A Novel Two-Enzyme Amperometric Electrode for Lactose Determination

Handan GÜLCE,*† Ahmet GÜLCE,* and Attila YILDIZ**

**Department of Chemistry, Suleyman Demirel University, Isparta, 32260, Turkey*
***Department of Chemistry, Hacettepe University, Beytepe, Ankara, 06532, Turkey*

Coimmobilization of β-galactosidase and glucose oxidase in a redox polymer, polyvinylferrocenium perchlorate ($PVF^+ClO_4^-$), led to the development of an enzyme electrode for the determination of lactose. The amperometric response of the electrode was measured at +0.70 V *vs.* SCE, which was due to the electrooxidation of enzymatically produced H_2O_2. The effects of the substrate and buffer concentrations as well as the pH on the electrode response were elucidated.

(Received May 25, 2001; Accepted October 22, 2001)

Introduction

A quantitative determination of lactose, an essential carbohydrate in milk and milk products, is important in the food industry. Low levels of milk are measured for cows suffering from mastitis. The amount of lactose is an indicator of lactosemia in clinical biochemistry. An excessive amount of lactose in human blood is caused by gastro-intestinal malignancy. Although various methods, such as polarimetric, chromatographic, spectrophotometric and titrimetric, are available for the analysis of lactose, these are usually tedious, time consuming and expensive. Enzyme immobilization techniques used in conjuction with electrochemical sensors provide much-faster, less-expensive reliable analysis methods.[1,2]

The use of a redox polymer, polyvinylferrocenium perchlorate ($PVF^+ClO_4^-$), as an immobilization matrix allowed the development of novel amperometric sensors for glucose,[3] galactose[4] and sucrose.[5] A single enzyme glucose oxidase or galactose oxidase was immobilized in the first two enzyme sensors. The determination of sucrose was accomplished using two coimmobilized enzymes, glucose oxidase and invertase. It is known that the reduced form of this redox polymer film, PVF, is a homogeneous compact film, whereas the oxidized form, PVF^+, is an inhomogeneous film.[6] Pores or pinholes exist in PVF^+ film, through which dissolved reactants could diffuse to the underlying metal surface.[7]

For the analysis of lactose immobilization of either a single enzyme, galactose oxidase, or coimmobilization of several suitable combinations of the following enzymes, β-galactosidase or lactase, mutarotase, glucose oxidase and galactose oxidase is required. A single-enzyme reaction produced H_2O_2 directly, whereas a multienzyme process produces the same electroactive product sequentially.[1,7-12] When the analysis is carried out in a medium which contains phosphate ions, the immobilization of invertase is no longer necessary, since the conversion of α-glucose to a glucose oxidase-specific substrate, β-glucose, is accomplished by phosphate ions.

$$\text{Lactose} + O_2 \xrightarrow{\text{galactose oxidase}} H_2O_2 + \text{oxidation product} \quad (1)$$

or,

$$\text{lactose} + H_2O \xrightarrow[\text{or lactase}]{\beta\text{-galactosidase}} \alpha\text{-glucose} + \text{galactose} \quad (2)$$

$$\alpha\text{-glucose} \xrightarrow{\text{mutarotase}} \beta\text{-glucose} \quad (3)$$

$$\beta\text{-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{gluconolactone} \quad (4)$$

$$\text{galactose} + O_2 \xrightarrow{\text{galactose oxsidase}} H_2O_2 + \text{gluconolactone} \quad (5)$$

A multienzyme electrode, whose amperometric response is due to the electroreduction of O_2, uses two coimmobilized enzymes, β-galactosidase and glucosidase.[13] Enzyme electrodes for the simultaneous determination of sucrose and glucose, lactose and glucose and starch and glucose have also been reported.[14]

A novel enzyme electrode for the determination of lactose is described in this work. β-Galactosidase and glucose oxidase were coimmobilized in a redox polymer matrix, $PVF^+ClO_4^-$, and an enzymatically generated H_2O_2 oxidation current was measured. Phosphate ions were used instead of a third enzyme, mutarotase. The reason for using phosphate ions in test solutions is to avoid using a third immobilized enzyme, mutarotase, which catalyzes the conversion of α-glucose to β-glucose in the presence of phosphate ions in a medium without the need for mutarotase.[5] The optimum working conditions with respect to the buffer and substrate concentrations and the pH were investigated.

