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Amperometric determination of bonded glucose with an MnO_2 and glucose oxidase bulk-modified screen-printed electrode using flow-injection analysis

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Abstract

A screen-printed amperometric biosensor based on carbon ink double bulk-modified with MnO_2 as a mediator and glucose oxidase as a biocomponent was investigated for its ability to serve as a detector for bonded glucose in different compounds, such as cellobiose, saccharose, (-)-4-nitrophenyl-β-D-glucopyranoside, as well as in beer samples by flow-injection analysis (FIA). The biosensor could be operated under physiological conditions (0.1 M phosphate buffer, pH 7.5) and exhibited good reproducibility and stability. Bonded glucose was released with glucosidase in solution, and the free glucose was detected with the modified screen-printed electrode (SPE). The release of glucose by the aid of glucosidase from cellobiose, saccharose and (-)-4-nitrophenyl-β-D-glucopyranoside in solution showed that stoichiometric quantities of free glucose could be monitored in all three cases.

The linear range of the amperometric response of the biosensor in the FIA-mode flow rate 0.2 mL min^{-1}, injection volume 0.25 mL, operation potential 0.48 V versus Ag/AgCl) extends from 11 to 13,900 μmol L^{-1} glucose in free form. The limit of detection (3σ) is 1 μmol L^{-1} glucose. A concentration of 100 μmol L^{-1} yields a relative standard deviation of approximately 7% with five injections. These values correspond to the same concentrations of bonded glucose supposed that it is liberated quantitatively (incubation for 2 h with glucosidase).

Bonded glucose could be determined in beer samples using the same assay. The results corresponded very well with the reference procedure.

Keywords: Bonded glucose; Screen-printed electrode; Manganese dioxide; Glucose oxidase; Glucosidase; Flow-injection analyses

1. Introduction

We have recently developed a biosensor for glucose [1–3] incorporating glucose oxidase as a biocomponent and manganese dioxide as a mediator for carbon ink screen-printed (SPE) and carbon paste electrodes (CPE). The reaction mechanism of glucose at MnO_2/GOD modified sensors is shown in Fig. 1(B).

Glucose is enzymatically oxidized with molecular oxygen forming gluconolactone and hydrogen peroxide as an intermediate (I). The latter reacts chemically with MnO_2 producing manganese species at lower oxidation states (II), which can be electrochemically reoxidized to MnO_2 (III). The oxidative current flow is directly related to the glucose concentration. Besides this rapid electrochemical process, a kinetically slower chemical reoxidation of MnO/Mn_2O_3

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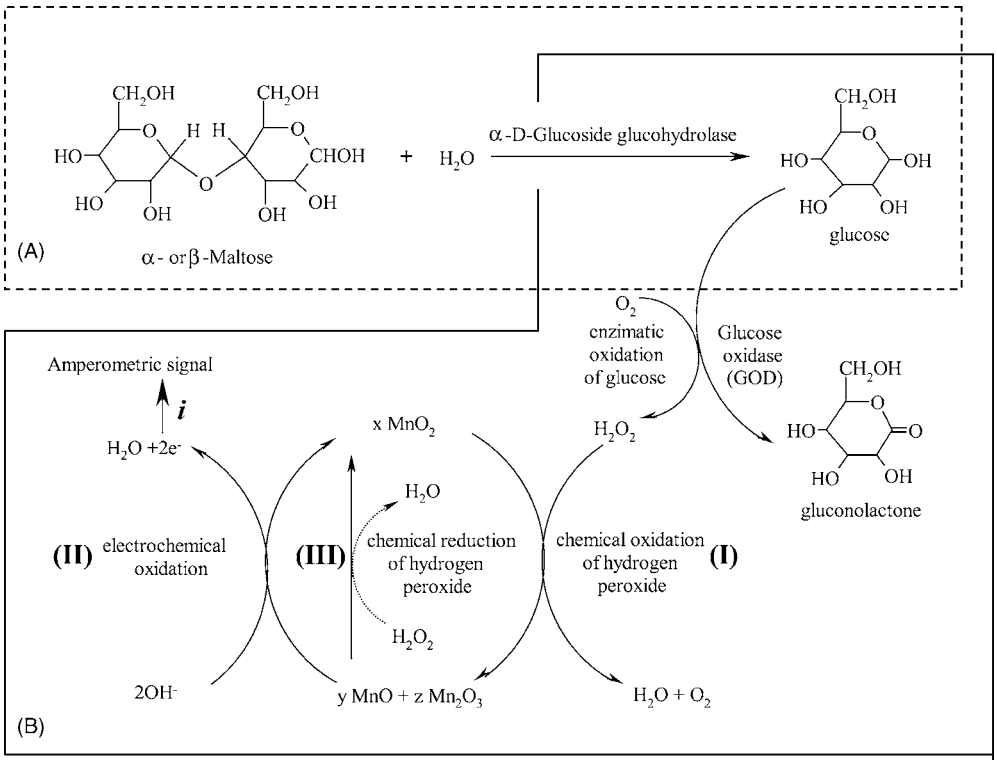

