

β -galactosidase Determination by an Electrochemical Biosensor Mediated with Ferrocene

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Abstract: In this paper, a new viewpoint on the activity determination of β -galactosidase is reported. Glucose oxidase was directly immobilized on a glassy carbon electrode and mediated by ferrocene. The biosensor's performance was based on mediated electron transfer by ferrocene, which reduced via glucose oxidase reaction. In this reaction, substrate of glucose oxidase, glucose was provided by the activity of β -galactosidase in the sample. The parameters of the fabrication process for the electrode were optimized. Experimental conditions influencing the biosensor performance, such as pH, ferrocene and lactose concentrations, and temperature, were investigated and assessed. Finally, the biosensor was successfully applied to determination of β -galactosidase activity of artificial intestinal juice.

Keywords: β -galactosidase, biosensor, glucose oxidase, ferrocene, enzyme activity determination

INTRODUCTION

The enzyme β -galactosidase is a tetramer of four identical amino acids chains and hydrolyzes lactose and other galactosides into monosaccharides. *E. Coli* β -galactosidase has two catalytic activities. First, it hydrolyzes the disaccharide lactose to galactose plus glucose. Second, it converts lactose to another disaccharide, allolactose, which is the natural inducer for the *lac* operon (Scheme 1).

β -galactosidase is related to very important diseases such as sphingolipidoses. Mucopolysaccharidoses, mucopolipidoses, glycoprotein, and glycogen storage diseases are all of the sphingolipidoses belonging to the lysosomal storage diseases (LSDs) [1–5], GM1-gangliosidosis, and Morquio disease type B are included in LSDs. The primary defect in patients with these diseases is deficiency in lysosomal β -galactosidase activity [6]. When the lysosomal degradation of ganglioside GM1 is fallen into decay ganglioside GM1 is stored in lysosomes. Storage of these substances may cause many different serious symptoms such as developmental arrest, progressive deterioration of the nervous system, startle responses to sound, a macular cherry-red

spot, hepatosplenomegaly, rigospasticity associated with seizures, and generalized skeletal dysplasia [7–9].

In addition, a deficiency of the enzyme β -galactosidase, which is produced by the cells lining the small intestine, causes lactose intolerance because β -galactosidase also catalyses to hydrolyze lactose into glucose and galactose, which are then absorbed into the bloodstream. People with lactose intolerance may feel uncomfortable 30 minutes to 2 hours after consuming milk and milk products. Symptoms range from mild to severe, based on the amount of lactose consumed and the amount a person can tolerate. Common symptoms include abdominal pain, abdominal bloating, gas, diarrhea, and nausea [10].

Thus far various detection methods based on scanning electrochemical microscopy [11], voltammetric [12], non-viral transfection [13], F^{19} NMR [14], chromogenic and fluorogenic substrates based [15–23], HPLC methods [24, 25] have been developed for β -galactosidase monitoring. Many of these methods are highly technical, time-consuming, and expensive.

This article describes a new biosensor system for the activity determination of β -galactosidase. The system is

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based on a glucose biosensor, which used ferrocene as an electron mediator. Glucose oxidase was immobilized by the help of a very simple immobilization procedure on glassy carbon electrodes. The measurement of glucose, which was the product of the reaction of β -galactosidase substrate, lactose, built up the theory of β -galactosidase activity determination. The biosensor was simply fabricated by covering of glucose oxidase with dialysis membrane by the help of an o-ring. The activity of β -galactosidase was determined via the measurement of current by ferrocene mediation (Scheme 2).

A key aspect of this biosensor is the integration of the glucose signal with the activity of β -galactosidase. The reaction rate of glucose oxidase immobilized on the electrode was proportionally increased with the increase of activity of β -galactosidase, which added to the reaction cell. In other words, enzymatic reaction rate of glucose oxidase in the bioactive layer of the biosensor was increased by the glucose released by β -galactosidase injected to the reaction cell. Herewith, the oxidation of ferrocene on the electrode surface was directly related to the β -galactosidase activity added to the reaction cell as sample or standard solution.

