

Sulfite Determination by an Inhibitor Biosensor-based Mushroom (*Agaricus Bisporus*) Tissue Homogenate

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Abstract: The aim of the study presented here is to develop a biosensor based on mushroom (*Agaricus bisporus*) tissue homogenate for sensitive and economical determination of sulfite in foods. The working principle of the biosensor is based on an inhibition effect of sulfite on polyphenol oxidases in mushroom. Mushroom tissue homogenate was immobilized by gelatin and glutaraldehyde on a Clark-type oxygen electrode. Some optimization studies related to the bioactive layer components and working conditions were identified. The biosensor was applied to the food samples. The biosensor reported here was successfully allowed to analyze sulfite, which was a food additive in real food samples.

Keywords: sulfite, food additives, inhibitor biosensors, polyphenol oxidase, tyrosinase, mushroom tissue

INTRODUCTION

Sulfites are used as bleaching, antioxidant, and preserving additives in food. Sulfites have been implicated as allergens [1]. A typical sulfite reaction involves flushing, dizziness, shortness of breath or wheezing. Asthmatic attacks can be provoked by sulfites and a few deaths have been attributed to them [2]. Sulfite sprays have been widely used on fresh produce in stores and restaurants to prevent browning with air exposure. French-fried potatoes are also treated this way. As preservatives, sulfites have been found in processed food, alcoholic beverages (wines and beer), and drugs. Even aerosols used to treat asthmatics contained sulfites as preservatives. The FDA has banned the use of six sulfite preservatives in fresh fruit and vegetables. Although there are restrictions on the use of sulfites, they are still used extensively by the manufacturers of processed foods, dried fruits, wines and beer [3,4].

In determining the sulfite content of foods, several procedures have been reported. The commonly used method is the Monier-Williams's procedure that quantifies sulfite in beverages and foods [5]. Ion chromatography [6,7], HPLC [8], and capillary electrophoresis [9] have been used as analytical methods for sulfite determination. However, these procedures were based on several steps which are time-consuming and tiring. In recent years, a few studies including

sulfite determination have also been reported. These methods were based on the spectrophotometric or electrochemical biosensors. For example, Abass and co-workers reported a biosensor utilizing sulfite oxidase with cytochrome c, as electron acceptor, and a screen-printed transducer [10]. In another method, sulfite oxidase was immobilized galvanostatically into polypyrrole film [11]. Yilmaz and Somer reported an enzymeless electrochemical method for determination of trace sulfite by using differential pulse polarography [12]. Zhao and colleagues developed a novel method based on an amperometric fill and flow channel biosensor employing sulfite oxidase for sulfite determination in beer samples [13]. Spricigo and his colleagues developed a similar method based on human sulfite oxidase co-immobilized with cytochrome c in a polyelectrolyte-containing multilayer [14].

In this study, a biosensor based on mushroom tissue homogenate for sulfite monitoring is presented. Mushroom tissue homogenate was used for sulfite determination for the first time by the presented study. In the optimization studies of bioactive membrane of the biosensor, detailed studies such as mushroom tissue homogenate amount, glutaraldehyde percentage as crosslinking agent, and gelatin amount were carried out. Then, some working conditions and characterization parameters were identified. Finally, the applicability of the biosensor to some food samples was demonstrated successively.

MATERIALS AND METHODS

Apparatus

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

Procedures

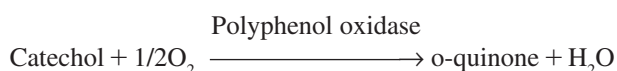
Dissolved Oxygen Probe. The dissolved oxygen probe was basically an amperometric cell that was polarized around 800 mV by an oxygenmeter. Because the reduction of oxygen is achieved between 400 to 1200 mV, there is a need for a voltage of around 800 mV. In order to construct the biosensor a dissolved oxygen probe was covered with high-sensitive teflon membrane by using an O-ring and then the teflon membrane (which is selective for oxygen) was pretreated with 0.5% sodium dodecylsulphate in phosphate buffer (50 mM, pH 7.5) to reduce the tension on the membrane surface.

Preparation of the Bioactive Layer Material. The bioactive layer of the biosensor was prepared by using mushroom tissue homogenate, which contained polyphenol oxidase. For this purpose, 100 mg mushroom was weighed and homogenized with 400 μ L working buffer (50 mM, pH 7.5, phosphate buffer) by a manual glass homogenizer. Then, 10 mg gelatin was weighed and added to a test tube. 300 μ L mushroom tissue homogenate was pipetted into the test tube. The mixture of mushroom tissue homogenate and gelatin was incubated at 38°C for 10–15 minutes to dissolve the gelatin.