Experimental

A $PVF^+ClO_4^-$ modified Pt surface was prepared by electrooxidizing polyvinylferrocene (PVF) at +0.70 V *vs.* Ag/AgCl in a methylene chloride solution containing 0.10 M tetrabutylammonium perchlorate (TBAP). PVF was prepared using a method of chemical polymerization[15] of vinylferrocene

† To whom correpondence should be addressed.

(Alfa products). The electroprecipitation of $PVF^+ClO_4^-$ was carried out under a nitrogen atmosphere. The purification of methylene chloride was accomplished according to a method proposed in the literature.[16] TBAP was prepared by a reaction of tetrabutylammonium hydroxide (40% aqueous solution) (Merck) with $HClO_4$ (Merck), crystallized from an ethanol–water mixture (9:1) several times and kept under a nitrogen atmosphere after vacuum drying at 120˚C. Buffer solutions were prepared using NaH_2PO_4 (AnalaR BDH) and NaOH (Merck). A lactose (Sigma L3625) solution was prepared in a 0.30 M phosphate buffer solution of pH = 7.8. A solution containing β-galactosidase and glucose oxidase was prepared by dissolving 3.8 mg β-galactosidase (E.C. 3.2.1.23 Sigma G6008) and 20.0 mg glucose oxidase (E.C. 1.1.3.4 Sigma G6125) in 2.0 mL of a 0.010 M phosphate buffer solution of pH = 7.8.

The preparation of the enzyme electrode was accomplished by immersing the $PVF^+ClO_4^-$ coated Pt electrode in a solution of β-galactosidase and glucose oxidase. The polymer coated-electrode was kept in the enzyme solution for 30 min without stirring. The pH of the enzyme solution was kept at pH = 7.8. At this pH, because both of the enzymes are in the form of anions they can be ion-exchanged and electrostatically held by the redox polymer. The isoelectric points of individual enzymes are 5.5[17] and 4.2,[18] respectively.

Enzymes (E) were incorporated into the polymer matrix by immersing a $PVF^+ClO_4^-$ coated Pt electrode in an enzyme solution according to the following ion exchange process:

$$PVF^+ClO_4^- + E^- \longrightarrow PVF^+E^- + ClO_4^- \quad (6)$$

The enzyme is held electrostatically in the polymeric structure. The enzyme electrode was then rinsed with a buffer solution of the working pH to remove any excess enzyme which was not held electrostatically. In a previous study, the amount of enzyme incorporated in the modified electrode was determined by following the decrease in the absorbance of the enzyme solution at 277 nm during the surface-immobilization procedure. The amount of enzyme adsorbed onto the glass surface that is lost by washing was measured spectrophotometrically, and found to be negligible. Furthermore, the amount of the enzyme immobilized within the polymer could then be determined using the same absorption peak after desorbing the enzyme in a solution having a pH value less than the isoelectric point of the corresponding enzyme.[3] The activity of the enzyme electrode was determined with a jacketed electrochemical cell, which kept the solution at the desired temperature. Oxygen was introduced into the solution in this cell at a constant flow rate to obtain an oxygen-saturated solution. Oxygen flow was continued above the solution to keep it saturated with oxygen during the measurements.

A constant potential of +0.70 V *vs*. SCE was applied to the enzyme elecrode to measure the amperometric response due to the electrooxidation of H_2O_2 produced enzymatically. A steady state background current was first measured at this potential with a blank buffer solution of the working pH. After the steady state background current value was reached, certain volumes of a lactose solution of known concentration were added and the currents for each added amount of the substrate were recorded.

A three-electrode system was used as an electochemical cell with separate compartments for the counter and reference electrodes. SCE was used in aqueous solution; an Ag/AgCl electrode immersed in a 0.10 M TBAP solution containing a saturated amount of AgCl was used in methylene chloride as a reference electrode. A Pt foil electrode (A = 0.5 cm^2) was used as a working electrode.

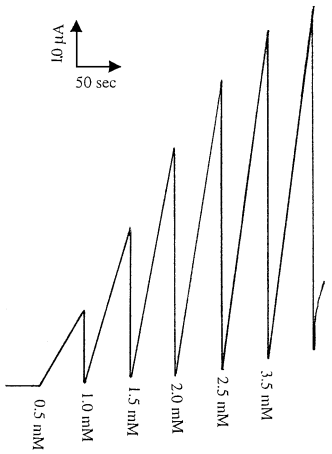

Fig. 1 Current–time curves for different lactose concentrations (0.3 M phosphate concentrations, 25˚C).

