the H_2O_2 concentration in μmol L^{-1}; the R^2 was 0.995. Reproducibility was also calculated and was not larger than 3% (for all the concentrations tested by three different electrodes). The response time needed to reach 90% of the steady state response was 10 s. The results obtained are in agreement with previous work (Ricci et al., 2003).

3.2. Glucose biosensor

The PB-modified electrodes were used as support for GOD immobilization, and the analytical properties of these SPE biosensors were studied. The biosensor for glucose detection showed a good linearity in the range between 1×10^{-6} and 5×10^{-4} mol L^{-1} with a detection limit of 8×10^{-7} mol L^{-1} (signal/noise = 3). The regression equation of the linear part of the curve was $Y = 4.0X - 0.4$: where Y represents the current in nA and X the glucose concentration in μmol L^{-1}. The reproducibility of the biosensor obtained was quite good. A intra-electrode RSD% of 4% was observed for three replicates using the same biosensor. Three different biosensors were also tested with the same concentration of glucose, resulting in inter-electrode RSD% value of 7%.

3.3. Trehalose measurements

Amperometric detection of trehalose is based on the trehalase enzyme reaction that hydrolyses the substrate (trehalose) into two α,D-glucose molecules. It is known in literature that the substrate of glucose oxidase is the β,D-glucose, so, firstly, a tri-enzymatic system based on trehalase enzyme, mutarotase enzyme and glucose oxidase enzyme was adopted and compared with the bi-enzymatic system where the mutarotase enzyme was not used. In the tri-enzymatic system, the mutarotase enzyme was co-immobilized with glucose oxidase on the Prussian Blue-modified screen-printed electrode. Under the same working operative conditions, no difference in the trehalose detection was observed using the tri-enzymatic system or the bi-enzymatic one (data not reported). These results are also in agreement with the work of Amine et al. (1995), where the mutarotase enzyme was not employed due to the spontaneous non enzymatic mutarotation of α,D-glucose to β,D-glucose. On the basis of these results, the more simple bi-enzymatic system was adopted.

The spontaneous non enzymatic mutarotation of α,D-glucose to β,D-glucose occurs while the glucose produced in the trehalose reactor reaching the GOD biosensor in the electrochemical cell. Then, the glucose oxidase converts the β,D-glucose molecules into gluconic acid and H_2O_2. The H_2O_2 was detected at low potential 0.05 V versus the screen-printed internal silver pseudoreference electrode using PB as electrochemical mediator.

$$\text{Trehalose} + H_2O \rightarrow 2\alpha, \text{D-Glucose}$$

$$\alpha, \text{D-Glucose} \rightarrow \beta, \text{D-Glucose}$$

$$\beta, \text{D-Glucose} + O_2 \rightarrow H_2O_2 + \text{gluconolactone}$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

The trehalase enzyme was immobilized in two different ways: by physical entrapment between two membranes of nitrocellulose or by chemical immobilization using Immobilon-AV membrane or Immunodyne ABC membrane. The results obtained are reported in the following paragraphs.

3.3.1. Physical immobilization

In order to obtaining a low detection limits, different amounts of trehalase enzyme of a 3.7 IU mL^{-1} enzyme solution, (4, 8 and 12 μL) corresponding at 14.8, 29.6 and 44.4 mIU, respectively were entrapped between the two membranes of nitrocellulose. The best results in terms of reproducibility and sensitivity were obtained using 29.6 mIU (data not shown).

3.3.1.1. Effect of flow rate. Fig. 2a shows the influence of the flow rate on the response to trehalose. Increasing the flow rate until 100 μL min^{-1}, the current increases, and a sharply decrease was observed at 150 μL min^{-1}. The choice of the optimal flow rate should take into consideration also the peak broadening and the sampling rate; therefore, 100 μL min^{-1} was selected as the most advantageous value for the determination of trehalose in the presented measurements.

3.3.1.2. Effect of pH. It is known that the Prussian Blue is stable and highly active at acidic pH, while its activity declines in alkaline media. Our deposition procedure, however, has demonstrated the maintenance of its activity and working stability in a large range of pH (Ricci et al., 2003) However, the system employs also two enzymes with different operative pH conditions: the glucose oxidase has its best at pH 5.5, while the trehalase enzyme at pH 6.5, then the working pH was selected evaluating the response of the whole system.

