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A novel combined thermometric and amperometric biosensor for lactose determination based on immobilised cellobiose dehydrogenase

Maria Yakovleva[a,*], Orsolya Buzas[a], Hirotoshi Matsumura[a,b,1], Masahiro Samejima[b], Kiyohiko Igarashi[b], Per-Olof Larsson[c], Lo Gorton[a], Bengt Danielsson[c,2]

[a] Department of Biochemistry and Structural Biology, Lund University, PO Box 124, 22100 Lund, Sweden
[b] Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[c] Department of Pure and Applied Biochemistry, Lund University, Getingevägen 60, 22100 Lund, Sweden

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ABSTRACT

A novel method for lactose determination in milk is proposed. It is based on oxidation of lactose by cellobiose dehydrogenase (CDH) from the basidiomycete *Phanerochaete chrysosporium*, immobilised in an enzyme reactor. The reactor was prepared by cross-linking CDH onto aminopropyl-silanised controlled pore glass (CPG) beads using glutaraldehyde. The combined biosensor worked in flow injection analysis (FIA) mode and was developed for simultaneous monitoring of the thermometric signal associated with the enzymatic oxidation of lactose using *p*-benzoquinone as electron acceptor and the electrochemically generated current associated with the oxidation of the hydroquinone formed. A highly reproducible linear response for lactose was obtained between 0.05 mM and 30 mM. For a set of more than 500 samples an R.S.D. of less than 10% was achieved. The assay time was ca. 2 min per sample. The sensor was applied for the determination of lactose in dairy milk samples (milk with a fat content of 1.5% or 3% and also "lactose free" milk). No sample preparation except dilution with buffer was needed. The proposed method is rapid, suitable for repeated use and allows the possibility to compare results from two different detection methods, thus providing a built-in quality assurance. Some differences in the response observed between the methods indicate that the dual approach can be useful in mechanistic studies of redox enzymes. In addition, a dual system opens up interesting possibilities for studies of enzyme properties and mechanisms.

1. Introduction

Cow milk is one of the most important bulk consumer products from the dairy industry containing high quality compounds such as protein, lactose, magnesium and calcium, as well as various vitamins and antioxidants. An increasing number of food safety regulations and legislation on authenticity require extensive control of the milk containing products. Each year dairy production suffers from great economical losses due to cow mastitis. It is a serious disease in dairy animals causing reduction in milk yield as well as lowering its nutritive value. One of the criteria used for determining whether milk is acceptable for human consumption is the level of the lactose content (Sharif et al., 2007). Lactose is a disaccharide and its concentration in milk of healthy cattle is approximately 4.1–5.0% (w/v). Milk from cows suffering from mastitis has a lower lactose level, so its concentration is basic for determination of milk quality. Effective control of lactose levels in dairy products is also important for the cheese industry in order to control the fermentation processes. Additionally, many people, especially adults, suffer from lactose intolerance. Therefore, a variety of methods have been developed for determination of lactose including spectrophotometry, polarimetry, infrared spectroscopy, titrimetry by the chromatine-T method and chromatography (Tkac et al., 2000). However, the present instrumental methods suffer from some general drawbacks. These include time-consuming sample preparation steps (extraction and filtration), high cost and need to be operated by well trained personnel.

* Corresponding author. Tel.: +46 76 2228261; fax: +46 46 2224611.
E-mail addresses: Maria.Yakovleva@gmail.com (M. Yakovleva), Orsolya.Buzas@gmail.com (O. Buzas), matsumuh@ebs.ogi.edu (H. Matsumura), amsam@mail.ecc.u-tokyo.ac.jp (M. Samejima), aquarius@mail.ecc.u-tokyo.ac.jp (K. Igarashi), Per-Olof.Larsson@tbiokem.lth.se (P.-O. Larsson), Lo.Gorton@biochemistry.lu.se (L. Gorton), bdacromed@gmail.com (B. Danielsson).
[1] Present address: Division of Environmental & Biomolecular Systems, Oregon Health & Science University, Beaverton, OR 97006, United States.
[2] Present address: Acromed Invest AB, Magistratsvägen 10, 226 43 Lund, Sweden.

