

A Biosensor Based on Bay Leaf (*Laurus nobilis* L.) Tissue Homogenate: Improvement of the Stability Characteristics by a Simple Bio-imprinted Technique

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Abstract: Although enzymes are effective biocatalysts that are widely used in biosensors, a major drawback that hampers many of these biotechnological applications of enzymes is their limited stability. Applications that use very pure, high value proteins need to employ effective stabilization technology, primarily due to cost considerations and availability of the proteins used. For this purpose, interest in bio-imprinting techniques increases because it allows stability characteristics of enzymes to be improved.

In this study, a bio-imprinted Bay leaf (*Laurus nobilis* L.) tissue homogenate biosensor was devised by a very simple way. For this purpose, the enzymes, polyphenol oxidases in the bay leaf tissue, were first complexed by using their competitive inhibitor, thiourea, in aqueous medium and then this enzyme was immobilized on gelatin by crosslinking with glutaraldehyde on a Clark-type oxygen electrode surface. Similarly, noncomplexed polyphenol oxidase with thiourea was also immobilized on a Clark-type oxygen electrode in the same conditions. The aim of the study was to prepare a new biosensor-based Bay leaf tissue homogenate and to improve the stability characteristics such as thermal stability, pH stability, and storage stability, of the biosensor by bio-imprinting method. The results showed that this simple technique should be effectively used to improve the stabilities of a biosensor.

Keywords: Bio-imprinted biosensors, polyphenol oxidase, biosensor stability, Bay leaf, *Laurus nobilis* L, bio-imprinting

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INTRODUCTION

Polyphenol oxidase (PPO) catalyzes both the o-hydroxylation of monophenols and the oxidation of the o-diphenols into o-quinones (diphenolase or catecholase activity). PPO is widely distributed among plant species, and many studies have been conducted on PPO, describing its purification and characterization [1–5]. Phenolic compounds (PCs) are one of the most widely products present in biological degradation processes. In food, the presence of phenols is an alert to lack of freshness. Some phenolic products in plastic, paint, and pharmaceutical industries can be found in their wastewater [6,7]. Several methods to detect phenols are available. Among them are biosensors, where the bio-component is tyrosinase, which are isolated from mushrooms and are commercially available [8,9].

Molecular imprinting technique was mainly confined to the separation of organic molecules and to nonenzymatic catalysis [10]. In order to improve its applicability, new variations in the conventional imprinting technique have emerged, such as “bio-imprinting,” metal-chelate imprinting, and a combination of immobilization and “bio-imprinting” [11–14]. It is reported that the activity of enzymes has been increased by bio-imprinting them with their inhibitors [15]. The techniques of combinatorial chemistry or by the approach of Keyes et al. employed a different kind of methodology to alter the catalytic properties of enzymes [16,17]. In this technique, the enzyme complexed with ligands was crosslinked by using glutaraldehyde. We demonstrated the combinatorial crosslinked imprinting approach. In our previous studies we reported that this technique for improvement of the stabilities of the biosensors based urease, ascorbate oxidase and mushroom tissue homogenate (PPO) was successfully applied [18–20].

In the present work, a biosensor based on Bay leaf (*Laurus nobilis* L.) tissue homogenate was developed for the determination of some phenolic compounds and improvement of some stability parameters of the biosensor was surveyed. Bay leaf was selected as a source of the enzyme because it contains polyphenol oxidase abundantly [21] and is economic. This plant is an evergreen shrub indigenous to the southern parts of Europe and the Mediterranean area. Bay leaf tissue homogenate was first mixed with gelatin and then this mixture was dispersed over a Clark-type oxygen electrode surface. The biomembrane was complexed with thiourea, which was the competitive inhibitor of the enzyme. Thus the active site of the enzyme was temporarily blocked from the negative effects of the immobilization procedure, especially from glutaraldehyde, by the competitive inhibitor. After the bioimprinting process, the enzyme-thiourea complex was crosslinked with glutaraldehyde. Finally, thiourea in the enzyme active site was removed by washing. Similarly, noncomplexed Bay leaf tissue homogenate biosensor was also immobilized without thiourea on an electrode under the same conditions.

Optimization and characterization studies of two biosensors were carried out. Optimum pHs and temperatures, and pH, temperature, and operational stabilities of two biosensors were compared. Besides repeatabilities, linear ranges and substrate specificities of the biosensors were also discussed in this paper.

MATERIALS AND METHODS

Materials

Sodium dihydrogenphosphate (NaH_2PO_4), disodium hydrogenphosphate (Na_2HPO_4), phenol, pyrogallol, naphthol, thiourea and catechol were purchased from E. Merck (Germany). Glutaraldehyde (Grade II, 25% aqueous solution), gelatin (type 3, 225 Bloom), ferulic acid, sinapinic acid, ellagic acid, caffeic acid were purchased from Sigma (St. Louis, USA). Bay leaves (*Laurus nobilis* L.) used in the experiments were collected from the shrub of a certain variety cultivated in the garden of Ege University at Izmir in Turkey. All reagents used were of analytical grades.

