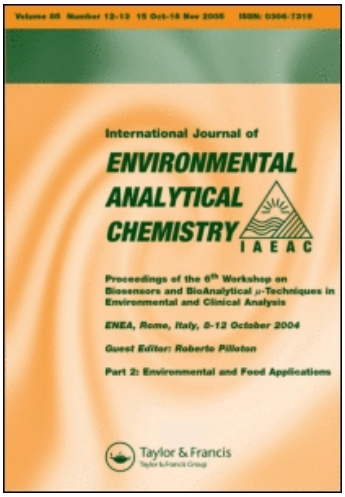

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A bio-imprinted ascorbate oxidase biosensor

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A bio-imprinted ascorbate oxidase biosensor

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Interest in bio-imprinting techniques has increased because it allows some stability characteristics of enzymes to be improved. In this study, we developed a simple way to improve the thermal and pH stabilities of ascorbate oxidase biosensor. The membrane of a Clark oxygen electrode was coated by a bioactive layer containing ascorbate oxidase and gelatin cross-linked by glutaraldehyde. Citrate was used to imprint the ascorbate oxidase molecularly. The optimum temperature and pH of both unmodified and citrate modified biosensors were investigated, by comparing their resulting stability. Also, calibration graphs and operational stabilities were compared with each other. The results showed that this simple way should be used to improve the stabilities of a biosensor.

Keywords: MIP biosensor; Ascorbate oxidase; Biosensor stability; Stability improvement; Bio-imprinting

1. Introduction

Biosensors are used extensively in diagnostics, environmental monitoring, and food processes. They utilize various biological molecules, such as microorganisms, immunological agents such as antibodies or antigens, and enzymes, which are capable of recognizing a specific target molecule [1–3]. However, many difficulties, such as instability at high temperatures, different pHs, and in the presence of organic solvents, exist for their practical use. Furthermore, in many cases, it is hard to find and purify a natural candidate which possesses the desired properties. The operational stability of a biosensor is a very important parameter affecting the biosensor performance [4]. Operational stability may be defined as the retention of activity of a protein or enzyme when in use. The operational stability of a biosensor response may vary considerably, depending on the sensor geometry, method of preparation, as well as the applied

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receptor and transducer. Finally, it may vary considerably depending on the operational conditions. For operational stability determination, it may be recommended consideration of the analyte concentration, the continuous or sequential contact of the biosensor with the analyte solution, temperature, pH, buffer composition, presence of organic solvents, and sample matrix composition [5].

This is often the most quoted parameter in biosensor publications, and it relates to the operating lifetime and reusability of a device [6]. In many cases, the lifetime of use of a sensor can be important, either in an environment where monitoring of an analyte is required or when an analytical device incorporates a reusable sensor in the measuring process.

In this study, we discuss potential applications of the technique of molecular imprinting with an emphasis on an enzyme with its competitive inhibitor and its using for the construction of a biosensor. In this technology called 'bio-imprinting', the enzyme is first complexed by using its substrate analogues or competitive inhibitors [6–9]. In the following step, the ligand uncomplexed with enzyme is removed.

The partially purified ascorbate oxidase enzyme was first complexed by using citrate, which is a competitive inhibitor of ascorbate oxidase. Therefore, the enzyme–citrate complex was immobilized with gelatin with the help of glutaraldehyde on a Clark electrode tip. The influence of the imprinting of ascorbate oxidase by its competitive inhibitor on pH, temperature, and the operational stability of the resulting biosensor is discussed in this article.

2. Experimental

2.1 *Reagents*

Ascorbate oxidase was purified partially including steps of ammonium sulfate precipitation gradiently, dialysis and lyophilization. Sodium dihydrogenphosphate (NaH_2PO_4), disodiumhydrogenphosphate (Na_2HPO_4), citrate and ascorbic acid were purchased from E.Merck (Germany). Glutaraldehyde (25%), and gelatin (type 3, 225 Bloom) were purchased from Sigma (St. Louis, MO). All reagents used were of analytical grade.