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Introduction of biosensors for estimation of lactose in dairy products allowed future development in this analytical field. Biosensors are a subgroup of chemical sensors, in which the analytical device is composed of a biological recognition element (such as enzyme, antibody or receptor) integrated with a physico-chemical transducer (electrochemical, mass, optical or thermal). These devices are promising tools for food analysis due to high selectivity and specificity, relatively low cost, potential for miniaturisation and automation and possibility for high throughput analysis (Mello and Kubota, 2002). Various lactose biosensors based on immobilised enzymes with electrochemical detection have been described in the literature. They belong to three different generations of amperometric biosensors (Adányi et al., 1999; Lourenço et al., 2003; Rajendran and Irudayaraj, 2002; Sharma et al., 2004; Tkac et al., 2000; Liu et al., 1998; Ludwig et al., 2010; Safina et al., 2010; Stoica et al., 2006). Cellobiose dehydrogenase (CDH) based lactose biosensors, belonging to the third generation, showed a much higher stability and sensitivity for lactose compared to other amperometric biosensors. They utilise direct electron communication between the redox enzyme and the electrode exemplified by CDH adsorbed on a solid spectrographic graphite electrode or other carbon based electrodes (Ludwig et al., 2010; Safina et al., 2010; Stoica et al., 2006).

CDH is an extracellular enzyme produced by various filamentous fungi (Ludwig et al., 2010). It consists of two domains, corresponding to one FAD containing dehydrogenase domain, DH_{CDH}, and one heme containing cytochrome domain, CYT_{CDH}, which are linked together by a linker region consisting a hydrophilic polypeptide (Stoica et al., 2005). The DH_{CDH} carries the catalytic activity and oxidises the reducing-ends of sugar substrates efficiently to their corresponding δ-lactones. The two electrons produced in the oxidative reaction from the DH_{CDH} pass one by one to the CYT_{CDH} (Igarashi et al., 2002, 2005). The heme cofactor is located close to the surface of the CYT_{CDH} (Hallberg et al., 2000; Henriksson et al., 2000), which facilitates an efficient electron transfer directly to a polarised electrode (Safina et al., 2010). CDH from basidiomycetes, such as *Phanerochaete chrysosporium*, is highly specific to soluble cellodextrins and lactose but it discriminates monosaccharides, which makes this enzyme especially attractive for biosensor development for lactose measurements as cellodextrins should not be present in dairy products (Safina et al., 2010).

In this work a novel combined thermometric/amperometric biosensor based on immobilised CDH was developed. The sensor is based on the use of an immobilised enzyme reactor placed in a flow system, which incorporates two flow through detectors, one thermistor (Ramanathan and Danielsson, 2001) and one amperometric flow through cell (Appelqvist et al., 1985). The sensor combines two different measurement principles, *viz.* thermometric detection of the heat produced in the enzymatic oxidation of lactose and reduction of BQ and electrochemical reoxidation of the reduced form of benzoquinone (BQ) used as mediator. The proposed biosensor is an improvement over previously reported lactose biosensors as it combines the advantages of two different measurement techniques and diminishes drawbacks (interferences and short operational stability). The combination of widely differing detection modes should improve the overall reliability of the system. In addition, a dual detection system opens up the interesting possibility for studies of enzyme properties and mechanisms. Previous studies on hybrid sensors involved electrically conducting enzyme reactors that permitted simultaneous amperometric and thermometric measurements at the same site (Xie et al., 1993, 1997). In this case investigations on direct electron transfer may also be possible. Such studies using CDH are underway.

2. Material and methods

2.1. Chemicals

Lactose was purchased from BDH Chemicals (Poole, UK), sucrose from ICN Biochemicals (Cleveland, USA), D(+)-glucose and glacial acetic acid from Merck (Darmstadt, Germany), β-D(+)-cellobiose and ethanolamine hydrochloride from Sigma–Aldrich (Stockholm, Sweden), *p*-benzoquinone (BQ) from Janssen Chimica (New Jersey, USA), glutaraldehyde solution 50% from Fluka Chemika (Buchs, Germany), controlled pore glass (CPG, Trisoperl® particle size 125–140 μm and 54.3 nm pore size) from VitraBio (Steinach, Germany). Recombinant cellobiose dehydrogenase (CDH) from the basidiomycete *P. chrysosporium* (7 U mg^{-1}) was heterologously expressed in *Pichia pastoris* and purified as described previously (Yoshida et al., 2001).

