Proceedings of the Estonian Academy of Sciences,
2011, **60**, 2, 136–140
doi: 10.3176/proc.2011.2.08
Available online at www.eap.ee/proceedings

CHEMISTRY

Calculating the output signal parameters of a lactose bienzymatic biosensing system from the transient phase response

Artur Gornischeff and Toonika Rinken*

Institute of Chemistry, University of Tartu, Ravila 14a, 50411 Tartu, Estonia

Received 5 May 2010, revised 14 September 2010, accepted 12 January 2011

Abstract. We constructed for the determination of lactose a bienzymatic biosensing system based on a fibre-optical oxygen sensor and two enzymes – β-galactosidase (β-gal, from *Aspergillus oryzae*, Sigma Aldrich, EC 3.2.1.23) and glucose oxidase (GOD, from *A. niger*, Sigma Aldrich, EC 1.1.3.4) and analysed how the calculation of biosensor output signal parameters, used for the calibration of lactose biosensors, is influenced by the data collection period during the transient phase of the signal rising in case no preliminary incubation period with β-gal was applied. The calculation of reaction steady state and kinetic parameters from the biosensor signal revealed that longer data collection periods resulted in more accurate biosensor calibration curves with bigger slopes, while in case of slower reactions the calculated reaction parameters had their maximal values already if data were collected for 600 seconds. For reactions where enzyme concentrations were higher (0.027–0.071 IU/mL β-gal and 2.03–5.33 IU/mL GOD), the steady state signal was not achieved even within 1 hour from the initiation of the reaction and the calculated reaction parameters continued to change. Although the sensor signal was decreasing continuously, the reaction parameters calculated from the transient phase data were suitable for biosensor calibration if the data of at least 500 seconds were taken into consideration.

Key words: lactose biosensor, transient phase output, reaction parameters, calibration.

INTRODUCTION

Lactose or milk sugar is the major carbohydrate in milk with its concentration ranging around 5 g/dL in cow milk. As about 75% of adults experience lactose intolerance, the measurement of lactose in milk and milk products has been thoroughly studied. For the estimation of lactose content in milk, numerous analytical methods, such as spectrophotometric, titrimetric, gravimetric, and chromatographic, are used [1]. Although these methods give reliable results, they are time consuming and require sample pre-treatment. A good alternative to traditional analytical methods for a rapid determination of lactose is the application of biosensors. The time required for lactose determination with biosensors depends on the bio-recognition element as well as the construction and properties of the applied signal transducers and it can vary from a few minutes to several hours. Signal transducers used in biosensors are mainly electrochemical or fibre-optical, the latter are also used for the construction of biosensors of acetylcholine [2], penicillin [3], or oligonucleotides [4].

Most lactose biosensors are based on two [5,6] or more [7–9] enzymes, forming a shorter or longer cascade of enzyme-controlled reactions enabling to turn lactose into some detectable products. The linear range of lactose detection of this kind of biosensors is typically up to 15 mM (0.5%) [10,11]. Biosensors in which Langmuir–Blodgett films are used for enzyme immobilization show linearity from 30 to 175 mM (1–6 g/dL of lactose) [12]. As several reactions are going on simultaneously in the system, the measurable output signal of a biosensor depends on the kinetics of more than one reaction. When milk is analysed the response of lactose biosensors based on lactose hydrolysis and consequent product oxidation suffers also from the interference of glucose and galactose, present in milk at concentrations of up to 0.1 mM [9,13]. Lactose hydrolysis by β-galactosidase (β-gal, EC 3.2.1.23) is very slow at room temperature; therefore temperatures as high as 45 °C are used to accelerate this process in biosensing systems [7]. At lower temperatures the response time of lactose biosensors can subsequently be far over 15 min [14]. In some cases, an