Biosensor Preparation. In order to construct the biosensor the mixture of gelatin and mushroom tissue homogenate should be crosslinked by glutaraldehyde on an oxygen electrode. Thus, the bioactive layer should become insoluble. For this purpose, 200 μ L of gelatin-mushroom tissue homogenate mixture was dispersed over the dissolved oxygen probe membrane surface and allowed to dry at 4°C for 15–30 minutes. For crosslinking with glutaraldehyde, the probe-carrying bioactive layer was immersed into 2.5% (v/v) glutaraldehyde solution and was allowed to wait 5 minutes. Then, the biosensor

was washed with distilled water to remove the excess glutaraldehyde.

Measurement Procedure. The biosensor based on mushroom tissue homogenate was put into the thermostatic reaction cell containing the working buffer (pH 8.0, 50 mM sodium-phosphate buffer) and the magnetic stirrer was fixed at a constant speed. A few minutes later, dissolved oxygen concentration was equilibrated because of the diffusion of dissolved oxygen between working buffer and dissolved oxygen probe. At this time, a phenolic compound was injected into the thermostatic reaction cell. The dissolved oxygen concentration started to decrease and a few minutes later it reached constant dissolved oxygen concentration due to the enzymatic reaction equilibration below.



At this moment, dissolved oxygen concentration was recorded and then sulfite standard or sample was injected into the cell. DO concentration started to increase because of the inhibition of polyphenol oxidases by sulfite. Five minutes later, after injection of sulfite standard or sample, DO concentration at five minutes was recorded. Measurements were carried out by the change of dissolved oxygen concentration related to sulfite concentration added to the reaction cell.

RESULTS AND DISCUSSION

Optimization Studies

Two main modes of performance of the biosensor presented and analytical characteristics of sulfite determination are considered: the optimization of the immobilization of the mushroom tissue homogenate and the fitting of working conditions. In order to reveal optimum immobilization conditions for the biosensor, the effects of the quantity of mushroom tissue homogenate (22.12 mg tissue homogenate/cm², 44.25 mg tissue homogenate/cm², 66.37 mg tissue homogenate/cm²), the quantity of gelatin (2.95 mg gelatin/cm², 4.43 mg gelatin/cm², 5.9 mg gelatin/cm², 8.85 mg gelatin/cm²), and the crosslinking agent glutaraldehyde (1.25, 2.5, 5.0%) percent on the biosensor were investigated. Measurements were accomplished by using each of the standard curves obtained under these conditions. The optimization studies of the immobilization revealed that optimum mushroom tissue homogenate quantity, optimum gelatin quantity, and glutaraldehyde percentage were 44.25 mg tissue homogenate/cm², 5.9 mg gelatin/cm², and 2.5%, respectively. Through all experiments, mushroom tissue homogenate quantity, gelatin quantity, and glutaraldehyde

percentage were kept constant at optimum as mentioned above.

The Effect of pH

As a rule the maximum of the enzyme activity is evaluated as most appropriate, both for substrate and inhibitor determination. Indeed, the pH dependence of the observed inhibiting effect often corresponds to that of the response of a biosensor.

As can be seen in Figure 1, the best biosensor response for sulfite was obtained at pH 6.5. The response decreased above pH 6.5. In addition, below pH 6, at more acidic pH, biosensor response was just about 60% of its initial activity. These results showed that the pH of the working buffer could be regarded as an important factor determining sensitivity of the biosensor toward sulfite. At higher and lower pH values than 6.5 there could be some parameters that affected the biosensor response. For example, the electrical charge of the active site of the enzyme should be affected negatively. Consequently, at these pH values the performance of the biosensor should be decreased. Moreover, the inhibition effect of sulfite should be decreased at these pHs. As a result, the optimum pH for the mushroom tissue homogenate biosensor was determined to be pH 6.5 phosphate buffer.