In this study, the trehalase concentration used was 29.6 mIU, the concentration of trehalose added was 1×10^{-4} mol L^{-1} and the current due to the formation of H_2O_2 was recorded in about 10 min. The signals obtained are shown in Fig. 3a. At pH value of 6.5 we observed the highest current response, thus this value of pH has been chosen for further experiments.

3.3.1.3. Calibration curve. To build the calibration curve useful for the determination of trehalose in the samples, many measurements were performed by determining the current values obtained with different standard solutions of trehalose (three measurements for each concentration value).

Each current value inserted into the curve was calculated as explained before: $I_A - I_B$.

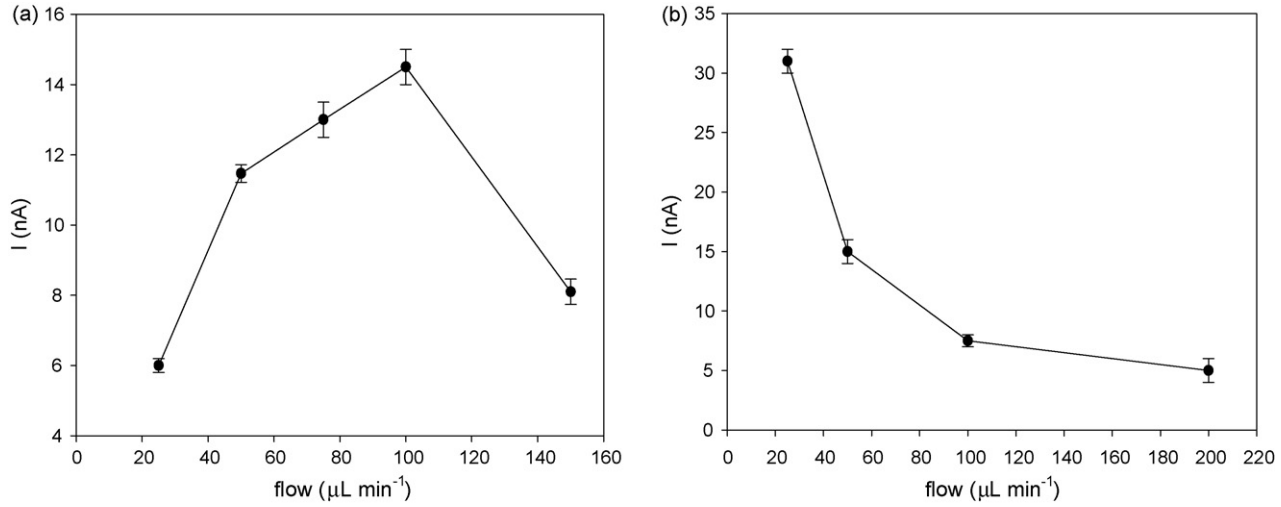

Fig. 2. Current response in function of flow rate in the system (pH 6.5, $T = 25\,^{\circ}C$, 1×10^{-4} mol L^{-1} trehalose solution) using physical immobilization of trehalase enzyme in the reactor (a) or chemical immobilization on Immunodyne ABC membrane (b). Values are the averages of three different measurements.

With the trehalase enzyme immobilized on the nitrocellulose membrane, the equation of the calibration curve was: $Y = 0.468X + 0.16$ ($R^2 = 0.994$). Under the optimised conditions, the system was able to detect trehalose at concentrations between 1.5×10^{-5} and 7×10^{-4} mol L^{-1}. The intra-membrane RSD% is quite good, equal to 4%. The detection limit obtained with the proposed method, using the physical immobilization of trehalase was 0.8×10^{-5} mol L^{-1}, lower than that obtained by Meyer zu Duttingdorf et al. (1997) with a spectrophotometric flow injection analysis system using trehalase, hexokinase and glucose-6-phosphate dehydrogenase enzymes.

3.3.1.4. Stability study. Investigations on the storage stability of the trehalase membrane coupled with the glucose biosensor were carried out by measuring the current response to a 1.0×10^{-4} mol L^{-1} trehalose solution. In order to control the working stability, measurements were repeated 10 times, the current response recorded showed a 40% decrease after the 8th measurement.