* Corresponding author, toonika.rinken@ut.ee

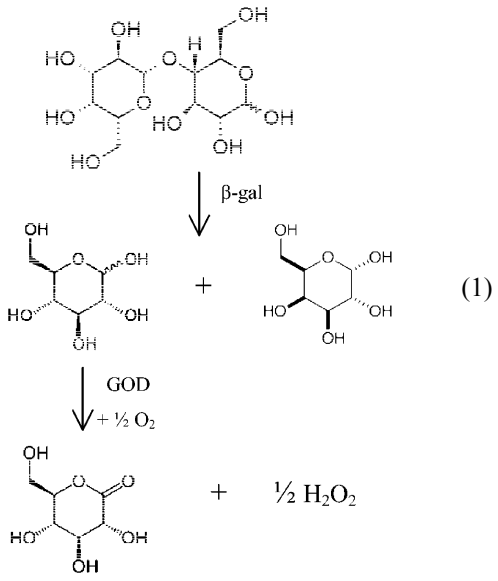
additional preliminary incubation of lactose containing probes with β-gal is carried out [15]. Detection of lactose with biosensors sometimes requires pre-treatment of milk samples to remove fat and proteins [16]. The response of a lactose biosensor may be additionally affected by calcium chloride, ascorbic and/or uric acids [17].

The aim of the present work is to study the pre-steady state signal of a fibre-optical lactose biosensor in which lactose hydrolysis and the consequent oxidation of the hydrolysis products are going on in parallel with lactose hydrolysis being the limiting step of the system, and to find optimal conditions for data acquisition and calculation of parameters that can be applied for biosensor calibration based on the transient phase response of the system.

EXPERIMENTAL

The lactose biosensing system was based on a fibre-optical oxygen sensor, constructed in the Institute of Physics of the University of Tartu, and two soluble enzymes: β-gal and glucose oxidase (GOD, EC 1.1.3.4). The oxygen sensor comprised optical fibre covered with an oxygen-sensitive Pt porphyrin-doped membrane, a source of excitation (λ = 405 nm), and a detector of fluorescent light (λ = 700 nm). The luminescence parameters of the oxygen-sensitive membrane depend on the concentration of dissolved oxygen according to the Stern–Volmer relationship [18].

The enzyme β-gal catalyses the hydrolysis of lactose into the monosaccharides glucose and galactose. The forming glucose is oxidized by dissolved oxygen into glucono δ-lactone and H_2O_2. This reaction is catalysed by GOD, whose specificity is 1000 times higher towards glucose than to galactose and lactose:



(1)

Because of the oxidation of glucose the concentration of dissolved oxygen decreases in the reaction medium. The decrease is proportional to the concentration of glucose. The degradation of the forming H_2O_2 is slow in comparison with the speed of glucose oxidation and does not influence the output of the oxygen sensor [19].

The kinetics of reactions (1) was followed with the oxygen sensor in air-saturated 0.14 M (5 g/dL) lactose solutions in a 0.1 M acetate buffer (pH = 5.60) at constant stirring at 25 °C. The reaction process was started with the injection of enzymes into the air-tight reaction cell (volume 28 mL).

The output of the oxygen sensor was registered automatically at 1 s intervals. Oxygen concentrations were calculated with the original software OxySens1.8.

We calculated from every output curve the characteristic process parameters, and used these for the characterization of the biosensor output. The calculations were carried out according to the biosensor model taking into account enzyme kinetics, diffusion of substrates to the sensor, and system inertia. This enabled calculation of steady state parameters from the sensor transient phase data according to the following equation [20,21]:

$$\frac{c_{O_2}(t)}{c_{O_2}(0)} = A\exp(-Bt) + (1-A) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_s}{n^2/B - \tau_s}\left[\exp(-Bt) - \exp\left(-n^2\frac{t}{\tau_s}\right)\right],$$

where $c_{O_2}(t)$ is the oxygen concentration at time moment t, $c_{O_2}(0)$ is the oxygen concentration at the start of the reaction, t is time, A is the total signal change parameter and B is the kinetic parameter, τ_s is the lag period that includes the inertia of the oxygen sensor and the lag period of the enzyme-catalysed reactions, and n is the number of terms.

All reagents used in the study were of analytical grade.