Temperature Dependence of the Biosensor

The effect of temperature on the biosensor was investigated. The temperatures of 20, 25, 30, 35, 40, and 45°C

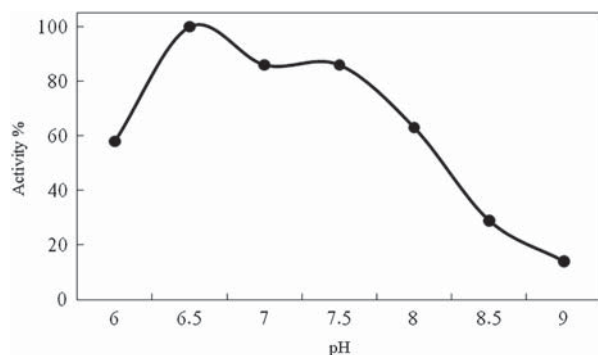


Figure 1. Optimum pH. [Phosphate buffers with pHs 6, 6.5, 7, 7.5 and 8, and tris-HCl buffers with pHs 8.5 and 9 were used. Buffer concentrations were 0.05 M. Catechol, substrate, and sulfite, as an inhibitor, concentrations were 100×10^{-6} and 150×10^{-6} M, respectively. $T = 35^\circ \text{C}$. (S.D. values for data points are given as differentiation in dissolved oxygen concentration (mg/mL): pH 6 (0.005), pH 6.5 (0.007), pH 7 (0.004), pH 7.5 (0.005), pH 8 (0.005), pH 8.5 (0.007), pH 9 (0.0025)).]

were studied as working temperatures. These studies showed that the temperature strictly influenced the biosensor response (Figure 2).

The highest biosensor response was observed at a temperature of 40°C . Above and below this temperature there was a decrease in the biosensor response of about 15–20%. Our system was based on the inhibition effect of sulfite on polyphenol oxidase. At higher temperatures this effect should be more significant. However, it is to be expected that the operation in higher temperatures would greatly affect the biosensor stabilities negatively. Moreover, a decrease in stability of the biosensor related to high working temperature should make the results unclear. In this condition, it would be difficult to say whether the activity decreased by inhibition effect of sulfite or by higher working temperature. Because of this, the working temperature was chosen as 35°C .

The Effect of Buffer System and Concentration

The effects of a working buffer system and its concentration were also investigated. In order to determine the appropriate buffer compound different buffer solutions, phosphate, citrate, and Hepes with pH 6.5 were tested. The results showed that there were slight differences between the signals obtained for different buffer systems. For this reason, phosphate buffer was chosen as the working buffer. In the optimization studies of the best buffer concentration, results showed that in the concentration range between 0.025 and 0.15 M the biosensor response was not affected by the buffer concentration. These results were good for both catechol signals and the signals obtained for sulfite inhibition.

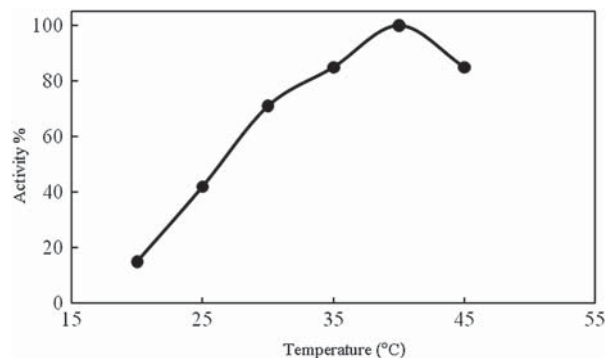


Figure 2. Optimum temperature. [Working conditions: pH 6.5, 0.05 M phosphate buffer was used. Catechol, substrate, and sulfite, as an inhibitor, concentrations were 100×10^{-6} and 150×10^{-6} M, respectively. (S.D. values for data points are given as differentiation in dissolved oxygen concentration (mg/mL): 20°C (0.002), 25°C (0.005), 30°C (0.005), 35°C (0.0025), 40°C (0.005), 45°C (0.0075)).]

Dependence on Substrate Type of the Biosensor

One of the most important parameters was the kind of substrate. For this purpose we used three different compounds as the substrates, which were catechol, ascorbic acid, and pyrogallol. This parameter affected the biosensor signals dramatically. The best results were obtained when catechol was used as the substrate. The results are given in Figure 3.

Ascorbic acid was also used as a substrate, but the signals obtained were about 85% lower than that of catechol. Moreover, when we used pyrogallol as the substrate, no signal was observed. In fact, this result was expected because the biosensor performance was maximum only when catechol was used as a substrate. In other words, the results showed that to obtain a relatively high inhibition effect on the biosensor the performance of the biosensor for substrate should be relatively high. Consequently, catechol was the appropriate substrate for the biosensor, because the biosensor signals obtained for catechol were higher than all other substrates used in the experiments.