The electrochemical instrumentation consisted of a PAR Model 362 Potentiostat–Galvanostat. Current–time curves were recorded on a Model 16100-II Linseis recorder.

Results and Discussion

As discussed concerning enzyme electrodes for a glucose, sucrose and galactose[3–5] PVF^+ matrix in which the enzymes are immobilized show a catalytic effect for the oxidation of H_2O_2. The ferrocene centers act as covalently bonded electron-transfer mediators which oxidize H_2O_2 chemically,

$$2Fc^+ + H_2O_2 \longrightarrow 2Fc + O_2 + 2H^+ \quad (7)$$

(Fc: ferrocene moiety of PVF)

in addition to the electrochemical oxidation at the applied potential.

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e \quad (8)$$

The reduced form of the polymer, PVF, is also electrooxidized at the same applied potential, regenerating the oxidized form of the polymer, PVF^+.

$$2Fc \longrightarrow Fc^+ + 2e \quad (9)$$

$$Fc^+ + X^- \longrightarrow Fc^+X^- \quad (10)$$

X^-: counter ion

This catalytic process is the reason for the sensitivity increase in these sensors which use PVF^+ modified electrodes.[3–5]

The response curves of the two-enzyme lactose sensor used in this study is shown in Fig. 1. The current rose upon each addition of the substrate. The slow steady increase in the recorded current values is due to the fact that the phosphate ions are not able to convert α-glucose to β-glucose rapidly. A similarly slow rise in current was also encountered with an enzyme electrode developed for sucrose.[5]

A calibration plot was constructed with current values measured after 50 s following each substrate addition. As can

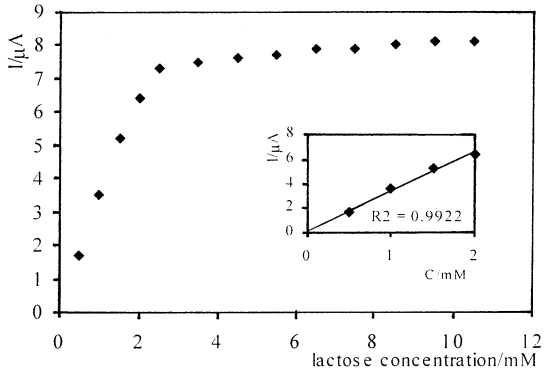

Fig. 2 Changes in the response of the enzyme electrode with the substrate concentration (0.3 M phosphate concentrations, 25˚C).

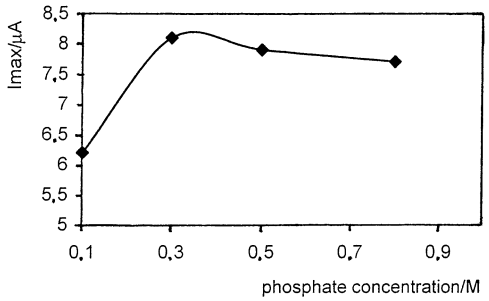

Fig. 3 Effect of the phosphate concentration on the response of the enzyme electrode (10.5 mM lactose concentrations, pH = 7.8, 25˚C).

be seen in Fig. 2, the response of the electrode reached a plateau after about a 7.5 mM substrate concentration. The upper limit of the linear working portion in the calibration plot (Fig. 2) was found to be 2.0 mM lactose concentration. Each point in Fig. 2 corresponds to the average of at least three measurements. The RSD value for successive assays of lactose was calculated be 4.2%. The response time of the electrode is comparable to those already published in the literature. The μA current response of the electrode should allow the determination of lactose concentrations lower than 0.5 mM.

The effect of the buffer concentration on the response of the electrode should also be investigated, since the response time is governed by the rate of conversion of α-glucose to β-glucose by phosphate ions. The effect of the phosphate concentration on the response of the enzyme electrode was tested at a substrate concentration of 10.5 mM. The electrode response was independent of the amount of the substrate around this concentration. Figure 3 shows that the response increased up to a phosphate concentration of 0.3 M. A further increase in the phosphate concentration did not cause any significant improvements in the electrode response.