RESULTS AND DISCUSSION

Two processes were running simultaneously in the system: the hydrolysis of lactose and the consequent oxidation of glucose formed in the course of hydrolysis. The hydrolysis of lactose catalysed by β-gal from *Aspergillus oryzae* is a relatively slow process with k_{cat} (catalytic constant) value of 63 s^{-1} [22], which can be described with the Michaelis–Menten equation with competitive product inhibition by galactose [23]. The value of k_{cat} for GOD-catalysed β-D-glucose oxidation is around 300 s^{-1} [22]. With a sufficient amount of GOD present in the system in comparison with β-gal, the oxidation of glucose can be considered to be proceeding

in line with its formation and the decrease of oxygen in the system is the indicator of the hydrolysis of lactose. In our system, the ratio of GOD and β-gal activities was 75 : 1 (counted in IUs), so the oxidation of glucose was approximately 350 times faster than the lactose hydrolysis, and the latter was the limiting step of the system.

The decrease in the concentration of dissolved oxygen in the system at different concentrations of the enzymes (the ratio GOD/β-gal was kept constant) and lactose concentration of 0.14 M (similar to that in raw milk) in time is shown in Fig. 1. The concentration of dissolved oxygen decreased in nonlinear mode and no stationary state was achieved within 1 h. At higher enzyme concentrations (0.027–0.071 IU/mL, as β-gal) the oxygen available in the system was totally consumed in the oxidation process of glucose and the sensor output signal reached its limiting value.

As the measurable oxygen decrease was nonlinear, it was necessary to select an appropriate model to characterize these curves. We used an integrated biosensor model allowing the calculation of the characteristic parameters of the curves where the steady state is not achieved and the accuracy from the transient phase data is high [19]. Each output curve was characterized with two independent parameters: the steady state and the kinetic parameter. The accuracy of these calculated parameters depended on the depth of the limiting reaction during which the data were collected.

In the initial phase, after the injection of enzymes into the reaction medium, there was a lag period during which the signal decreased slowly in nonlinear mode and the reactions going on in the system at different speeds due to different concentrations of enzymes were practically indistinguishable (Fig. 1). We calculated the length of the initial lag period for each curve using the biosensor model [21] and found that it depended on total enzyme concentrations. At lower GOD/β-gal concentrations (0.009–0.022 IU/mL, as β-gal) the lag period was approximately 150 s; at higher concentrations (0.027–0.071 IU/mL, as β-gal), up to 400 s. During this lag time, the model deviation from experimental curves was considerable, and the calculated process parameters did not enable the calibration of the system. Accordingly, simple and widely used system calibration options measuring the sensor output at a certain fixed time moment will also lead to systematic mistakes because the factors determining the course of the output signal curve depend on the speed of the measured processes.

For the characterization of the lactose bio-sensing system we calculated the signal parameters from different selections of the sensor transient phase data, always including the start of the reaction (the injection of enzymes) and following the reaction to different depths. The calculated values of the total signal change parameter for the studied reactions were decreasing almost linearly along with the prolongation of the data collection periods used for calculations (Fig. 2a) and did not reach any stable value within 3600 s. At the same time, the values of kinetic parameters increased up to 4 times at higher enzyme concentrations (Fig. 2b). The effect of the length of the data collection period on the

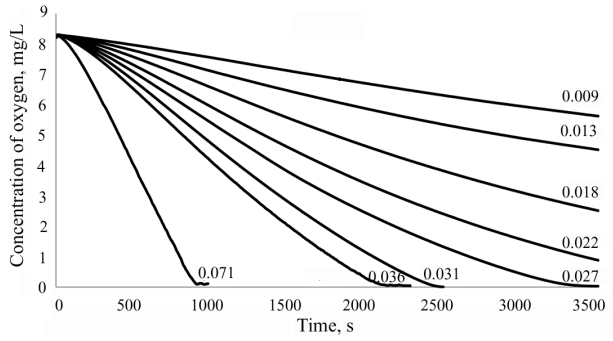


Fig. 1. Decrease in the dissolved oxygen concentration in time in the lactose biosensor at different enzyme concentrations in case the ratio of glucose oxidase and β-galactosidase (concentration shown in the graph) activities was kept constant at 75 : 1. The measurements were carried out in 0.14 M lactose solutions in an air-saturated 0.1 M acetate buffer (pH 5.60) at 25 °C.