The proposed system, using such a physical immobilization, reaches a low detection limit but suffers of poor stability, probably due to an enzyme loss during the measurements; therefore other immobilizations were investigated to overcome this drawback.

3.3.2. Chemical immobilization

Two different chemical immobilizations were developed using either Immobilon-AV or Immunodyne ABC membrane. The preliminary results obtained investigating the reproducibility and the sensitivity of the systems (see Section 3.3.1.3) indicates that the calibration curve preformed with the trehalose immobilized on Immunodyne ABC membrane is characterised by the best analytical performance, and thus this immobilization was chosen for the present work.

3.3.2.1. Effect of flow rate. The effect of flow rate was also investigated using the system developed with the trehalase chemically immobilized. Fig. 2b shows the influence of the flow rate on the response to trehalose. The current response decreases by increas-

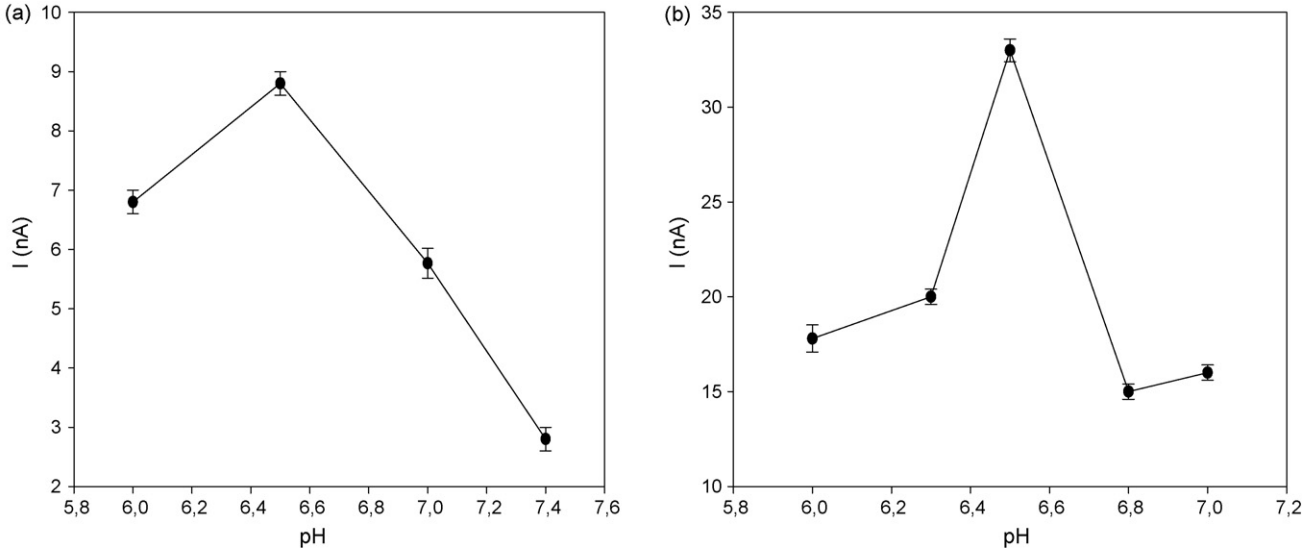

Fig. 3. Current response in function of pH ($T = 25\,^{\circ}C$, 1×10^{-4} mol L^{-1} trehalose solution) using: physical immobilization of trehalase enzyme in the reactor at a flow rate = 100 μL min^{-1} (a) or chemical immobilization on Immunodyne ABC membrane at a flow rate = 50 μL min^{-1} (b). Values are the averages of three different measurements.

ing the flow rate, showing a different behaviour than the system with the trehalase physically immobilized. As already stated for the physical immobilization, the choice of the optimum flow rate was investigated and a flow rate equal to 50 μL min^{-1} was selected, resulting as the best compromise between a sensitive measurement and a fast response.