For the actual experiments degassed 20 mM acetate buffers with different pHs were used. The pH was adjusted with 1 M sodium hydroxide from Eka Nobel AB (Bohus, Sweden). All solutions were prepared in ultra pure deionised water (18.2 MΩ) purified with an Elga Maxima water purification system (Elga Processed Water Ltd., Marlow, UK). Milk from the Swedish company "Skånemejerier" (Malmö, Sweden) with different fat contents (0.1%, 0.5% and 3%) and "lactose-free" milk were purchased from a local store.

2.2. Biosensor device

The flow-injection device for determination of lactose with immobilised CDH is shown in Fig. 1. Thermometric measurements were performed with an enzyme thermistor (ET). The instrumentation of the ET has been elaborated elsewhere (Ramanathan and Danielsson, 2001). The flow-injection device for determination of lactose is described in details in Supplementary data.

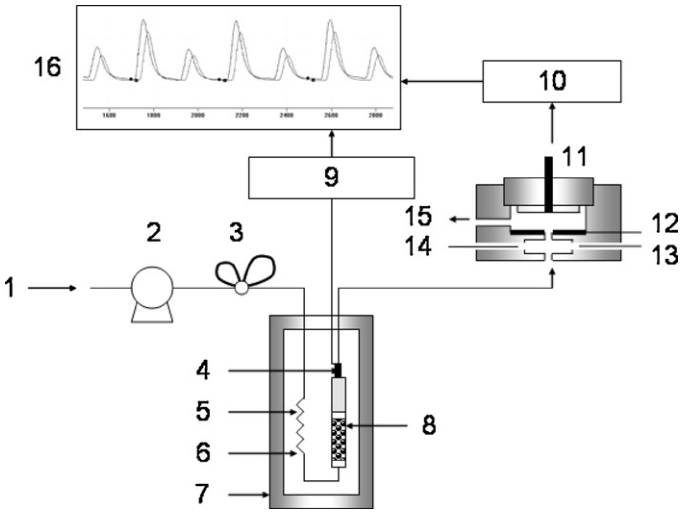

Fig. 1. Schematic diagram of the combined biosensor set up. Working parts: buffer (1), peristaltic pump (2), injection valve with two loops (30 μL and 50 μL) (3), thermistor (4), heat exchanger (5), aluminium block (6), polyurethane insulation (7), enzyme reactor (8), electronic interface (9), potentiostat (10), working electrode (11), counter electrode (12), reference electrode (13), syringe with 0.1 M KCl (14), outlet (15), recorder (16). Explanation in the text (Section 2.2).

2.3. Working principle

The principle behind the thermometric estimation of lactose is based on the following enzymatic reaction, where ΔH is the enthalpy change:

$$\text{lactose} \underset{-2e^- -2H^+}{\overset{\text{CDH}}{\longrightarrow}} \text{lactosobionolactone} + \Delta H \quad (1)$$

The catalytic oxidation of lactose by CDH (reaction (1)) takes place inside of the reactor and is associated with heat production (ΔH) that is registered immediately after the reactor.

The current measurements are based on a reaction cascade. When lactose in oxidised, 2 electrons are transferred from the sugar to the DH_{CDH} domain. In the proposed device CDH is immobilised inside the enzyme reactor with no direct contact with the surface of the working electrode. Therefore, direct electron transfer cannot take place and a mediator such as BQ should be introduced to facilitate the electron transfer between the active site of the enzyme and the working electrode. In that case electrons produced in the oxidation reaction of lactose are transferred from the DH_{CDH} domain of the enzyme to BQ in the following reaction:

$$BQ + 2e^- + 2H^+ \overset{\text{reduced CDH}}{\longrightarrow} H_2BQ \quad (2)$$

The formed reduced mediator (H_2BQ) subsequently reaches the electrochemical cell with the buffer flow and transfers the electrons onto the surface of the graphite electrode at the applied potential (E_{appl}), which is set more positive than the formal potential of the BQ/H_2BQ redox couple ($E^{\circ\prime}_{BQ/H_2BQ}$) according to:

$$H_2BQ \overset{E_{appl} > E^{\circ\prime}_{BQ/H_2BQ}}{\longrightarrow} BQ + 2e^- + 2H^+ \quad (3)$$

2.4. Immobilisation of CDH

CDH from *P. chrysosporium* was immobilised on controlled pore glass (CPG) according to a previously described procedure (Raghavan et al., 1999). Briefly, two different enzyme reactors were prepared by covalent binding of CDH (20 U and 50 U) onto 150 mg of GA-activated aminopropyl-silanised CPG beads (see Supplementary data).