The Effect of Substrate Concentration on the Biosensor

For an inhibitor biosensor the substrate concentration has to be adjusted carefully in order to obtain correct results. All calibration curves seen in Figure 4 have been recorded under the same conditions but with the presence of different substrate concentrations.

As can be seen in Figure 4, the best results were obtained by using 100 μM catechol concentration. So, in all

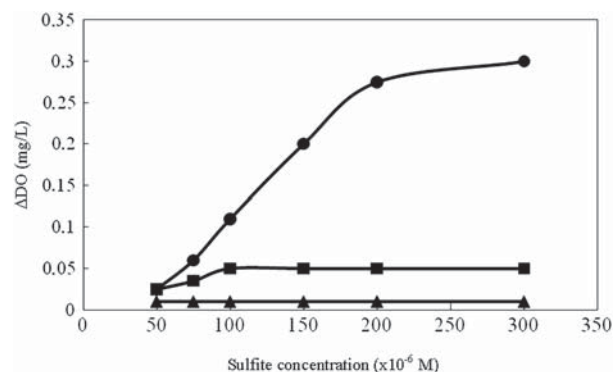


Figure 3. The effect of substrate type on sulfite determination. [Substrate types used: $\bullet$ - $\bullet$ -: Catechol, $\blacksquare$ - $\blacksquare$ -: Ascorbic acid and $\blacktriangle$ - $\blacktriangle$ -: Pyrogallol. The concentrations of these substrates were constant as 100×10^{-6} M. Working conditions: pH 6.5, 0.05 M phosphate buffer and $T = 35^\circ\text{C}$. (S.D. values for data points of catechol: 50×10^{-6} M (0.0025), 75×10^{-6} M (0.0025), 100×10^{-6} M (0.0025), 150×10^{-6} M (0.005), 200×10^{-6} M (0.005), 300×10^{-6} M (0.005). S.D. values for data points of ascorbic acid: 50×10^{-6} M (0.002), 75×10^{-6} M (0.002), 100×10^{-6} M (0.0015), 150×10^{-6} M (0.0025), 200×10^{-6} M (0.0025), 300×10^{-6} M (0.0025)].

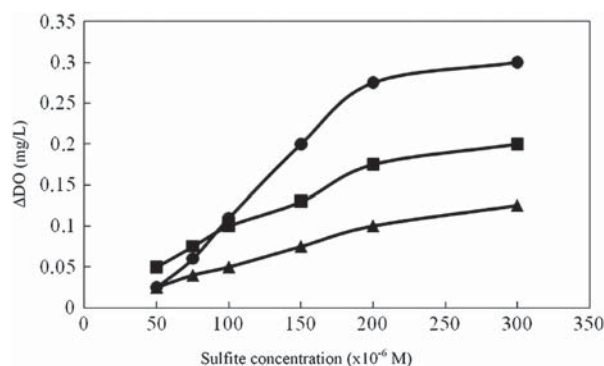


Figure 4. The effect of substrate concentration. [Substrate concentrations tested: $\bullet$ - $\bullet$ -: 100×10^{-6} M, $\blacksquare$ - $\blacksquare$ -: 200×10^{-6} M, and $\blacktriangle$ - $\blacktriangle$ -: 50×10^{-6} M. Working conditions: pH 6.5, 0.05 M phosphate buffer and $T = 35^\circ\text{C}$. (S.D. values for data points of 100×10^{-6} M catechol: 50×10^{-6} M (0.005), 75×10^{-6} M (0.005), 100×10^{-6} M (0.004), 150×10^{-6} M (0.004), 200×10^{-6} M (0.005), 300×10^{-6} M (0.0075). S.D. values for data points of 200×10^{-6} M catechol: 50×10^{-6} M (0.005), 75×10^{-6} M (0.005), 100×10^{-6} M (0.0075), 150×10^{-6} M (0.0075), 200×10^{-6} M (0.0075), 50×10^{-6} M (0.0075). S.D. values for data points of 50×10^{-6} M catechol: 50×10^{-6} M (0.0075), 75×10^{-6} M (0.0075), 100×10^{-6} M (0.01), 150×10^{-6} M (0.01), 200×10^{-6} M (0.01), 50×10^{-6} M (0.025)].

experiments, catechol concentration was kept constant as 100 μM .

