

DEVELOPMENT OF AN IMMOBILIZED ENZYME ELECTRODE FOR THE DETERMINATION OF SULFITE ION

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(Received: July 26th 1990 , Accepted: Mar. 16th 1992)

ABSTRACT: *An enzyme electrode was developed using the enzyme sulfite oxidase (EC1.8.3.1) immobilized onto pig intestine. Hydrogen peroxide produced was monitored amperometrically. All experimental parameters such as pH, temperature, buffer constituent concentration, were thoroughly investigated and optimized when appropriate. The response to sulfite concentration was linear in the range 5.2×10^{-6} to 1.02×10^{-3} M. The proposed method was found to have a correlation coefficient of 0.952 when evaluated in comparison with the standard titration method.*

KEY WORDS : *Ion selective electrode, Sulfite ion, Immobilized enzyme*

INTRODUCTION:

Biosensors generally consist of two parts. One part is the recognition element, which is usually an enzyme that is immobilized onto a membrane. Enzymes (naturally occurring bio-catalysts) have played an important role in the development of analytical methods for clinical, immunochemical, environmental, and biotechnological analyses [1]. Interference effects from similar "pseudo-substrates" are generally minimal. The second part, the transducer, converts the recognition reaction into electrical or optical signals that can

be measured. The substrate to be measured diffuses into the biocatalyst layer in which the reaction occurs, generating or consuming an electroactive species. The electrode-measurable species is monitored either potentiometrically or amperometrically, and can, in turn, be correlated back to the effective concentration (activity) of the substrate (or enzyme) [2]. The current research topics are usually focussed on two main efforts: first, improvement of the biocatalytic (recognition) element; and second, performance

enhancement and miniaturization of the electrode- sensing device. Such immobilized bioreagent electrochemical sensors have been developed for a wide range of applications.

Our research focuses on the determination of sulfite in foods and feeds. Sulfite is used extensively in the pharmaceutical and food industries. It acts as an anti- oxidant and also inhibits bacterial growth. However, sulfite is a pollutant in large quantities. In fact, *the Food and Drug Administration* (FDA) has recently ruled to revoke what was generally recognized as a safe level for sulfiting agents used on fruits and vegetables sold raw. The FDA has also established 10.0 ppm as the threshold for the declaration of sulfites in the labelling of foods, wine products and non alcoholic beverages [3]. These actions were taken after life- threatening reactions in sulfite sensitive individuals occurred as a result of eating foods with these agents [4]. There are various methods used for sulfite analysis, including gas chromatography [5], liquid chromatography [6], mercuric sulfide / mercurous chloride electrodes [7,8], ion chromatography [9-11], enzymatic UV [12,13], flow injection analysis [13], titration by the *Monier-Williams* method and spectrophotometry. The FDA reference method is the *Monier- Williams* procedure. This method is, however, time consuming and impractical for routine determinations.

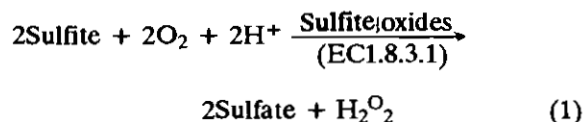
This paper describes the preparation of a sulfite enzyme electrode based on the amperometric monitoring of increase in hydrogen peroxide concentration. The experimental parameters were all optimized. The enzyme sulfite oxidase (EC1.5.3.1) is used in this biochemical procedure. The main advantage in using enzymes is that they are specialized to carry out a specific reaction, thus making it a reliable analytical reagent. There are, however, various disadvantages, such as high cost and short stability in aqueous assay conditions when soluble enzymes are used. These disadvantages can be overcome by immobilization. This procedure makes the enzyme more efficient making it available for

use up to several hundred times and extending the stability for several weeks. Also, there is less contamination of the reaction mixture and, because of these factors, the problems associated with the cost are also minimized.

There are various types of immobilization procedures for enzymes used in the construction of enzyme electrodes. Two specific methods will be discussed.

1. Physical entrapment of the enzyme: Many enzymes are bound to polyacrylamide gels which are cross- linked polymers with the enzyme trapped inside the matrix. The stability can be as long as two to three weeks (50-100 assays). A net is placed on top of the electrode for support of the enzyme gel which is then added. After being left to dry, the electrode is then stored in buffer solution at 5.0°C.
2. Chemically bound enzymes: This method was used here. The products are very stable and can be used for 200-10,000 assays. There are various coupling agents that can be used for chemical immobilization. The best method depends on the individual enzyme. In this case, glutaraldehyde / bovine serum albumin (BSA) coupling was used [14].

The biochemical reaction is as follows:



EXPERIMENTAL:

Chemicals

The enzyme was initially immobilized onto a synthetic dialysis membrane (3500 MW cut off). Although the results obtained were successful to some extent, it was not stable for prolonged periods. A physiological membrane, pig intestine, was therefore used as described previously [15-17]. This is a lipoprotein membrane with available sites for attachments. The procedure involves 25 μl of the enzyme EC1.5.3.1 sulfite oxidase (20IU) with 10 μl of 5% BSA and 1.5 μl of 6.25% glutaraldehyde. After being dried at room temperature for three

hours, it was washed in 0.05 M tris buffer, pH = 8.50, and stored at 4°C when not in use. After the enzyme electrode is prepared, there must be a way to measure the enzymatic reactions.