2.5. Lactose determination

For construction of calibration curves stock solutions of lactose and cellobiose were prepared in Milli-Q water at a concentration of 100 mM and stored at 4 °C for a maximum of two weeks. Concentrations from 0.05 to 50 mM were obtained by mixing a calculated amount of stock solution with buffer. Two sample volumes were compared (30 and 50 μL). The effect of the flow rate, pH, mediator concentration, thermostating of the ET unit on thermometric/amperometric responses was investigated. For study of cross-reactivity separate standards of glucose, sucrose, and galactose were prepared in 20 mM acetate buffer at a concentration of 50 mM.

Different sample pretreatment procedures were tested for milk with 0.1, 0.5 and 3% fat content. In the first approach milk samples were centrifuged at 13,000 rpm for 5 min. The supernatant was collected, diluted in assay buffer 150 or 300 times and analysed. The second approach did not include any centrifugation step. Samples were analysed directly after dilution 150 or 300 times. "Lactose-free" milk samples were treated in the same way with and without spiking by addition of aliquots of 0.5 mM lactose.

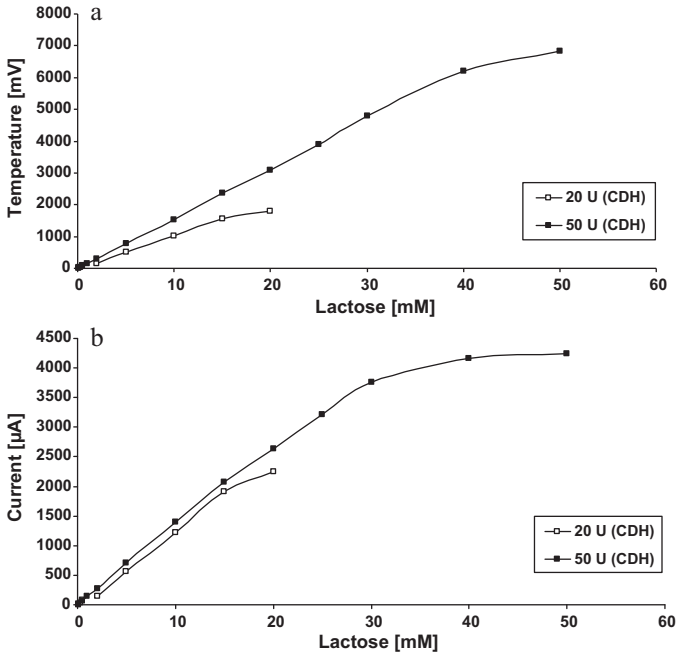

Fig. 2. Influence of immobilisation efficiency on the thermometric (a) and the amperometric (b) responses measured with two enzyme reactors (20 U and 50 U of immobilised CDH, respectively). Acetate buffer (20 mM, pH 5.0) with 2 mM BQ was introduced at flow rate 1.0 mL min^{-1}. Lactose between 0.05 mM and 50 mM was injected with a 50 μL loop. The thermostating temperature was 30 °C.

3. Results and discussion

3.1. The efficiency of immobilisation

Immobilisation efficiency plays a critical role for future practical applications of a biosensor, since it influences important factors, such as operational stability, sensitivity, and linear range. Thus, the immobilisation efficiency was studied by preparation of reactors with different amounts of immobilised enzyme. Enzyme capturing was achieved by covalent coupling of CDH to the solid support (20 and 50 U per 150 mg of CPG). Calibration curves for the two different reactors were constructed and the analytical properties were compared (Fig. 2). It can be observed from Fig. 2 that with 50 U of immobilised CDH a higher concentration of lactose can be oxidised. That enables determination of lactose in a wider concentration range and with a higher sensitivity. Thus, the reactor with 50 U of immobilised CDH was used in all further investigations reported on below.