MATERIALS AND METHODS

Apparatus

A Metrohm VA 746 Trace Analyzer (Switzerland) and VA 747 Stand (Switzerland) were used through the chronoamperometric measurements controlled with a personal computer running the electrochemical software package of Metrohm Trace Analyzer for parameter set-up, data acquisition, and processing. A three electrode configuration was employed in all experiments, with potentials referred to an Ag/AgCl reference electrode. A glassy carbon electrode (Metrohm, Switzerland) and a platinum electrode (Metrohm, Switzerland) were employed as a working and an auxiliary electrode (2.0 ± 0.1 mm in diameter), respectively. All measurements were carried out at a constant temperature by the help of a cryostat (Lauda RE106, Germany). Before every experiment the glassy carbon electrode tip was polished by alumina polishing kits (Metrohm, Switzerland).

Biosensor Construction

The surface of the glassy carbon electrode was first polished with $0.05 \mu\text{m}$ Al_2O_3 powder and washed ultrasonically in deionized water for 5 minutes. After that, an electrochemical pre-treatment was carried out by using multi-cycle voltammetric scanning ($100\times$) between $(-100) - (-1200)$ mV at the speed of forward 1 V s^{-1} , and at the speed of backward 10 V s^{-1} in 0.1 M HNO_3 solution. These polishing

and pretreatment procedures were repeated before every electrode preparation step. A certain amount of glucose oxidase was pipetted onto electrode surface as a total volume of $50 \mu\text{L}$ (in pH 4.8, 50 mM citrate buffer solution). Then, the electrode surface was dried at room temperature for 15 minutes. In the last step of immobilization the electrode tip was carefully covered with a piece of dialysis membrane by the help of an o-ring.

Assembly of the Biosensor and Determination

The biosensor was immersed into a stirred 19 mL citrate buffer solution containing lactose in order to make chronoamperometric measurements for β -galactosidase activity. Then it was allowed to purge for 15 minutes for removing of dissolved oxygen. β -galactosidase standard solutions or samples were injected into the cell as a final volume of 1 mL . Glucose occurred by the reaction of β -galactosidase was oxidized via glucose oxidase immobilized on the electrode surface. In the second reaction the electrons were transferred to ferrocene from FAD center of glucose oxidase. Finally, reduced ferrocene was again oxidized by giving electrons to electrode. These current changes were monitored chronoamperometrically.

RESULTS AND DISCUSSION

The Effect of Glucose Oxidase Activity on the Biosensor Performance

The activity of glucose oxidase (GOD) used in the biosensor slightly affected the response. In order to assess the effect of the glucose oxidase activity the biosensors were constructed using different GOD activities such as 22.5, 45, 67.5, and 90 U . The results showed that the chronoamperometric signals of the biosensor increased as the glucose oxidase activity increased. However, neither the limit of detection nor linear range for β -galactosidase was markedly improved by the help of this increase. At 22.5 U , the linear range was narrowed because of the insufficient activity of GOD. As a result the best biosensor performance was obtained when we used 45 U GOD activity for the construction of biosensor.

Optimization of Ferrocene Mediator Concentration

The behavior of the biosensor was studied against ferrocene that was involved in the enzymatic reaction as a mediator. Thus, sets of chronoamperometric measurements were taken for comparison in different ferrocene concentrations. The obtained sets of calibration graphs for β -galactosidase are shown in Figure 1.