Fig. 1. Reaction mechanism of bonded glucose measurement with the biosensor. Step (A) cleavage of bonded glucose by glucosidase in the sample solution. Step (B) action of MnO_2 and glucose oxidase at the surface of the biosensor.

with H_2O_2 (corresponding to a catalytic decomposition of hydrogen peroxide) is possible [2].

The reaction proceeds only with free glucose as a substrate. Bonded glucose can be cleaved with glucosidase as shown in Fig. 1(A) with maltose as an example.

Bonded glucose is present in almost all complex matrices together with free glucose. Investigations and quantitative determinations of bonded glucose can yield valuable information on the chemical composition of a system and applied to the analysis of human samples, they can supply parameters on long-term glucose levels in blood. Usual methods for the determination of glucosylated compounds comprise their direct detection via spectrophotometry [4,5], imunoassays [6], HPLC [7], or electrochemical methods, such as potentiometry or amperometry [8–11]. As a physiologically important example, glucosylated hemoglobin HbA1c is photometrically, imunologically or chromatographically determined [12–18]. These methods are either quite straightforward imposing problems in more complex matrices, or they involve hydrolysis of the glucose ether via determination of the liberated glucose [19].

The aim of this work was to investigate the basic possibilities for the determination of bonded glucose in some model analytes with the biosensor modified with manganese dioxide and to investigate the experimental conditions necessary that the biosensors complies with the glucosidase action. Practical applicability shall be demonstrated by determination of bonded glucose in beer. Beer is a rather complex matrix because several thousand compounds have been already identified in it [20].

2. Experimental

2.1. Apparatus

2.1.1. Flow-injection system

The flow-injection system consisted of a high-performance liquid chromatographic (HPLC) pump (Model 510, Waters, Milford, MA, USA), a sample injection valve (U6K, Waters), and a thin-layer electrochemical cell (CC5, BAS Bioanalytical Systems Inc., West Lafayette IN, USA). Teflon spacers (MF-1047, MF-1048, BAS) were used to adjust the thickness of the flow-trough cell. An Ag/AgCl electrode (3 M KCl, model RE-1, BAS) served as the reference. The counter electrode was the back plate of the cell, made of stainless steel. The currents obtained were recorded with the electrochemical workstation BAS 100 B and evaluated with the corresponding software (BAS 100 W, version 2).

2.1.2. Reagents and solutions

Deionized water was distilled twice in a quartz still and then purified with an ion exchange system (Nanopure, Bernstead). Phosphate buffer (0.1 M, pH 7.5) was prepared by mixing aqueous solutions (0.1 M) of sodium

dihydrogenphosphate (approximately 38 mL, Fluka, Buchs, Switzerland) and di-sodium hydrogenphosphate (approximately 200 mL, Merck) to achieve the desired pH. Glucosidase (EC 3.2.1.21, from almonds, #G4511, ca. 20–40 U mg^{-1}) and glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, #49180, ca. 200 U mg^{-1}) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical purity grade (p.a., Merck).

2.1.3. Fabrication of the working electrode

Manganese dioxide and glucose oxidase double bulk-modified screen-printed electrodes were prepared by thoroughly mixing of 4.50 g carbon ink (C50905DI, Gwent, Pontypol, UK) with 0.25 g manganese dioxide (805958, Merck) and 0.25 g glucose oxidase (EC 1.1.3.4). The MnO_2/GOD-modified carbon ink was sonicated for 20 min and used immediately for electrode fabrication. The working electrodes were screen printed on inert laser pre-etched ceramic supports (Coors Ceramic, Chattanooga, TN, USA). Printing was done by applying thick layers (0.05 mm) of the ink onto the substrates through an etched stencil with the aid of a screen printing device (SP-200, MPM, Franklin, Ma, USA). The resulting plates were dried at 40 °C for 1 h. Electric contact was made with a crocodile clamp.