3.2. Effect of pH on thermometric/amperometric responses

As shown previously, *P. chrysosporium* CDH exhibits maximum catalytic activity in a very narrow acidic pH range similar to several other basidiomycete CDHs (denoted class I CDH in contrast to the recently discovered CDHs of ascomycete origin, denoted class II CDH). In the present work 2 mM lactose was used as substrate for the determination of optimal working conditions and the response at various pHs between 3.5 and 6.0 was studied. It can be observed from Fig. 3 that *P. chrysosporium* CDH has different pH profiles for the thermometric and the amperometric responses. The pH dependence of the reducing activity of CDH observed in homogenous solution using a two-electron acceptor (BQ) shows a maximum at pH 5.0 for amperometric measurements. Then the activity slowly decreases with increasing pH up to 6.0. This pH dependence has been observed and explained previously (Igarashi et al., 2002, 1999). By reconstitution experiments

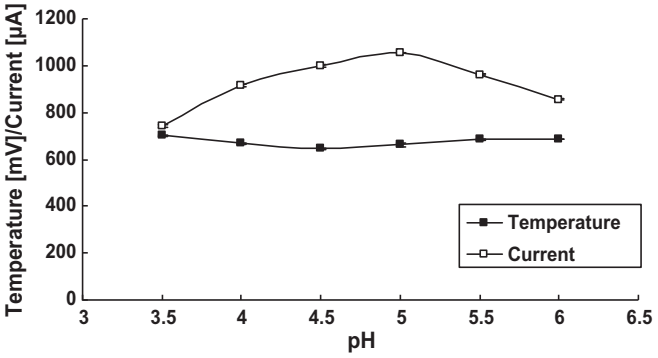

Fig. 3. Influence of pH on the thermometric and the amperometric responses with an enzyme reactor (50 U of immobilised CDH). Acetate buffer (20 mM) with 2 mM BQ was introduced at a flow rate of 1.0 mL min^{-1}. Lactose (2 mM) was injected with a 50 μL loop. The thermostating temperature was 30 °C.

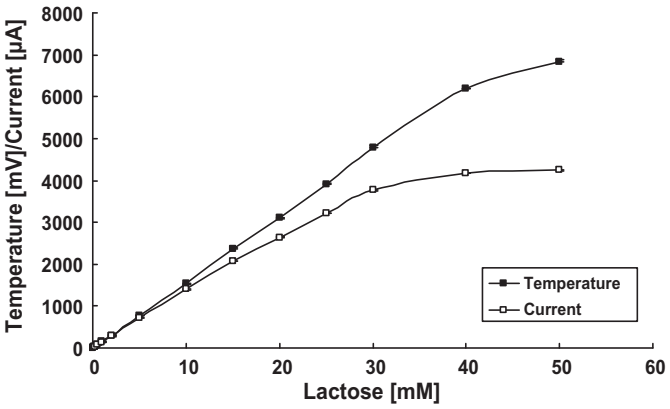

Fig. 4. A plot of the thermometric and the amperometric responses vs. lactose concentration between 0.05 mM and 50 mM measured with an enzyme reactor (50 U of immobilised CDH). The corresponding equations are $y = 136.32x - 0.8496$ ($R_2 = 0.9999$) for the thermometric response and $y = 151.87x + 2.4933$ ($R_2 = 0.9998$) for the amperometric response. Acetate buffer (20 mM, pH 5.0) with 2 mM BQ was introduced at a flow rate of 1.0 mL min^{-1}. The sample loop was 50 μL. The thermostating temperature was 30 °C.

with 6-hydroxy-FAD or FAD it was proven that this pH dependence is not caused by the flavin cofactor but is intrinsic to the protein molecule. However, reversible changes in the peptide backbone were found to have no influence on the ability of CDH to convert lactose into lactosobionolactone judged by the heat production. Thus, thermometric measurements can be performed in a wide range of pH. Taking into account the pH dependence of the current response, pH 5.0 was utilised in all further experiments.

3.3. Effect of flow rate on the thermometric/amperometric responses

The flow rate greatly influences the performance of the thermometric/amperometric system in a rather complex manner (see Supplementary data). In the present work as the flow rate was increased from 0.5 to 1.04 mL min^{-1} the thermometric and amperometric responses were also increased (Fig. S1 in Supplementary data). Higher flow rates resulted in sharper peaks and reduction of the time required for the assay. Taking into account all factors mentioned above a flow rate of 1.0 mL min^{-1} was found to be optimal to get an efficient mass transfer and good operational stability. This resulted in a measurement time of 2 min per sample.