Apparatus

YSI 54 A, 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). A 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.

Methods

Preparation of the Bioactive Layer Material

One hundred milligrams of Bay leaves were weighed and homogenized with 1 ml working buffer (0.05 M, pH 7.5, sodium phosphate buffer) in porcelain bowl. Then, 10 mg gelatin was weighed and added to a test tube and Bay leaf tissue homogenate (300 μl) was pipetted into the test tube. The mixture of Bay leaf tissue homogenate and gelatin was incubated at 38°C for 15 min to dissolve the gelatin.

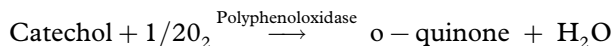
Preparation of the Bio-imprinted Biosensor

Bay leaf tissue (200 μL) homogenate-gelatin mixture for preparation of bio-imprinted biosensor was dispersed over the dissolved oxygen probe membrane surface and allowed to dry at 4°C for 1 h. The biomembrane was complexed with thiourea (0.5 M) for 1 h, after this complexation process, for crosslinking with glutaraldehyde, the probe carrying bioactive layer was immersed into 2.5% (v/v) glutaraldehyde solution and was allowed to wait for 5 min. Finally, thiourea in the bioactive layer was removed by washing for 1 hour. Similarly, the other biosensor (noncomplexed Bay leaf tissue homogenate biosensor) was prepared in the same way. But there was no treatment with thiourea. The tissue homogenate, gelatin amount, and glutaraldehyde percentage were the same as the bio-imprinted biosensor.

Thiourea is bound to the active center of the enzyme. Thus the active site of the enzyme is temporarily blocked by the “false substrate.” The inhibition is called competitive. Characteristically increasing the concentration of substrate reduces the effect of the inhibitor. Inhibition can be overcome. Consequently, in our system, by the help of thiourea, the active site of polyphenol oxidases in the tissue homogenate can be protected from the negative effects of the immobilization procedure during this process.

Measurement Procedure

The biosensors based on Bay leaf tissue homogenate was put into the thermostatic reaction cell containing working buffer (pH 7.5, 0.05 M 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, phenolic compound 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.



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

RESULTS AND DISCUSSION

Firstly, optimum immobilization conditions of the biosensor-based Bay leaf tissue homogenate were carried out. The bio-imprinted biosensor was compared with non-complexed biosensor in their stability characteristics because the aim of this work was to improve the stabilities of the biosensor based on bay leaf tissue contained polyphenol oxidase by imprinting technique. For this purpose, optimum temperatures and pHs, thermal, pH, storage, operational stabilities, linear ranges, and substrate specificities for both biosensors were discussed in the following sections.

Optimization Studies

In order to reveal optimum immobilization conditions for the biosensors, effects of the quantity of Bay leaf tissue (50, 100, and 150 mg) and the quantity of gelatin (5 and 10 mg) on the biosensor, and the effect of the crosslinking agent glutaraldehyde (1.25, 2.5, and 5.0%) percent on the biosensor were investigated. Measurements were accomplished by plotting each of the standard curves obtained under these conditions. Optimum Bay leaf tissue quantity, optimum gelatin quantity and glutaraldehyde percentage were revealed as 100 mg, 10 mg, and 2.5%, respectively. Through all experiments, Bay leaf tissue quantity, gelatin quantity, and glutaraldehyde percentage were kept constant at optimum as mentioned above.

pH Effect on the Biosensors

In order to compare optimum pHs of two biosensors buffer solutions with different pH values were used in the experiments. The results showed that optimum pHs of both biosensors were the same as 8.5. As can be seen in Figure 1, however, at more acidic and more basic pHs than 8.5, there was a decrease in the activity of the modified biosensor.

It is clear that the imprinted biosensor was more sensitive to change in the pH value than the other biosensor.

pH Stabilities of the Biosensors

For investigation of pH stabilities of the biosensors, both were incubated in pH 8.50, 50 mM sodium phosphate buffer for 20 hours.