The results presented in sections of 3.5 and 3.6 show that the inhibitor biosensor depended on the parameters of the kind of substrate and its concentration. This can be caused by K_m values of the polyphenol oxidases for different substrates and by their K_i values for different inhibitors. Also, the relationship between K_m and K_i values of the enzymes immobilized on the biosensor may effect the results. Moreover, at higher substrate concentrations than 100 μM , the inhibition effect of sulfite on the biosensor should be masked; when there was a lot of substrate in the cell, the inhibition effect of sulfite on the polyphenol oxidase should not be much more significant. When the substrate concentration was decreased to 50 μM , the biosensor performance was also decreased. This was probably caused by insufficient operation of polyphenol oxidase in the bioactive layer of the biosensor.

Calibration Graph for Sulfite

The combination of *Agaricus bisporus* with an oxygen electrode and its use for inhibitor determination led to a biosensor with a linear response for sulfite concentrations between 5×10^{-5} and 2×10^{-4} M with a linear correlation coefficient of 0.9924. The limit of detection (LOD) was 5×10^{-5} M. The linear calibration curve of the biosensor is given in Figure 5.

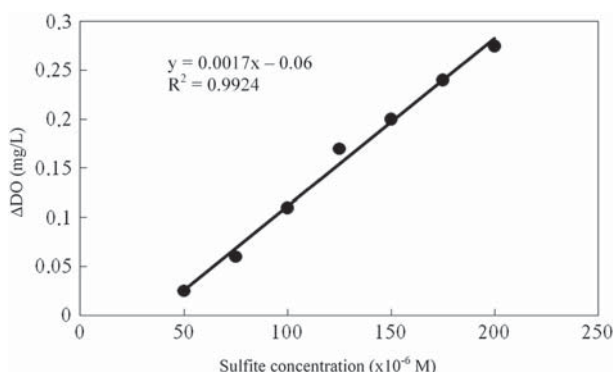


Figure 5. Sulfite calibration graph. [Working conditions: pH 6.5, 0.05 M phosphate buffer and T = 35°C. Catechol concentration was 100×10^{-6}].

The linear detection range for sulfite was sufficient to operate the biosensor for analysis of sulfite in real food samples.

Repeatability

Repeatability was evaluated by performing seven ($n = 8$) successive injections of sulfite in concentration of 1.5×10^{-4} M. In these studies, the concentration of the substrate, catechol, was 0.1×10^{-4} M. Acceptable precision, average value: 1.48×10^{-4} M, the standard deviation (S.D.): ± 0.0046 and the variation coefficient (C.V.): 3.1 % were obtained. The results revealed that the repeatability of the biosensor was very good. This indicated that a biosensor should successively be used for analysis of a series of samples without a loss of activity.

Sample Analysis

The biosensor based on *Agaricus bisporus* homogenate, at the end of these optimization and characterization studies, was adopted for the determination sulfite in some real samples. The results are given in Table 1 comparatively with a reference method [15] including enzymatic and spectrophotometric detection.

As follows from Table 1, the results of the biosensor presented are in good agreement with those of the reference method.

CONCLUSION

In our system, an inhibitor biosensor based on *Agaricus bisporus* homogenate had been constructed to electrochemically detect sulfite in food samples. It has been revealed that the use of the inhibition properties of polyphenol

Table 1. Sulfite analysis in some real samples by the biosensor and by the enzymatic reference method [10].

Sample	Found sulfite (ppm)		% Relative error
	Reference ^a	Biosensor ^a	
Pickle water 1	4200	4320	102.9
Pickle water 2	5440	5520	101.5
Pickle water 3	4756	4801	101
Beer	182	193	106
Vinegar	903	955	105.8

^aMean of three determinations.

oxidases by sulfite as a measurement principle leads to determine sulfite successively. It was clear that the biosensor was simply prepared from inexpensive and commercially available materials through a general method. The analytical performances of the presented biosensor were very good for monitoring of sulfite. Beside the direct determination of sulfite in food samples without any pretreatment, separation and derivatization steps were very important advantages of the biosensor. Finally, the accuracy of the biosensor for sample analysis was validated by using an enzymatic reference method and the results obtained with the biosensor were in good agreement with the reference method. Although the lowest detection limit of the biosensor is sufficient for analyzing sulfite in real food samples, it would be difficult to detect sulfite in concentrations lower than 50 μ M.

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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