3.3.2.2. Effect of pH. Because the optimal pH of enzymes generally changes depending of the immobilization procedure, we again tested the pH effect of this covalent enzymatic bonding in the range $6 \leq pH \leq 7$. In this study, 96.2 mUI of trehalase and 1×10^{-4} mol L^{-1} of trehalose were adopted. Fig. 3b shows that the best operative conditions correspond to a pH 6.5, so this value has been chosen.

3.3.2.3. Standard calibration curves. The calibration curve obtained with the trehalase immobilized on Immobilon-AV is described by the following equation: $Y = 0.468X + 0.16$ ($R^2 = 0.994$). With the trehalase immobilized on the Immunodyne ABC, the calibration curve showed the subsequent equation: $Y = 1.812X - 3.46$ ($R^2 = 0.997$). This curve was obtained by performing three measurements at the same concentration values using the same membrane disk with the immobilized enzyme (intra-membrane curve). For all the chemical immobilization the same concentration range was examined: $2.5 \times 10^{-5} \leq [\text{trehalose}] \leq 30 \times 10^{-5}$ mol L^{-1}.

As it can be observed by comparing the equations of the calibration curves, the system with the trehalase, either physically or chemically (Immobilon-AV) immobilized, gave rise to similar results, while by using the Immunodyne ABC immobilization, a higher slope was obtained, so permitting more sensitive analyses.

The immobilization of trehalase on Immunodyne ABC membrane has been chosen and the reproducibility using the same membrane was 9% (intra-membrane RSD%, $n = 3$).

A new calibration curve was then plotted, by measuring the current involved when each measurement was repeated for the same concentration value by using new membrane disks with the immobilized enzyme, and obtaining a inter-membrane reproducibility equal to 11% ($n = 3$). The relative equation was: $Y = 1.662X - 1.08$ ($R^2 = 0.993$). The comparison of the two intra- and inter-membrane curves shows that the immobilization method is reproducible.

3.3.2.4 Stability study

The working stability has been evaluated by recording the response of the enzymatic system during 7 successive measurements. The results indicated a working stability better than that obtained using physical immobilization; in fact after seven consecutive measurements, no decrease of signal was observed. The proposed system then is characterised not only by good working stability but also reaches a lower detection limit than that of the reported method (Meyer zu Duttingdorf et al., 1997). Thus, the trehalase Immunodyne ABC immobilization was adopted for the analysis of trehalose in mushrooms real samples.

3.4. Interferences

The possible interference from other saccharides has been controlled for by measuring the current involved when standard solutions of 1.0×10^{-4} mol L^{-1} saccharose or 1.0×10^{-4} mol L^{-1} of fructose sugars were pumped into the flow measuring system, although no interferences were reported in the literature, owing to the high specificity of the trehalase enzyme (Kalf and Rieder, 1957). In agreement with these results, no current variation was recorded, therefore we can assert that the trehalase activity is not influenced by the presence of these disaccharides.

3.5. Application to real samples

As an example of the application of the proposed flow system method for the trehalose determination in food samples, a *B. edulis* mushroom sample has been analyzed. The sample was in the dessicated form and the extraction procedure was that described in Section 2.8. The found concentration of trehalose was 15.9×10^{-5} mol L^{-1}, mean value of three measurements with RSD% of 4.1%. The concentration of trehalose found is in good agreement with the range of concentrations reported in the literature for the mushrooms content, depending on the different kinds of mushrooms (Yang et al., 2001).

3.5.1. Study of matrix effect and recovery

To control the matrix effect on real samples, after the extraction procedure, four portions were prepared and each was spiked with a different amount of a standard solution of trehalose. The obtained curve showed this equation: $Y = 1.819X - 2.695$ ($R^2 = 0.995$), which is similar and parallel to the already reported calibration curve obtained with standard solutions (Section 3.3.2.3), showing that the matrix effect, due to other compounds eventually present in the sample, is negligible.