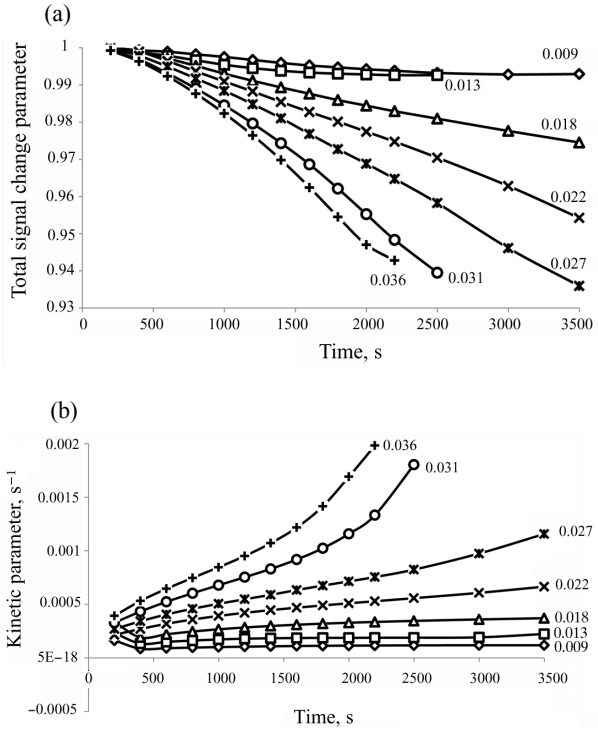


Fig. 2. Values of the total signal change (a) and kinetic (b) parameters for data sets of different length at different β-galactosidase activities in case the ratio of glucose oxidase and β-galactosidase activities was kept constant at 75 : 1. The measurements were carried out in 0.14 M lactose solutions in an air-saturated 0.1 M acetate buffer (pH 5.60) at 25 °C.

kinetic parameter was greater in comparison with the effect on the total signal change and its impact was dependent on the speed of the measured reaction.

From the curves shown in Fig. 2 we constructed biosensor calibration curves (the value of the calculated parameters vs enzyme concentration) for different data collection periods (0–400 s, 0–500 s, 0–600 s, etc.). The slopes of these calibration curves indicated the sensitivity of the system to definite data collection periods (Fig. 3). The biosensor sensitivity rose exponentially along with the increase of the length of the data collection period when we used the total signal change to characterize the system (Fig. 3a). The parameters calculated from the data of less than 500 s from the start of the reaction were not applicable for system calibration as the slope of the calibration curve was very small (below 0.002 $conc^{-1}$). When data of 800 s were used the slope of the calibration curve was over two times bigger than with data of 500 s, and the sensitivity of the system was sufficient to differentiate between processes going on at various speeds. After 500 s we could characterize the function as a linear regression with a slope of $(1.364 \pm 0.033) \times 10^{-5}$ s^{-1} (squared correlation coefficient $R^2 = 0.994$). For longer data collection periods the sensitivity was higher, but as the measuring time was very long, these are not suitable for practical applications. In case very long measuring periods are used, it should be remembered that the amount of dissolved oxygen in the reaction medium is an ultimate quantity and the system can run out of oxygen at higher enzyme concentrations (reactions at higher speed).