3.4. Effect of sample volume on thermometric/amperometric responses

The effect of the size of the sample loop size on the thermometric/amperometric responses was briefly studied (Fig. S2 in Supplementary data). The linear range was extended when using the 30 μL loop. However, the sensitivity was higher for the 50 μL loop. Since the sensitivity plays a major role in milk analysis, the 50 μL sample loop was used in all further experiments.

3.5. Effect of thermostating on the thermometric responses

The construction of the ET unit makes it possible to thermostat the column at 25, 30, and 37 °C, because the catalytic activity of the enzyme depends not only on pH but also on reaction temperature. The height of the temperature response peak can vary with different thermostating temperatures. Thermostating the reactor at higher temperatures (30 and 37 °C) exhibited higher sensitivities compared to thermostating at 25 °C. The same thermometric signal was achieved when thermostating at 30 or 37 °C. However, thermostating at 30 °C yielded a higher reproducibility in comparison with 37 °C (Fig. S3 in Supplementary data), since the entire solution introduced into the system at room temperature has enough time to be homogeneously heated before entering the reactor. Thus, thermostating at 30 °C was used for all further investigations.

3.6. Analytical properties of the combined biosensor

For optimisation of the amperometric response two concentrations of BQ (2 and 5 mM) were compared. A higher concentration of the mediator should improve the signal and widen the linear range. However, according to the results application of a higher mediator concentration causes an increase in the background noise and thus lowers the sensitivity (data not shown). Thus, 2 mM BQ was utilised in all experiments. The following optimised working conditions were used: amount of enzyme applied for immobilisation: 50 U, thermostat setting: 30 °C, flow rate: 1.0 mL min^{-1}, pH 5.0, sample volume: 50 μL, concentration of mediator in the buffer: 2 mM.

The response of the dual biosensor to lactose was investigated in the concentration range from 0.05 mM to 50 mM. A typical calibration profile of peak height versus lactose concentration for both thermometric and amperometric responses is shown in Fig. 4. The thermometric and current responses were reproducible and showed an intra-assay precision (R.S.D.%) of less than 4 and 3%, respectively. A linear correlation between lactose concentration and both thermometric and amperometric responses was achieved between 0.05 and 30 mM with a corresponding detection limit of 0.05 mM lactose for a 50 μL sample volume. The correlation coefficients were 0.9998 and 0.9999 at linear regression analysis of the thermometric and amperometric responses, respectively. With a sample volume of 30 μL the linear range was wider, the calibration curve was linear from 0.1 to 50 mM for the thermometric response and from 0.1 to 40 mM for the amperometric response but the LOD was higher – 0.1 mM. The results demonstrated that signals obtained for the thermometric and amperometric measurements are both valid and can be used as reference for each other.

The R.S.D. was less than 4% for the thermometric response and less than 3% for the amperometric responses. The inter-assay precision (within three days) was less than 10% for both the thermometric and the amperometric responses. As can be observed from the results of both the thermometric and the amperometric measurements they are characterised by a high accuracy and reproducibility.

Table 1
Results obtained for lactose analysis in milk samples using the combined thermometric/amperometric biosensor.

Product	Pretreatment	Dilution	Fat content, %	Claimed amount of lactose, g L^{-1}	Found amount of lactose, g L^{-1}	Found amount of lactose, g L^{-1}
					Thermometric response	Amperometric response
Milk[a]	Centrifugation for 5 min at 13,000 rpm	1:150	0.1	50.0	53.36 ± 0.39	50.19 ± 0.22
Milk[a]	Centrifugation for 5 min at 13,000 rpm	1:300	0.1	50.0	51.51 ± 1.41	49.65 ± 1.30
Milk[a]	–	1:150	0.1	50.0	49.87 ± 0.00	47.43 ± 0.38
Milk[a]	–	1:300	0.1	50.0	57.14 ± 0.00	54.43 ± 1.15
Milk[a]	Centrifugation for 5 min at 13,000 rpm	1:150	3	50.0	52.57 ± 0.34	47.64 ± 0.43
Milk[a]	Centrifugation for 5 min at 13,000 rpm	1:300	3	50.0	52.86 ± 0.78	56.36 ± 0.87
Milk[a]	–	1:150	3	50.0	50.32 ± 0.39	44.88 ± 0.38
Milk[a]	–	1:300	3	50.0	51.51 ± 1.03	46.57 ± 0.87

[a] Produced by Skånemejerier, Malmö, Sweden.