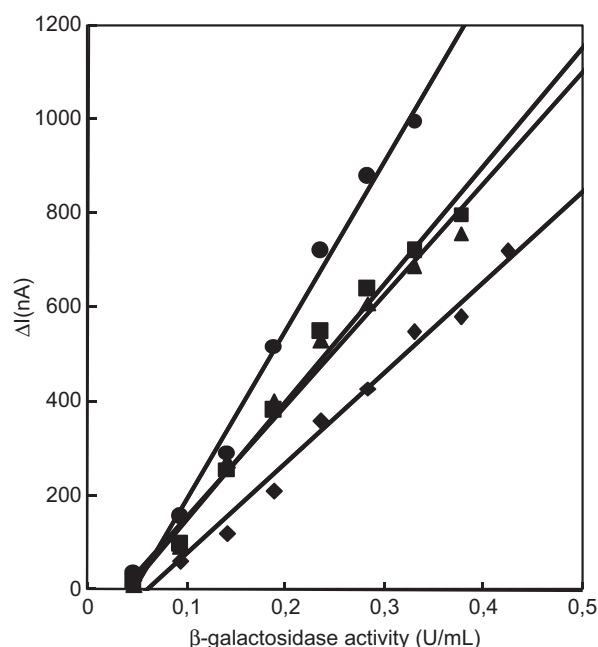


Figure 1. The effect of mediator, ferrocene concentration on the biosensor performance [Ferrocene concentrations used: $\bullet$ – $\bullet$ –: 1 mM, $\blacksquare$ – $\blacksquare$ –: 2.5 mM, $\blacktriangle$ – $\blacktriangle$ –: 5 mM, $\blacklozenge$ – $\blacklozenge$ –: 7.5 mM. Working conditions: The amount of glucose oxidase immobilized on the electrode was 45 U, 50 mM citrate buffer (containing 100 mM lactose, and of course containing ferrocene as indicated above, pH 4.8), $T = 35^\circ\text{C}$. Chronoamperometric medium: at a constant potential: 250 mV, t.puls:40 ms, t.meas:20 ms].

A decrease in ferrocene concentration resulted in an increase in chronoamperometric signals. However, linear detection range was the same in all ferrocene concentrations. The higher ferrocene levels than 1 mM probably resulted in a serious diffusion barrier between the electrode surface and dialysis membrane. Moreover, at the high ferrocene concentrations a little ferrocene deposition was also observed. Consequently, for the highest biosensor performance 1 mM ferrocene was used as optimum ferrocene concentration.

Lactose Content of the Working Buffer

One of the most important parameters was lactose concentration in the working buffer. For an enzyme-catalysed reaction, there is usually a hyperbolic relationship between the rate of reaction and the concentration of substrate as observed for our β -galactosidase-lactose system. The results are given in Figure 2.

In such a manner that, as the concentration of lactose increased, β -galactosidase became saturated with its substrate. The rate of formation of glucose now depended on the activity of the β -galactosidase itself, and adding more lactose didn't affect the rate of the reaction to any significant effect. For the assessment of the effect of lactose concentration

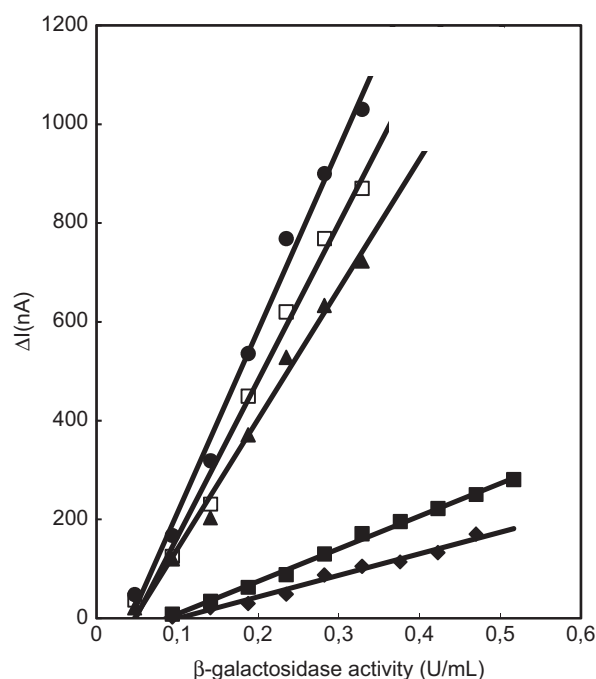


Figure 2. The effect of lactose concentration on the biosensor performance [Lactose concentration levels added to the working buffer: $\blacklozenge$ – $\blacklozenge$ –: 10 mM, $\blacksquare$ – $\blacksquare$ –: 25 mM, $\blacktriangle$ – $\blacktriangle$ –: 50 mM, GGGGGGGGGGGGGG: 75 mM, $\bullet$ – $\bullet$ –: 100 mM. Working conditions: The amount of glucose oxidase immobilized on the electrode was 45 U, 50 mM citrate buffer (containing 1 mM ferrocene, and of course containing lactose as indicated above, pH 4.8), $T = 35^\circ\text{C}$. Chronoamperometric medium: at a constant potential: 250 mV, t.puls:40 ms, t.meas:20 ms].