Apparatus

All measurements were made on an LC-4B electroanalyzer (BAS, West Lafayette, IN). The platinum electrode was a model 0-960 (Universal Sensors, Inc., Metairie, LA). The signals were monitored on a dual- pen chart recorder (Houston Instruments, Houston, TX).

Methods

Two methods were used in our research:

1. Total Steady State or Equilibrium Method. In this procedure, it is preferable to use large amounts of enzyme and small amounts of substrate to assure a rapid equilibrium. The reaction is allowed to go to completion and the change in current is measured for each assay. A calibration curve is then constructed where the change in current versus the concentration of sulfite is plotted.
2. Initial Rate Method. In this procedure, either the disappearance of substrate, or the change in the concentration of substrate or the product is monitored. The rate is a function of concentration of substrate, enzyme, and any other inhibitors or activators that may be present. This is a faster procedure because the reaction does not have to go to completion and hence is diffusion dependent. The change in current with time is monitored for each assay and a graph of initial rate versus sulfite concentration is plotted.

RESULTS AND DISCUSSION:

After the immobilization was complete the first calibration curve was obtained using dialysis membrane (3500 MWCO). It was observed that the enzyme membrane was not fully optimized. The linear dynamic range was from 1×10^{-6} to 2×10^{-4} M, the inhibitor response may be caused by the sulfite acting as an inhibitor at greater concentrations, blocking the enzyme

active site irreversibly. However, the enzyme was successfully immobilized onto pig intestine as described previously [17].

pH

A series of 1500 μ l samples with different pH were prepared, each containing a constant amount of sulfite (1×10^{-4} M). The graph obtained is shown below and has an optimum pH of 8.76, (Fig. 1).

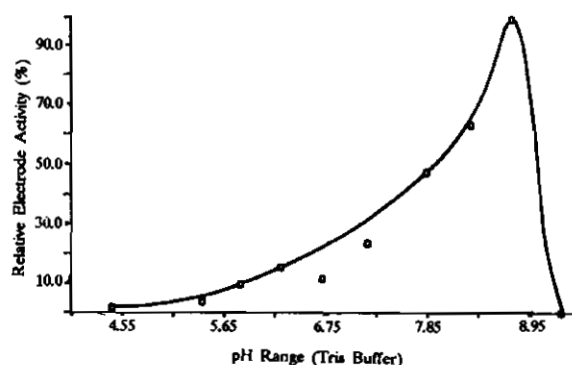


Fig. 1: Relative activity of sulfite electrode as a function of pH

Buffer concentration

The initial concentration employed was 0.12M. After using representative samples, it was observed that the response increased as the concentration of buffer decreased. The optimum concentration was 0.025M, which has a response six times greater than that at 0.12M, (Fig. 2).

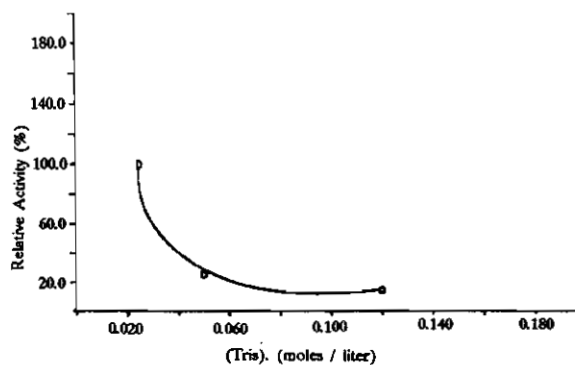


Fig. 2: Determination of optimal buffer constituent concentration ($pH = 8.76$; $[SO_3^{2-}] = 1.3 \times 10^{-4} M$)

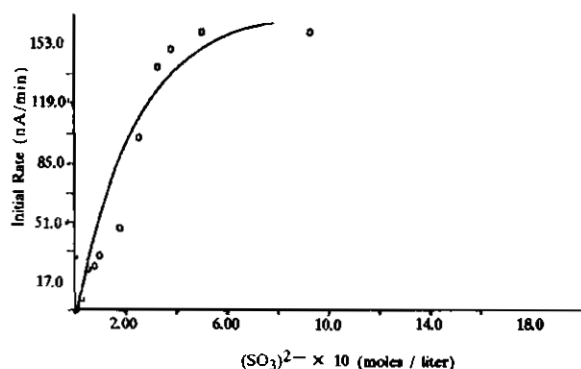


Fig. 3: Calibration curve for the determination of sulfite (initial rate method; 0.02M tris buffer, pH= 8.76)