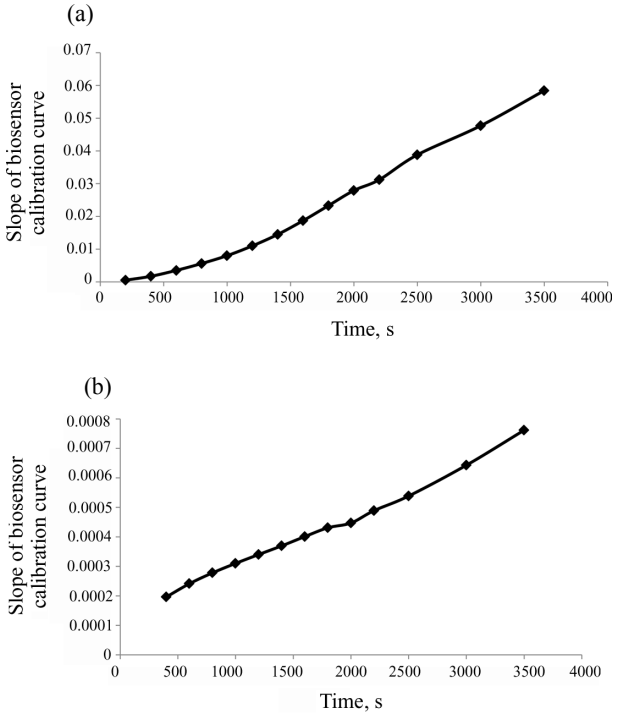


Fig. 3. The dependence of the slope of the lactose biosensor calibration curve or the biosensor sensitivity on the total signal change (a) and kinetic (b) parameters for data sets of different length. The ratio of glucose oxidase and β-galactosidase activities was kept constant at 75 : 1 with β-galactosidase activity ranging from 0.009 to 0.071 IU/mL. The measurements were carried out in 0.14 M lactose solutions in an air-saturated 0.1 M acetate buffer (pH 5.60) at 25 °C.

The sensitivity of the system calculated on the basis of the kinetic parameter rose linearly along with the increase of the data collection period (Fig. 3b), with the slope of $(1.716 \pm 0.044) \times 10^{-7}$ s^{-1} ($R^2 = 0.993$). The sensitivity of the sensor increased 3.5 times in case we used the data collection period of 3500 s instead of 500 s. The kinetic parameter was about 10 times less sensitive to the length of the data collection period than the signal total change parameter.

CONCLUSIONS

The calculation of reaction steady state and kinetic parameters from the biosensor signal revealed that it was possible to characterize lactose hydrolysis using biosensor transient phase output, but the sensitivity of the system was dependent on the length of the data collection period. Longer periods resulted in higher biosensor sensitivity, both by the signal total change and the kinetic parameter. For reactions where enzyme concentrations were higher (0.027–0.071 IU/mL, as β-gal), the steady state was not achieved even after 1 h from the start of the reaction and the values of the reaction parameters could not be fixed. The minimum data collection period enabling the calculation of reaction parameters applicable for biosensor calibration was 500 s.

ACKNOWLEDGEMENT

This research was supported by the Estonian Science Foundation (grant No. 7465).

REFERENCES

1. Newburg, D. S. and Neubauer, S. H. Carbohydrates in milks: analysis, quantities, and significance. In *Handbook of Milk Composition* (Robert, G. J., ed.). Academic Press, San Diego, 1995, 273–349.
2. Bronk, K. S., Michael, K. L., Pantano, P., and Walt, D. R. Combined imaging and chemical sensing using a single optical imaging fiber. *Anal. Chem.*, 1995, **67**, 2750–2757.
3. Healey, B. G. and Walt, D. R. Improved fiber-optic chemical sensor for penicillin. *Anal. Chem.*, 1995, **67**, 4471–4476.