different working buffers containing 10, 25, 50, 75, and 100 mM lactose were used. As can be seen from Figure 2, lactose concentration played a very important role in the β -galactosidase determination system. In fact, it was not a surprise to increase chronoamperometric signals with the increase in lactose concentration. At the levels higher than 25 mM, the signals increased hyperbolically as mentioned above. However, in the range of 50–100 mM lactose, the signals didn't significantly impress by the further increase in lactose concentration. This result agreed with the theory expressed above. In this range of lactose concentration, the biosensors were similar in their limits of detection and linear detection ranges for β -galactosidase. Therefore, in all experiments, a working buffer containing 100 mM lactose was used.

Investigation of Temperature Effect on the Biosensor Performance

The working temperature was very important because the performance of the biosensor was correlated with two different enzymes. That optimum temperature should be suitable for both two enzymes, glucose oxidase from biosensor

and β -galactosidase from the sample. In order to investigate the most suitable temperature, we made a lot of tests. First of all, the biosensor performance was evaluated in the temperature range from 20 to 50 °C at an interval of 5 °C. The sensitivity was increased as the temperature was increased up to 45 °C, after which it was declined (Figure 3).

Although the highest signals were obtained by working at 45 °C, this temperature probably was high and not suitable. Thermal stabilities of the enzymes and repeatability of measurements could negatively be affected at this temperature. Hence we assessed the thermal stability of the biosensor at the temperatures of 35, 40, and 45 °C. For this purpose two calibration graphs were prepared at these different temperatures. One of them was immediately drawn after construction of the biosensor. The other one was drawn after the incubation period for 5 hours at the related temperature. After the incubation at 35 °C the biosensor maintained 100 % of its initial activity. Moreover, the incubations at 40 and 45 °C caused activity loss of about 8.5 % and 16 %, respectively. It is clear that the activity decrease was the result of the thermal denaturation of the enzymes. Finally, in order to make a decision on optimal working temperature, after these experiments we studied the operational stability of the biosensor by using two different temperatures, 35 and 40 °C. When we worked at the temperature of 35 °C, 13 measurements were taken without an activity loss. In addition, at 40 °C we could make 7 measurements without an activity loss. It indicated again that the thermal denaturation of the enzymes occurred at relatively high temperatures. Also, it should be stated that this activity loss may be caused by enzyme escape from the joining points of the o-ring and dialysis membrane. As a result of all these investigations, we chose 35 °C as the optimum working temperature.

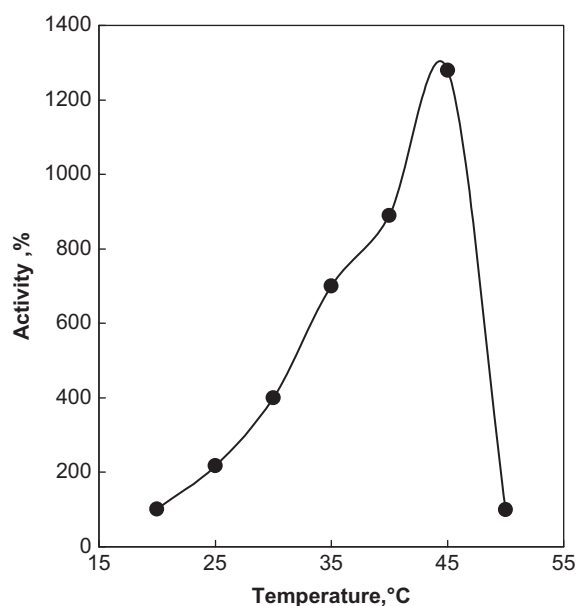


Figure 3. The effect of temperature on the biosensor performance. [The amount of glucose oxidase immobilized on the electrode was 45 U. β -galactosidase standard used in the experiments: 0.188 U/mL. Working conditions: 50 mM citrate buffer (containing 100 mM lactose, 1 mM ferrocene, pH 4.8). Chronoamperometric medium: at a constant potential: 250 mV, t.puls:40 ms, t.meas:20 ms.]