Finally, the recovery of analyte in the proposed procedure was evaluated by addition method. Three different quantities of a standard solution of trehalose were added to the mushroom sample before the extraction (50, 75 and 150 μL [trehalose] $\times 10^{-3}$ mol L^{-1}), the extracted solutions were then 25-fold diluted and then pumped into the flow system. By extrapolating the original value of trehalose concentration in the sample, a value of 14.9×10^{-5} mol L^{-1} was obtained. When the same mushroom sample was directly analyzed, a value of trehalose of 15.9×10^{-5} mol L^{-1} was found. The recovery (Γ) was calculated and showed a good performance of the proposed extraction procedure:

$$\Gamma = \frac{[\text{Trehalose}]\,\text{st.add}}{[\text{Trehalose}]\,\text{dir}} \times 100 = 93.7\%$$

4. Conclusion

The proposed biosensor/reactor flow system biosensor for the trehalose assay seems a very promising tool for the analyses on food samples, because of the high specificity of the two enzymes employed (glucose oxidase and trehalase). In particular a new and quite simple method of chemical immobilization of the trehalase enzyme is outlined here.

A concentration range of trehalose between 2.5×10^{-5} and 30×10^{-5} mol L^{-1} can be analyzed. The method appears free from disaccharides interferences and shows a good accuracy and reproducibility.

Finally, to evaluate the suitability of the analytical method, an application in real samples should need a comparison with certified products. However, to our best knowledge, neither trehalose certified reference material nor commercial material with a known trehalose concentration are available. In these cases, the trueness of the measurements assessed through recovery calculated by standard addition method on the unknown sample is acceptable. Recovery data are acceptable only when they are within 10% of the target value (Council of the European Communities, 2002). In this research work, a very good recovery through the standard addition method to that of the analyte (about 94%) has been obtained, using an original extraction procedure of trehalose from the *B. edulis* mushroom samples.

References

Amine, A., Cremisini, C., Pallescgi, G., 1995. MiKrochim. Acta 121, 83–190.
Campanella, L., Attioli, R., Colapicchioni, C., Tomassetti, M., 1999. Sens. Actuators B 55, 23–32.
Council of the European Communities, Council Directive EC/02/657, 2002. Off. J. Eur. Communities L. L221, 8–36.
Chuanbin, L., Jian, X., Fengwn, B., Zhiguo, S., 1998. Biotechnol. Tech. 12, 941–943.
El-Bashiti, T., Hamamci, H., Oketem, H.A., Yucel, M., 2005. Plant Sci. 169, 47–54.
Elekes, O., Moscone, D., Venema, K., Korf, J., 1995. Clin. Chim. Acta 239, 153–165.
Ferreira, J.C., Paschoalin, V.M.F., Panek, A.D., Trugo, L.C., 1997. Food Chem. 60, 51–254.
Kalf, G.F., Rieder, S.V., 1957. J. Biol. Chem. 230, 691–698.
Kelly, S., Compagnone, D., Guilbault, G., 1998. Biosens. Bioelectron. 13, 173–179.
Meyer zu Duttingdorf, H.D., Bachmann, B., Buchholz, M., Leuchtenberger, W., 1997. Anal. Biochem. 25, 8–12.
Moscone, D., D'Ottavi, D., Compagnone, D., Palleschi, G., Amine, A., 2001. Anal. Chem. 73, 2529–2535.
Murray, D.B., Hayashida, Y., Nishimura, K., 1997. Biotechnol. Tech. 11, 269–270.
Ricci, F., Palleschi, G., 2005b. Biosens. Bioelectron. 21, 389–407.
Ricci, F., Amine, A., Palleschi, G., Moscone, D., 2003. Biosens. Bioelectron. 18, 165–174.
Ricci, F., Moscone, D., Tuta, C.S., Palleschi, G., Amine, A., Poscia, A., Valgimigli, F., Messeri, D., 2005. Biosens. Bioelectron. 20, 1993–2000.
Richards, A.B., 2002. Food Chem. Toxicol. 40, 871–898.
Sampedro, J.G., Uribe, S., 2004. Mol. Cell. Biochem. 256–257, 319–327.
Sibanda, L., De Saeger, S., Van Peteghem, C., 1999. Int. J. Food Microbiol. 48, 203–209.
Terashita, T., Yoshida, K., Suzuki, A., Sakai, T., Yoshikawa, K., Nagai, M., 2003. Myoscience 44, 71–74.
Tudorache, M., Bala, C., 2007. Anal. Bioanal. Chem. 388, 565–578.
Yang, J.H., Lin, H.C., Mau, J.L., 2001. Food Chem. 72, 465–471.