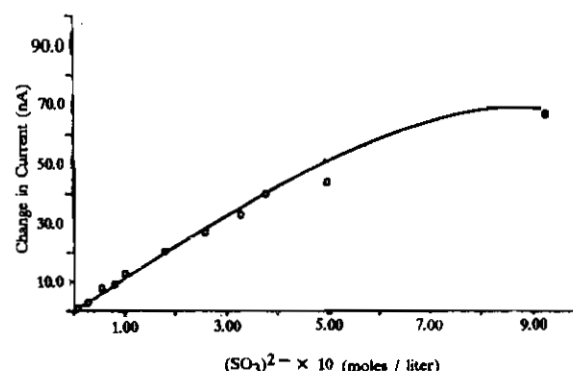


Fig. 4: Calibration curve for the determination of sulfite (steady-state method; 0.02M tris buffer, pH= 8.76)

Table 1: The enzyme electrode response to sulfite under optimal conditions (0.025M tris buffer, pH = 8.76)

Vol. sulfite added (μ l)	Total Assay Volume (μ l)	Sulfite (moles/litre)	Residual current (nA)	Steady state (nA)	Change in current (nA)	Initial rate (nA/min)
1	1501	5.3×10^{-6}	32.0	33.0	1.0	0.66
5	1505	2.6×10^{-5}	31.7	34.8	3.1	7.00
10	1510	5.2×10^{-5}	28.5	36.6	8.1	24.0
15	1515	7.9×10^{-5}	27.0	36.3	9.3	26.0
20	1520	1.0×10^{-4}	28.0	41.0	13.0	32.0
35	1535	1.8×10^{-4}	23.4	43.8	20.4	48.0
50	1550	2.6×10^{-4}	21.0	48.0	27.0	100.0
65	1565	3.3×10^{-4}	22.0	58.0	33.0	140.0
75	1575	3.8×10^{-4}	20.0	60.0	40.0	150.0
100	1600	5.0×10^{-4}	21.0	65.0	44.0	160.0
200	1700	9.3×10^{-4}	20.0	87.0	67.0	160.0

Temperature

Similar assays in thermostated cells were performed to determine the optimum temperature. Samples ranged from 20-40°C were used and the optimum temperature was found to be 35°C. All assays were therefore made at room temperature because there was less than 10% loss of activity. This eliminated the need to use a thermostated reaction cell.

A second calibration curve was constructed under optimum conditions using physiological pig intestine instead of synthetic dialysis

membrane. The results and graphs are shown in Table 1 and in Figs. 3 and 4.

- Using the initial rate method, the linear dynamic range extended from 5.5×10^{-6} to 5.0×10^{-4} M. There is also less irregularity in the results; that is, a broader linear dynamic range was obtained.
- Using the steady-state method, a linear dynamic range of 5.2×10^{-6} to 1.0×10^{-3} M extends over three decades of concentration. The difference between the initial rate and the steady-state methods is that there is a

steeper slope, that is higher sensitivity, in the initial rate method. Although the steady-state method is less sensitive, it does have a greater linear dynamic range.

Stability

Stability studies were also performed. There are two types of stability studies.

1- Storage stability: Where the immobilized enzyme membrane is stored at 0-5°C. It is then used for assay occasionally and mounted from time to time to see a trend in the electrode activity.

2- Operational stability: Where the enzyme membrane is used on a daily basis for at least 10 assays. This method was used in our research. Fig. 5 shows such a stability profile over a three-week period. The enzyme lost about 35% of its activity but then it started to level off. For approximately three months, about 60% of the initial activity was retained.

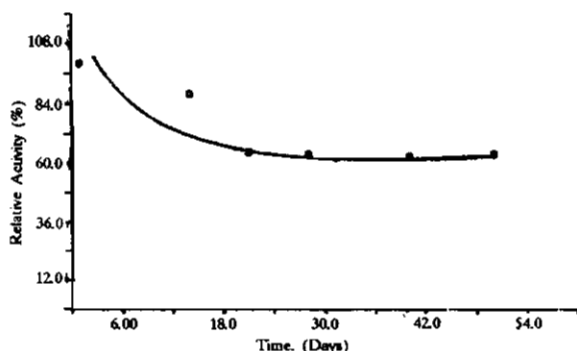


Fig. 5: Stability studies of sulfite electrode

Interference studies

Table 2 summarizes the interference studies of compounds analogous to sulfite. Sodium metasulfite and sodium bisulfite at high pH, that is, pH > 8.5 produce sulfite. This in turn results in comparable signals for these substances.

The proposed method of the enzyme electrode was finally evaluated against the reference *Monier-Williams* method for determination of sulfite. The correlation coefficient was found to be $r = 0.952$.

Table 2: Effect of possible interferences in the determination of sulfite using the enzyme electrode

Substance	Change in Current (nA)	Relative Activity (%)
Sodium sulfite	15.0	100
Sodium metabisulphite	16.5	110
Sodium bisulphite	10.2	68
Sodium nitrite	4.8	32
Sodium sulfate	0	0

CONCLUSIONS:

The enzyme electrode for the determination of sulfite has so far been optimized and evaluated against the reference method. The next objective is to use it for extensive real sample analyses. Such an approach could also be applied toward the assay of other chemically important analyses when a suitable specific enzyme is found.

Acknowledgement:

Some technical assistance provided by Fariba Eshaghzadeh is greatly appreciated.

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