Optimum pH and Buffer Concentration

To study the effect of pH on biosensor response, the pH of working buffer was varied from pH 4.0 to 6.0 at an interval of 0.2, using 0.05 M citrate buffer. The highest signals were obtained at pH 4.8 (Figure 4). The value of pH 4.8 was in between the optimum pH value of glucose oxidase (pH 5.1) and optimum pH value of β -galactosidase (pH 4.5)

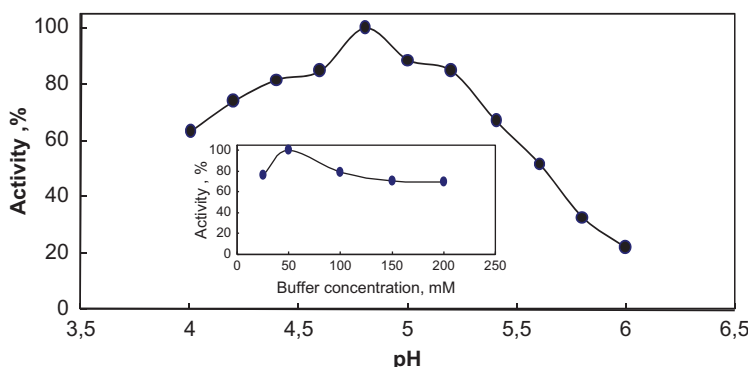


Figure 4. The effect of pH and buffer concentration on the biosensor performance. [For both parameters the amount of glucose oxidase immobilized on the electrode and β -galactosidase standard used in the experiments were same as 45 U and 0.188 U/mL, respectively. For optimum pH studies; Working conditions: 50 mM citrate buffers with differing pHs (containing 100 mM lactose, 1 mM ferrocene), T = 35 °C. For investigation of buffer concentration (inner graph); Working conditions: citrate buffers with differing concentrations (containing 100 mM lactose, 1 mM ferrocene), T = 35 °C. Chronoamperometric medium was same for two tests as follows; a constant potential: 250 mV, t.puls:40 ms, t.meas:20 ms.]

[26, 27]. Consequently, our results agreed well with the theoretical data on enzymes.

In the effect of buffer concentration studies the biosensor was subjected to 0.188 U/ml β -galactosidase at pH 4.8 in various concentrations of citrate buffers. The results are illustrated in the inner graph of Figure 5. It was found that the response decreased with the increase in buffer concentration. High buffer concentrations likely affected both the dissolved O_2 concentration and the biosensor's response time such that when the buffer concentration was above 0.05 M, the response time increased dramatically, which may be due to the slowing down of both enzymatic reactions. Accordingly, the optimum pH of the working buffer was selected to be 0.05 M.

Analytical Properties of the Biosensor

Calibration graph for β -galactosidase analysis by the biosensor based on glucose oxidase in the concentration range of 0.047–0.329 U/mL was plotted by injecting 100 μ L of standard β -galactosidase solution at concentrations of 0.047, 0.094, 0.141, 0.188, 0.235, 0.282, 0.329 U/mL of β -galactosidase and recording the increase in ferrocene reduction peak of the sample. A graph of concentration versus increase in ferrocene reduction current was plotted with β -galactosidase concentration on X-axis and ΔI (nA) drop on Y-axis (Figure 5).