2.2. Procedures

2.2.1. Flow-injection analyses (FIA)

Flow-injection analysis (FIA) was performed with an applied potential of 0.48 V. The typical flow rate was 0.2 mL min^{-1}, the injection volume 250 μL. Evaluation of responses was done by their peak heights. The height was enumerated by adjusting a tangent (identical to the baseline) to the base of the peak and determining the distance to the peak maximum.

2.2.2. Analyses of samples

Samples of beer in bottles were bought in a local shop and stored at 4 °C in a refrigerator. Prior to the determination of bonded glucose, the beer sample (MARKE CLAUSTHALER, CLASSIC, PREMIUM ALKOHOLFREI, BINDING-BRAUEREI AG, FRANKFURT AM MAIN) was deaerated with argon to remove dissolved gases, and appropriate volumes were diluted with 0.1 M phosphate buffer (pH 7.5) in a ratio of 1:50. These solutions were directly injected into the FIA-system.

Reference determinations were made with the glucose analyzer "Glucotrend" (Boehringer, Mannheim, Germany) using commercially available optical glucose biosensors.

3. Results and discussion

3.1. Analytical characteristics of the sensors

The screen-printed sensors were characterized and experimentally optimized for their analytical performance as described elsewhere [5]. They yield calibration curves (i [μA] = 0.55 c [μmol L^{-1}] + 0.03 with c as concentration and i as amperometric response) with a linear range between concentration and amperometric response in the flow-injection mode (with optimum conditions of 0.2 mL min^{-1} flow rate, 0.48 V operation potential, and 0.25 mL injection volume) between 11 and 13900 μmol L^{-1} with a detection limit (3σ)

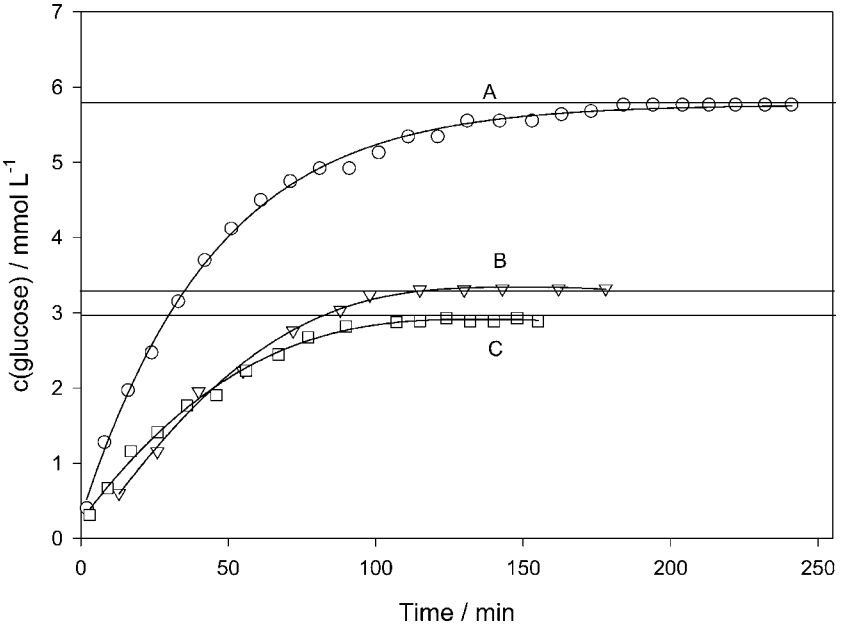

Fig. 2. Time dependence of the release of glucose from cellobiose (A), (-)-(4-nitrophenyl)-β-D-glucopyranoside (B), and saccharose (C). Initial concentrations of each substrate: 1 g L^{-1}; enzyme concentration: 50 mg 10 mL^{-1}; reaction medium: phosphate buffer (0.1 M, pH 7.5). The lines refer to the theoretical values.

of 1 μmol l^{-1}. The relative standard deviation (100 μmol L^{-1} glucose, five injections) is 7%.