4. Ferguson, J. A., Steemers, F. J., and Walt, D. R. High-density fiber-optic DNA random microsphere array. *Anal. Chem.*, 2000, **72**, 5618–5624.
5. Pfeiffer, D., Ralis, E. V., Makower, A., and Scheller, F. W. Amperometric Bi-enzyme based biosensor for the detection of lactose – characterization and application. *J. Chem. Technol. Biot.*, 1990, **49**, 255–265.
6. Ferreira, L. S., Trierweiler, J. O., De Souza, M. B., Jr., and Folly, R. O. M. A lactose FIA-biosensor system for monitoring and process control. *Braz. J. Chem. Eng.*, 2004, **21**, 307–315.
7. Adanyi, N., Szabo, E. E., and Varadi, M. Multi-enzyme biosensors with amperometric detection for determination of lactose in milk and dairy products. *Eur. Food Res. Technol.*, 1999, **209**, 220–226.
8. Eshkenazi, I., Maltz, E., Zion, B., and Rishpon, J. A three-cascaded-enzymes biosensor to determine lactose concentration in raw milk. *J. Dairy Sci.*, 2000, **83**, 1939–1945.
9. Liu, H., Li, H., Ying, T., Sun, K., Qin, Y., and Qi, D. Amperometric biosensor sensitive to glucose and lactose based on co-immobilization of ferrocene, glucose oxidase, β-galactosidase and mutarotase in β-cyclodextrin polymer. *Anal. Chim. Acta*, 1998, **358**, 137–144.
10. Ferreira, L. S., Souza, J., Trierweiler, J. O., Hitzmann, B., and Folly, R. O. M. Analysis of experimental biosensor/FIA lactose measurements. *Braz. J. Chem. Eng.*, 2003, **20**, 7–13.
11. Logoglu, E., Sungur, S., and Yildiz, Y. Development of lactose biosensor based on beta-galactosidase and glucose oxidase immobilized into gelatin. *J. Macromol. Sci. Pure*, 2006, **43**, 525–533.
12. Sharma, S. K., Singhal, R., Malhotra, B. D., Sehgal, N., and Kumar, A. Lactose biosensor based on Langmuir–Blodgett films of poly(3-hexyl thiophene). *Biosens. Bioelectron.*, 2004, **20**, 651–657.
13. Amarita, F., Fernandez, C. R., and Alkorta, F. Hybrid biosensors to estimate lactose in milk. *Anal. Chim. Acta*, 1997, **349**, 153–158.
14. Volesky, B. and Emond, C. Continuous-flow monitoring of lactose concentration. *Biotechnol. Bioeng.*, 2004, **21**, 1251–1276.
15. Husain, Q. Galactosidases and their potential applications: a review. *Crit. Rev. Biotechnol.*, 2010, **30**, 41–62.
16. Safina, G., Ludwig, R., and Gorton, L. A simple and sensitive method for lactose detection based on direct electron transfer between immobilised cellobiose dehydrogenase and screen-printed carbon electrodes. *Electrochim. Acta*, 2010, **55**, 7690–7695.
17. Brain, J., Collier, W., and Hart, A. The occurrence and nature of electrochemical activity in milk from a herd of dairy cows. *J. Dairy Res.*, 2006, **73**, 115–120.
18. Stern, O. and Volmer, M. Über die Abklingungszeit der Fluoreszenz. *Physikalische Zeitschrift*, 1919, **20**, 183–188.
19. Rinken, T., Rinken, A., Tenno, T., and Järv, J. Calibration of glucose biosensors by using pre-steady state kinetic data. *Biosens. Bioelectron.*, 1998, **13**, 801–807.
20. Rinken, T. Determination of kinetic constants and enzyme activity from a biosensor transient signal. *Anal. Lett.*, 2003, **36**, 1535–1545.
21. Rinken, T. and Tenno, T. Dynamic model of amperometric biosensors. Characterisation of glucose biosensor output. *Biosens. Bioelectron.*, 2001, **16**, 53–59.
22. Brenda. The Comprehensive Enzyme Information System. http://www.brenda-enzymes.info (Release 2011.1, January 2011).
23. Yang, S. T. and Tang, I. C. Lactose hydrolysis and oligosaccharide formation catalyzed by β-galactosidase kinetics and mathematical modelling. *Ann. NY Acad. Sci.*, 2006, **542**, 417–422.

Laktoosi biosensori väljundsignaali parameetrite arvutamine tasakaalueelsetest andmetest

Artur Gornischeff ja Toonika Rinken

Fiiberoptilise laktoosi biosensori väljundsignaali parameetrite arvutamise täpsuse sõltuvust mõõtmise ajast uuriti laktoosi biosensorsüsteemis, kus toimus üheaegselt kaks paralleelset protsessi: β-galaktosidaasi poolt katalüüsitud laktoosi hüdrolüüs ja glükoosi oksüdaasi poolt katalüüsitud tekkinud glükoosi oksüdeerimine lahustunud hapniku toimel. Tulenevalt ensüümide aktiivsuste suhtest lahuses on biosensori signaali limiteerivaks staadiumiks laktoosi hüdrolüüs. Kuna laktoosi hüdrolüüs on 25 °C juures suhteliselt aeglane, ei jõua biosensori signaal statsionaarsesse olekusse isegi ühe tunni jooksul. Biosensori väljundsignaali iseloomustamiseks kasutati reaktsiooni maksimaalse kogumuutuse ja kineetilist parameetrit, mis arvutati sensori tasakaalueelsest väljundsignaalist. Leiti, et biosensori piisava tundlikkuse saavutamiseks on minimaalne andmete kogumise periood 500 sekundit. Mida pikem on andmete kogumise aeg, seda suurem on sensorsüsteemi tundlikkus, kusjuures signaali kogumuutuse parameeter on andmete kogumise perioodi pikkuse suhtes kümme korda tundlikum kui kineetiline parameeter.