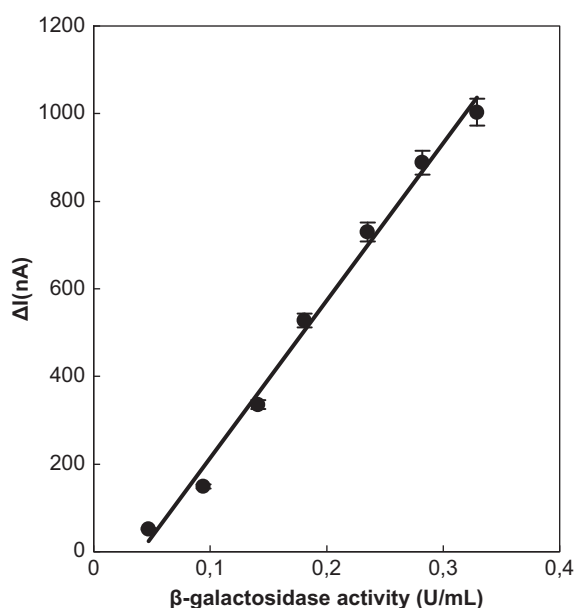


Figure 5. Calibration graph for β -galactosidase activity. [The amount of glucose oxidase immobilized on the electrode was 45 U. Working conditions: 50 mM citrate buffer (containing 100 mM lactose, 1 mM ferrocene, pH 4.8), $T = 35^\circ\text{C}$. Chrono-amperometric medium: at a constant potential: 250 mV, t.puls: 40 ms, t.meas: 20 ms.]

Assay variances were evaluated by the repeatability study. A standard sample was analyzed 10 times for β -galactosidase. The average value, standard deviation, and variation coefficient were 0.188 U/mL, ± 0.0017 U/mL, and 1 %, respectively. The results indicated that the biosensor-based technology is very reliable with regards to repeatability. The reproducibility of the biosensor preparation process was evaluated by preparing the calibration graphs and linear detection ranges for β -galactosidase carried out consecutively under the same experimental conditions. The results are summarized in Table 1.

Another analytical characteristic was storage stability of the biosensor. We also investigated this parameter in detail. For this purpose two different methods were tested on the biosensor. In one of them the biosensor was stored at $+4^\circ\text{C}$ for 2, 3, and 4 weeks, separately. The results revealed that for a period of 2 weeks the biosensor showed no loss of activity. At the end of 3 and 4 weeks, the biosensors remained 95.5 % and 86 % of their initial activities, respectively. In the other storage method the biosensor was used at particular intervals. The biosensor showed 100 % of its performance at the end of days 1, 4, 7, and 10. Three days later, at the thirteenth day, measurements showed that the biosensor lost 4.6 % of its beginning activity. From all investigations a very interesting conclusion can be drawn if the results are compared with each other. It is as follows: if the biosensor is used at intervals it saves its activity fine. The microbial growth on the biosensor surface is probably retarded in favor of the biosensor by this method.

Determination of β -galactosidase Activity in Artificial Intestinal Juice

The biosensor was used to determine the activity of β -galactosidase of artificial intestinal juice. All determinations were carried out using the standard addition method, with triple addition of a standard β -galactosidase solution (Table 2).

The results revealed that the determinations were carried out satisfactorily with good agreement between the determined value and the total assigned β -galactosidase content at the considered significance level.

Table 1. Results obtained from the reproducibility studies of the biosensor based on glucoseoxidase.

Biosensor	Linear range (μM)	R^2	y
1	4.7×10^{-3} – 3.29×10^{-1}	0.9885	$3620.2x - 131.87$
2	4.7×10^{-3} – 3.29×10^{-1}	0.9933	$3549.2x - 151.65$
3	4.7×10^{-3} – 3.29×10^{-1}	0.9894	$3629x - 131.9$

Table 2. β -galactosidase activity determination in artificial intestinal juice by the biosensor and by a spectrophotometric reference method.

β -galactosidase (U/ml) Added	Found by the biosensor	Found by the reference method	% Recovery	% Relative difference
0.188	0.18	0.195	96	8

CONCLUSION

This study describes a new aspect of the biosensors for β -galactosidase activity determination. The direct and very easy immobilization of glucose oxidase on a glassy carbon electrode was successfully performed and used. The biosensor had great capability in catalyzing the reduction of ferrocene and was able to be used as a chronoamperometric biosensor for the determination of β -galactosidase. Compared with the other determination systems mentioned in the introduction section, this biosensor exhibited a more rapid, sensitive, easier, and very economical way to create a new biosensor system for β -galactosidase. In addition, the biosensor reported here also showed a wider linear range and lower detection limit for β -galactosidase activity as compared to the biosensor systems for this purpose.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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