3.2. Flow injection analysis

The screen-printed biosensors modified with MnO_2/GOD were investigated on their ability to detect the release of bonded from cellobiose, saccharose and (-)-4-nitrophenyl-β-D-glucopyranoside using glucosidase (from *Aspergillus niger*) in the sample solution (Fig. 2). The results indicate clearly that the solutions, containing still glucosidase, can be injected directly without adversely influencing the expected signal responses. With saccharose and the glucopyranoside full cleavage is achieved after some 100 min when using 50 mg 10 mL^{-1} enzyme and a concentration of 1000 mg L^{-1} substrate. Cellobiose requires almost twice this time, which is evident because not all of the glucose–glucose bonds are immediately accessible for cleavage.

Additionally when transforming to molar relations, it can be seen easily that stoichiometric quantitative lysis can be achieved with glucosidase and all three model substrates.

Fig. 3 shows the influence of the enzyme content in the sample solution on the reaction kinetics. Curves A and B show the time dependence of the release of glucose from saccharose with 100 mg glucosidase 10 mL^{-1} and 50 mg of glucosidase 10 mL^{-1}, respectively, in a solution containing 1000 mg L^{-1} of saccharose.

As can be expected, a higher concentration of the enzyme will enhance the reaction speed of the hydrolysis.

Thus, there are principally two possibilities of determining bonded glucose in samples with the assay sketched above; either a kinetic study is performed over a period of time, or complete hydrolysis of glucoside is prompted, and the total concentration of glucose is measured. The second approach was used for the work here because it gave well-producible results. Kinetic investigations are also applicable but show higher standard deviations of the corresponding results.

As the method is depicted as a differential assay (difference of response signals after and before the action of glucosidase), the initial amount of glucose present in the sample is of minor importance only unless it is not too high to cause saturation effects at the sensor. In this respect, it is also not necessary to eliminate interferences (ascorbic acid, uric acid) because their influence on the signal will be the same before and after the action of the hydrolase. This was proven by treating standard solutions, spiked with ascorbate overnight with ascorbate oxidase and catalase immobilized on glass beads. The results for bonded glucose were identical for the treated and the untreated solutions.

Fig. 4 shows an exemplaric kinetic study on the release of glucose.

3.3. Analysis of a beer sample

The results for the beer sample are shown in Table 1.

As can be seen, there is a very good congruence between the results obtained with the electrochemical biosensor and the optical reference method. Both devices were calibrated with external standards. The optical reference method usually yielded results, which were slightly higher (5–10%) than the results of the electrochemical method, but they are still in the overlap of the experimental uncertainties of both methods (approximately 8% for each). In fact, the share of the bonded glucose, which was then calculated as a difference of the individual results (amount of free glucose, measured directly, subtracted from the total glucose amount, determined

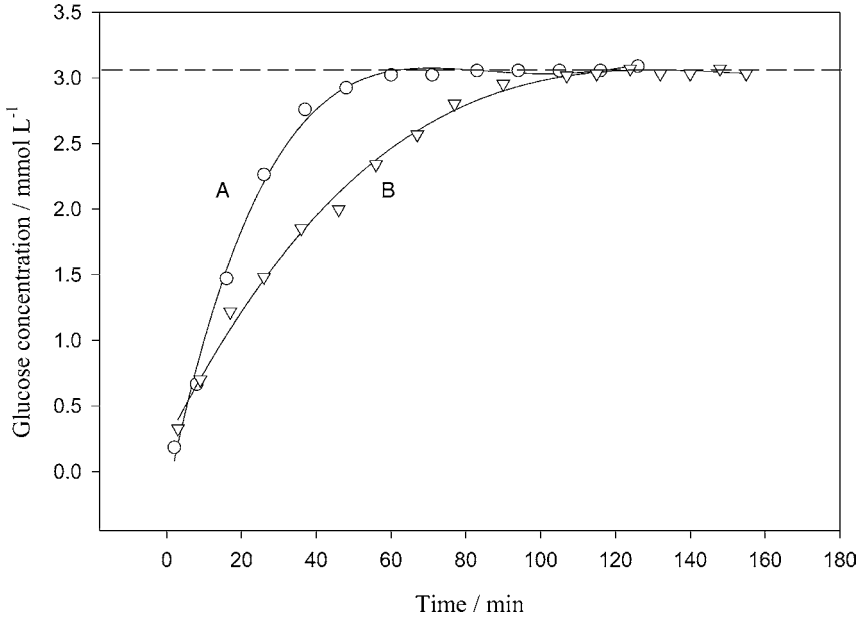

Fig. 3. Release of glucose from saccharose. Saccharose concentration: 1 g L^{-1} in phosphate buffer pH 7.5 (0.1 M); (A) 100 mg enzyme 10 mL^{-1}; (B) 50 mg enzyme 10 mL^{-1}.