Another important parameter on the response of the enzyme electrode was the solution pH. The response was measured with a subsrate concentration of 10.5 mM in a buffer solution which contained 0.3 M phosphate ions. It was found that the electrode response showed a maximum value at pH = 7.8 (Fig. 4).

No measurements related to the effect of potential interferant, such as ascorbic acid, was performed. We know from our earlier applications using the same electrode that the current due to the oxidation of ascorbic acid is not appreciable compared to the current due to the catalytic regeneration of PVF^+.[3,5] The

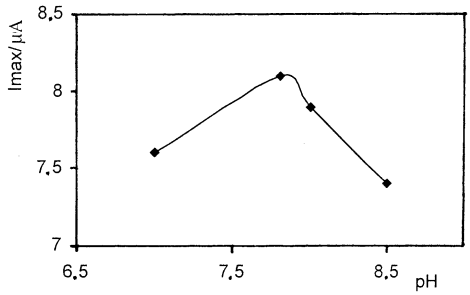

Fig. 4 Effect of the solution pH on the response of the enzyme electrode (10.5 mM lactose concentration, 0.3 M phosphate concentration, 25˚C).

operational stability was also investigated by recording over 60 – 70 assays of the current response to increasing concentrations of lactose. Each series of measurements relative to each substrate was carried out in a fresh electrolyte. Finally, we found that the enzyme electrode activity was not changed significantly.

It can be concluded that the redox polymer, PVF^+, possesses unique properties as an immobilizing medium for the enzymes and as a catalyst for H_2O_2 oxidation for the development of a simple and sensitive amperometric lactose sensor. The response time and the linear working range of the electrode are comparable to those already proposed in the literature. The response of the electrode is in the μA range which gave current values in the nA range.

References

1. J. Abdulhamid, G. J. Moody, and J. D. R. Thomas, *Analyst*, **1989**, *114*, 1587.
2. Y. Xu, G. G. Guilbault, and S. S. Kuan, *Enzy. Microb. Techn.*, **1990**, *12*, 104.
3. H. Gülce, H. Özyörük, S. S. Çelebi, and A. Yıldız, *J. Electroanal. Chem.*, **1995**, *397*, 63.
4. H. Gülce, İ. Ataman, A. Gülce, and A. Yıldız, *Enzy. Microb. Techn.*, **2001**, in press.
5. H. Gülce, S. S. Çelebi, H. Özyörük, and A. Yıldız, *J. Electroanal. Chem.*, **1995**, *397*, 217.
6. G. Inzelt and J. Bacskai, *Electrochim. Acta*, **1992**, *37*, 647.
7. P. J. Peerce and A. J. Bard, *J. Electroanal. Chem.*, **1980**, *112*, 97.
8. J. L. Garcia, A. Lopez-Mungia, and E. Galindo, *Enzy. Microb. Techn.*, **1991**, *13*, 672.
9. R. Pilloton and M. Mancini, *Food Chem.*, **1990**, *36*, 213.
10. V. Bilitewski, A. Jager, P. Ruger, and W. Weise, *Sens. Actuators*, **1993**, *15*, 113.
11. W. J. Albery, Y. N. Kalia, and E. Marger, *J. Electroanal. Chem.*, **1992**, *325*, 83.
12. A. Jager and V. Bilitewski, *Analyst*, **1992**, *119*, 125.
13. T. Katsu, X. Zhang, G. A. Reichnitz, and X. N. Zhang, *Talanta*, **1994**, *41*, 843.
14. M. Filipiak, K. Fludra, and E. Gosciminska, *Biosens. Bioelectron.*, **1996**, *11*, 355.
15. E. Watanabe, M. Takagi, S. Takei, M. Hoshi, and S. Cao, *Biotech. Bioengin.*, **1991**, *38*, 99.
16. W. T. Smith, J. Kuder, and E. Wychick, *J. Polym. Sci.*, **1976**, *14*, 2433.
17. D. D. Perrin and W. L. F. Armarego, "*Purification of Laboratory Chemicals*", **1980**, Pergamon Press, Oxford,

265.
18. M. Suzuki, T. Saito, and T. Satoh, *Biosci. Biotech. Biochem*., **1963**, *60*, 139.
19. P. D. Boyer, H. A. Lordy, and K. Murback, "*Enzyme Handbook*", 2nd ed., **1963**, Vol. 7, Academic Press, New York.