2.2 *Apparatus*

YSI 54 A, 57 A model oxygen meters and YSI 5700 series dissolved oxygen (DO) probes (YSI, Yellow Springs, OH) were used. A water bath was used for preparation of bioactive material (Linear Shaker bath SBS 35; Stuart Scientific, Redhill, UK). All measurements were carried out at a constant temperature using a thermostat (Haake JF, Germany). A magnetic stirrer (IKA-Combimag, RCO) and pH meter with an electrode (WTW pH 538, Germany) for preparing buffer solutions were used. The temperature was kept constant in the reaction cell by circulating water at the appropriate temperature around the cell compartment during the experiment.

2.3 *Procedure*

2.3.1 Dissolved oxygen probe. In order to construct the biosensor, a dissolved oxygen probe was covered with a highly sensitive Teflon membrane using an O-ring.

2.3.2 Preparation of the bioactive layer material. Two milligrams of ascorbate oxidase and 10 mg citrate were weighed. This mixture was solved in 200 μL of working buffer (50 mM, pH 7.5, phosphate buffer) and then left for 1 h at room temperature. At the end of this period, 100 μL of gelatin solution (10 mg/100 μL) was added to the enzyme–citrate mixture. This bioactive material was incubated at 38°C for 15 min. Then, 200 μL of bioactive material was dispersed over the dissolved oxygen probe and allowed to dry at 4°C for 45 min. For cross-linking with glutaraldehyde, the probe carrying bioactive layer was immersed in 2.5% (v/v) glutaraldehyde solution and left for 5 min. Finally, citrate in the bioactive layer was removed for 1 h by washing with bidistilled water. Moreover, the biosensor utilizing non-complexed ascorbate oxidase with citrate was prepared in the same way, without any pre-treatment step. No washing procedure was required, but there was no pretreatment step with citrate, and of course it was not necessary to wash the biosensor with bidistilled water for 1 h. The amount of enzyme and gelatin, and percentage glutaraldehyde were the same as those of the first biosensor.

2.3.3 Measurement procedure. The biosensors based on ascorbate oxidase were put into the thermostatic reaction cell containing working buffer (pH 7.5, 50 mM sodium phosphate buffer), and the magnetic stirrer was fixed at a constant speed. A few minutes later, the dissolved oxygen concentration was equilibrated because of the diffusion of dissolved oxygen between working buffer and dissolved oxygen probe. At this time, ascorbic acid was injected into the thermostatic reaction cell. The dissolved oxygen concentration started to decrease and a few minutes later, it reached the constant dissolved oxygen concentration due to the enzymatic reaction equilibration below:

$$\text{Ascorbic acid} + \frac{1}{2}O_2 \xrightarrow{\text{ascorbate oxidase}} \text{dehydro} - \text{ascorbic acid} + H_2O.$$

At this moment, dissolved oxygen concentration was recorded. Measurements were carried out by noting the decrease in dissolved oxygen concentration in relation to ascorbic acid concentration added into the reaction cell.

3. Results and discussion

3.1 *Optimization studies*

3.1.1 Optimum pHs of the biosensors. Biosensors are affected by changes in pH. The most favourable pH value – the point where the biosensor is most active—is known as the optimum pH. This is illustrated in figure 1.

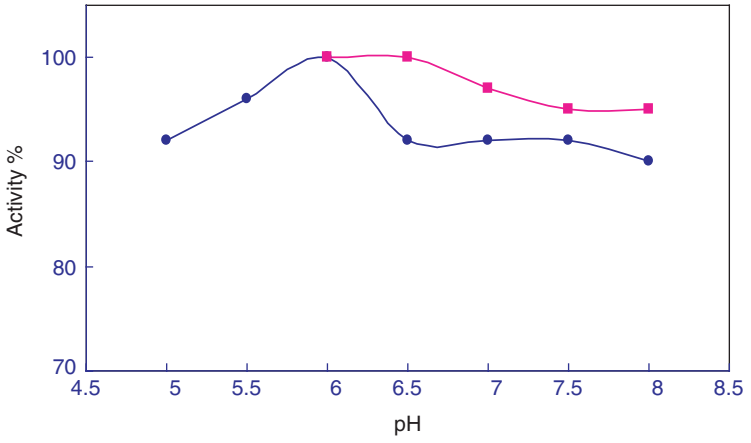

Figure 1. Optimum pHs of the biosensors (-●-●-: unmodified biosensor; -■-■-: modified biosensor). Working conditions: amounts of ascorbate oxidase, gelatin, and percentage glutaraldehyde were kept constant at 2 mg, 10 mg, and 2.5%, respectively. All buffers were 0.05 M and pH 5 and 5.5 citrate buffers and pH 6, 6.5, 7, 7.5, and 8 phosphate buffers were used in the experiments. All buffers were of 0.05 M conc. working temperature was 35°C. For bioimprinting 10 mg of citrate which was the competitive inhibitor of ascorbate oxidase was used.