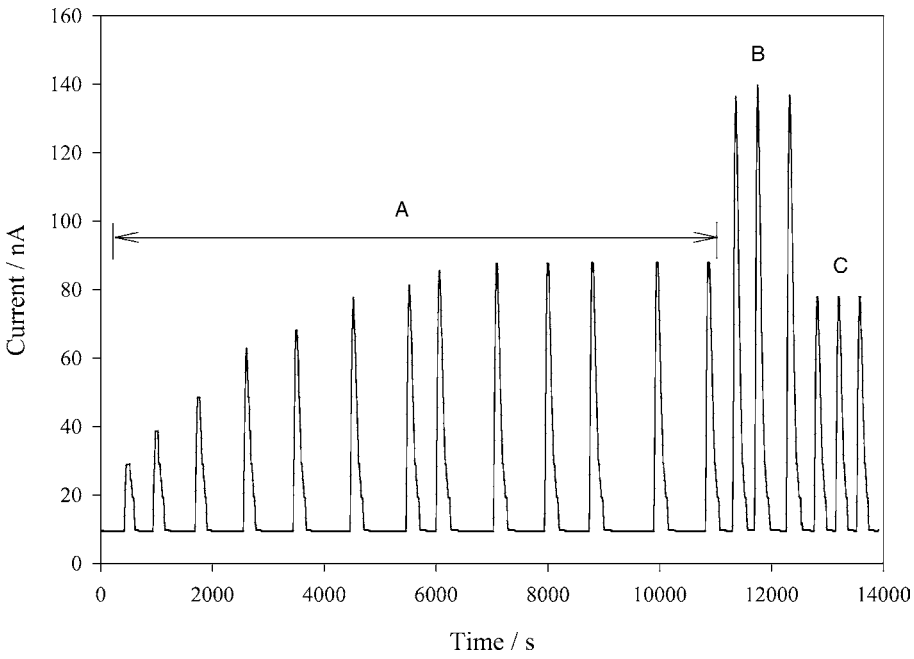

Fig. 4. Release of glucose from (-)-(4-nitrophenyl)-β-D-glucopyranoside by glucosidase. Substrate concentration: 1000 μg mL^{-1}; enzyme concentration: 50 mg 10 mL^{-1}; (A) kinetic study of the release of glucose; (B) 1000 μg mL^{-1} glucose; (C) 500 μg mL^{-1} glucose.

Table 1
Content of free and bonded glucose in a beer sample (given as mass portion in mass%) using a SPE biosensor and a standard optical reference method ("Glucotrend")

Type of glucose (%)	Methods	
	SPE-biosensor	Glucotrend
Total glucose	4.25 ± 0.02	4.55 ± 0.1
Free glucose	1.36 ± 0.02	1.45 ± 0.1
Bonded glucose	2.89 ± 0.02	3.10 ± 0.1
Bonded glucose (in % of total glucose)	68.0 ± 0.02	68.0 ± 0.1

after incubation with glucosidase) corresponded even much better (relative percent difference below 5), as also shown in Table 1. Based on this, it may be concluded that the suggested procedure with the amperometric glucose sensor with manganese dioxide is a proper sensor for the determination of bonded glucose. The enzyme glucosidase does not exert any negative influence on the determination when present in the sample solution.

4. Conclusion

The method presented here is a simple procedure for the determination of bonded glucose. Practical applications allow convenient determination of the bonded substrate in beer. No pre-treatment of the sample was needed except removing dissolved gases and dilution with carrier buffer. Work is in progress to apply this method to detect bonded glucose in form of glucoproteins in other complex matrices such as blood serum, because glucosylated hemoglobin (Hb A1c) is an important surveillence factor for patients suffering from diabetes.

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