Table 2
Recovery studies of lactose in lactose free milk samples spiked with 0.5 mM lactose.

Analyte	Pretreatment	Dilution	Fat content, %	Added, mM	Found, mM	
					Thermometric response	Amperometric response
Lactose free milk[a]	Centrifugation for 5 min at 13,000 rpm	1:300	0.5	0.50	0.51 ± 0.02	0.46 ± 0.00
Lactose free milk[a]	Centrifugation for 5 min at 13,000 rpm	1:150	0.5	0.50	0.53 ± 0.01	0.47 ± 0.00
Lactose free milk[a]	–	1:300	0.5	0.50	0.48 ± 0.02	0.46 ± 0.00
Lactose free milk[a]	–	1:150	0.5	0.50	0.51 ± 0.01	0.48 ± 0.00

[a] Produced by Skånemejerier, Malmö, Sweden.

3.7. Interferences

The proposed biosensor showed no response to high concentration (50 mM) of glucose, galactose and sucrose (see Supplementary data).

3.8. Application of the combined biosensor for milk analysis

The proposed combined biosensor was applied for the determination of lactose in milk with different fat contents (0.1% and 3%). Different approaches for sample preparation were tested in order to avoid any matrix effects. No difference in results was observed for samples prepared in various ways (Table 1). It was therefore concluded that no special pretreatment step is required and samples can be analysed directly after being diluted 150 or 300 times. An excellent agreement between estimated and claimed values of lactose in milk samples was achieved for both the thermometric and the amperometric responses. To verify the reliability of the technique, samples of lactose free milk with a fat content of 0.5% were also analysed by the "add-found" method (Table 2). Samples were spiked with 0.5 mM lactose after passing the pretreatment procedure described above. The found amount of lactose was in good correlation with the added amount, however, the amperometric response was slightly lower.

3.9. Operational and storage stability

Commonly used amperometric biosensors have disadvantages such as enzyme leakage and denaturation of the enzyme on the electrode surface, which affect the life time of enzyme modified electrodes (Salman et al., 2008). Our previous studies showed that the ET is a robust system and can work without loss of enzyme activity for prolonged periods of time (up to several months) (Ramanathan et al., 2001). In the present work amperometric measurements were combined with thermometric measurements in order to avoid storage stability problems, which commonly occur when the enzyme is immobilised directly onto the electrode surface. CDH was covalently bound to CPG in the enzyme reactor and the graphite electrode surface was free from enzyme. Therefore, a proper storage and operational stability was achieved. The enzyme reactor was used daily for two months at 30 °C without showing any change in response during that period. Moreover, stability studies are still under investigation in our laboratory and the expected life-time of the reactor could be up to 1–2 years as was found in previous investigations of various other enzyme reactors (Ramanathan et al., 1999). The operational stability was tested by injection of 2 mM lactose into the system for 8 h (more than 80 injections, Fig. S4 in Supplementary data). No decrease in either of the two response signals was observed. The combined biosensor gave reproducible responses indicating proper operational stability.

4. Conclusions

The results described in this paper demonstrate the applicability of a combined thermometric/amperometric biosensor based on immobilised CDH for the determination of lactose in milk. The most important operational parameters of the proposed combined biosensor were optimised. As a result a detection limit of 0.05 mM for lactose was achieved. The linear part of the calibration graph covers the range required for measurement of lactose in dairy products after proper dilution, i.e., between 0.05 mM and 30 mM. The system allows simple and rapid analysis of diluted samples, i.e., ca. 2 min per sample. No interferences on the analytical responses were observed indicating that the proposed biosensor is highly selective. The system was characterised by high repeatability, reproducibility and storage stability (at least two months at room temperature). The thermometric and amperometric results are in good correlation with each other and one can be applied as a reference for the other one. If one or both of the sensors would be adversely influenced by sample components, their response towards lactose would probably no longer agree, giving the operator a valuable warning. This built-in performance check obviously increases the reliability of the system. Based on the results it can be

concluded that the proposed combined biosensor is highly reliable for lactose determination in milk and a clearly competitive alternative to other available methods. The dual biosensor concept will also be useful in mechanistic studies of enzymes. Studies are underway using a hybrid biosensor that permits simultaneous amperometric and thermometric measurements on a electrically conducting enzyme reactor (Xie et al., 1993, 1997).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.10.027.

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