Extremely high or low pH values resulted in a partial loss of activity for the biosensors. The maximum response was obtained with pH 6 buffer. The working pH was accepted as 7.5 because of a possible negative effect of acidic pH.

3.1.2 Temperature dependence of the biosensors. The activity of the biosensors was strongly affected by changes in temperature. Both biosensors worked best at the same temperature, 45°C. The activity of enzymes decreased away from 45°C (figure 2).

However, this value was very high for a working temperature of biosensor. Consequently, 35°C was accepted as the working temperature for both biosensors.

3.1.3 Thermal and pH stabilities of the biosensors. To compare their thermal stability, modified and unmodified biosensors were incubated at 35°C. Both biosensors showed a different thermal stability. Unmodified biosensor had 92% activity of its initial activity after 32 h. However, the other biosensor showed no thermal deactivation after the 47th hour. This result was very good for the aim of our study. The improvement in stability can be ascribed to the modification of the enzyme ascorbate oxidase with citrate. A similar result was obtained with stability studies of buffer pH, keeping the pH at 7.5 in 50 mM phosphate buffer. As can be seen from table 1, the pH stability of the modified biosensor was higher than that of the unmodified biosensor.

The modified biosensor lost about 8% of its initial activity at 35°C after 40 h, and unmodified biosensor retained just 93% of its activity after 23 h when they were operated at the same conditions. Thus, the pH stability of the ascorbate oxidase biosensor was improved by this simple modification method.

3.1.4 Operational stability. In order to demonstrate the improved stability of the biosensor, repeated measurements were carried out for both biosensors, under the

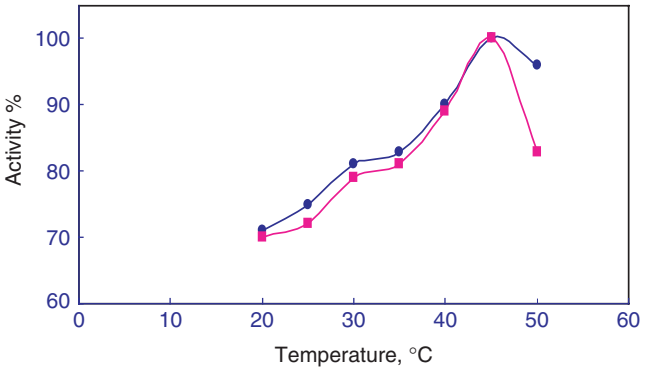

Figure 2. Optimum temperatures of the biosensors (-●-●-: unmodified biosensor; -■-■-: modified biosensor). Working conditions: amounts of ascorbate oxidase, gelatin, and percentage of glutaraldehyde were kept constant at 2 mg, 10 mg, and 2.5%, respectively. The working buffer was pH 7.5, 0.05 M phosphate buffer. For bio-imprinting, 10 mg of citrate which was the competitive inhibitor of ascorbate oxidase was used.

Table 1. Thermal and pH stabilities of the biosensors.

	Initial activity	Incubation period (h)	Residual activity (%)
Thermal stabilities (incubation temperature: 35°C)			
Biosensor 1[a]	100	32	92
Biosensor 2[b]	100	47	100
pH stabilities (buffer used for incubation: pH 7.5, 50 mM phosphate buffer)			
Biosensor 1[a]	100	23	93
Biosensor 2[a]	100	40	92

[a]Unmodified biosensor.
[b]Biosensor modified with citrate.

above optimized experimental conditions. The measurements were made sequentially. The results showed that the modified biosensor was more stable than the other in terms of the operational period (figure 3).

There was no decrease in activity at the end of the 30th measurement with the modified biosensor. However, after the 15th measurement, the activity of the unmodified biosensor started to decrease and lost just 2% of its initial activity. This unmodified biosensor lost about 17% of its initial activity at the end of the 20th measurement. This result also supported the effect explained in the thermal and pH stability sections.

3.1.5 Calibration graphs for the biosensors. At this stage of the study, the calibration graph of the modified biosensor was compared with that of the unmodified biosensor. The sensitivity obtained for the biosensor based bio-imprinted enzyme was slightly lower than that for the unmodified biosensor. The detection limits of both biosensors were as low as 100 μM ascorbic acid. Also, an excellent R^2 value was calculated for the modified biosensor. The calibration graphs are given in figure 4.

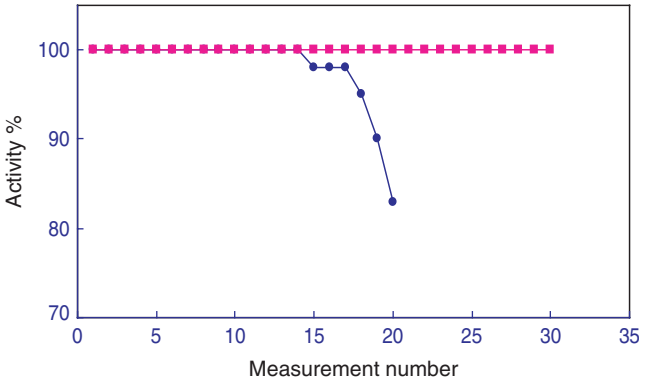

Figure 3. Operational stabilities of the biosensors (-●-●-: unmodified biosensor; -■-■-: modified biosensor). Working conditions: amounts of ascorbate oxidase, gelatin and percentage of glutaraldehyde were kept constant at 2 mg, 10 mg, and 2.5%, respectively. Working buffer: pH 7.5, 0.05 M phosphate buffer. In the experiments, 600 μM ascorbic acid standard solution was used, $T = 35°C$.

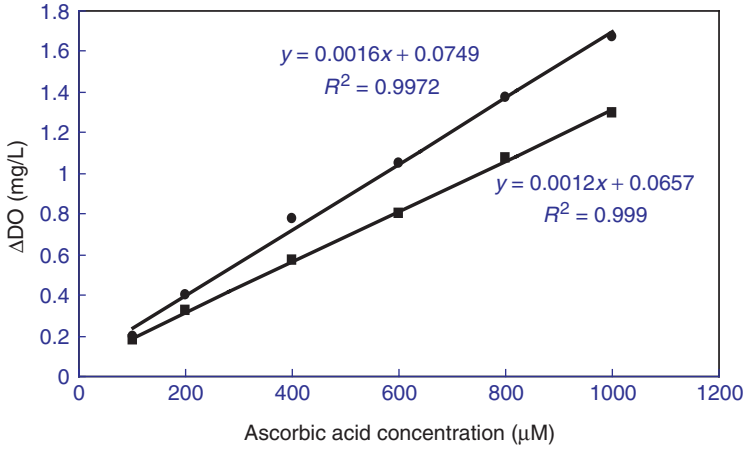

Figure 4. Calibration graphs of the biosensors for ascorbic acid (-●-●-: unmodified biosensor; -■-■-: modified biosensor). Working conditions: amounts of ascorbate oxidase, gelatin, and percentage of glutaraldehyde were kept constant at 2 mg, 10 mg, and 2.5%, respectively. Working buffer: pH 7.5, 0.05 M phosphate buffer, $T = 35°C$.

3.1.6 Repeatability of the biosensors. The precision of the biosensors was established by repeated assays ($n = 10$) using 600 μM standard of ascorbic acid. For the modified biosensor, the average value (x), standard deviation and coefficient of variation were 597 μM, ±7, and 1%, respectively. For the unmodified biosensor's values; these values were 575 μM, ±10, and 1.7%, respectively.

4. Conclusion

The biosensor exhibited a good sensitivity, selectivity, and repeatability for ascorbic acid. A higher stability was achieved for the ascorbic acid biosensor than

for the unmodified biosensor. Consequently, this method of improving the stabilities of the biosensors should be used with other enzymes and biosensors.

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