According to the optimum pH study, the activities of biosensors are optimum at pH 8.5. The stability of the unmodified biosensor decreases at the

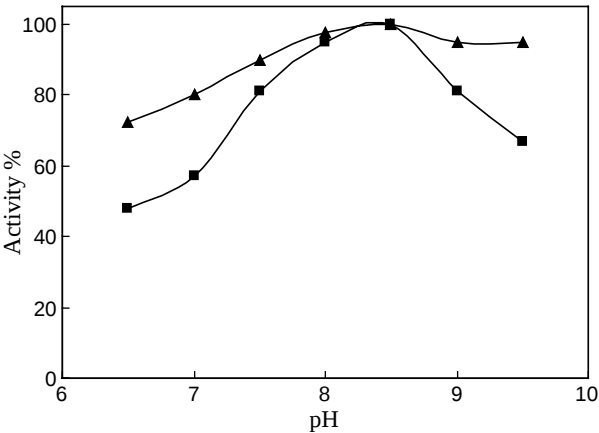


Figure 1. pH dependence of the biosensors [(■) imprinted PPO biosensor; (▲) PPO biosensor. Working conditions: Catechol concentration was injected to a final concentration of 200 μM. Sodium phosphate buffer with different pH values, 50 mM and 25°C].

end of the 20 hours incubation period at pH 8.5 sodium phosphate buffer. However, the polyphenol oxidase biosensor modified with thiourea was more stable at the same pH. As seen from Table 1, it kept 100% of its initial activity.

Temperature Dependence of the Biosensors

The effect of temperature on the biosensors was one of the most important parameters that affected the performance of the biosensors. Both biosensors worked best at the same temperature, 40°C. The biosensor bio-imprinted with thiourea was also presented an activity of 100% at 45°C in Figure 2.

Table 1. pH stabilities of the biosensors

	Initial activity	Incubation period (h)	Residual activity (%)
Biosensor 1 ^a	100	20	94
Biosensor 2 ^b	100	20	100

pH stabilities of the biosensors were carried out at pH 8.5, 50 mM sodium phosphate buffer.

^aThe biosensor with unmodified.
^bThe biosensor modified with thiourea.

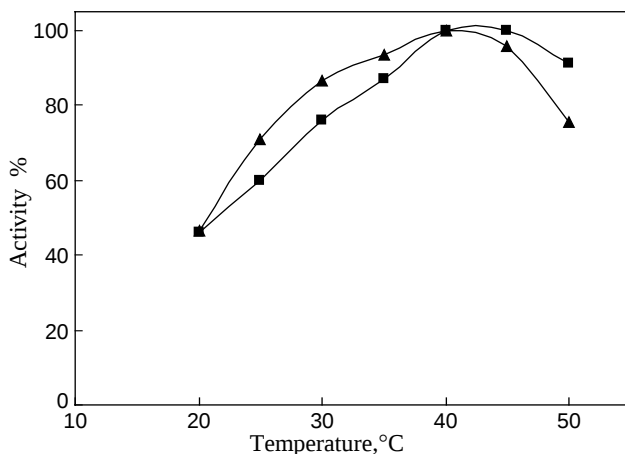


Figure 2. Temperature dependence of the biosensors [(■) imprinted PPO biosensor; (▲) PPO biosensor. Working conditions: Catechol concentration was injected to a final concentration of 200 μ M. Sodium phosphate buffer, 50 mM and pH 8.5].

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

Thermal Stabilities of the Biosensors

To compare thermal stabilities of the biosensor, modified and unmodified biosensors were incubated at 35°C for 20 hours. The biosensor unmodified lost 20% of its initial activity. However, the biosensor modified with thiourea retained 94% of its original activity. This result was very good for the goals of our study. This result is summarized in Table 2.

Table 2. Thermal stabilities of biosensors

	Initial activity	Incubation period (h)	Residual activity (%)
Biosensor 1 ^a	100	20	80
Biosensor 2 ^b	100	20	94

Thermal stabilities of the biosensors were carried out at 35°C and pH 8.5, 50 mM sodium phosphate buffer. The concentration of catechol used in the experiments was 200 μ M.

^aThe biosensor unmodified.

^bThe biosensor modified by thiourea.

The temporary blocked active site of polyphenol oxidase by thiourea decreased the possibility of deactivation of the enzyme in the step of crosslinking by glutaraldehyde. Consequently, because of the resistance to immobilization conditions, the bio-imprinted biosensor was more stable than the unmodified one.

Calibration Graphs for the Biosensors

In this study, the calibration graphs of two biosensors were also investigated. As shown from Figure 3, linear ranges of both biosensors were not identical in terms of their slopes. However, two biosensors showed the same linear concentration range from 25 μM to 400 μM catechol. Besides, for the two biosensors the minimum detectable concentration of catechol was estimated to be 25 μM .

The signals obtained for the substrate catechol by the bio-imprinted biosensor was higher than that of the other biosensor. Consequently, the results showed that the sensitivity of the biosensor constructed by using the bio-imprinted technique was higher than that of the unmodified biosensor.

Repeatabilities of the Biosensors

The repeatabilities of the biosensors were also studied for 200 μM catechol concentration ($n = 10$). The average values ($\bar{x}$), the standard deviations (S.D.),

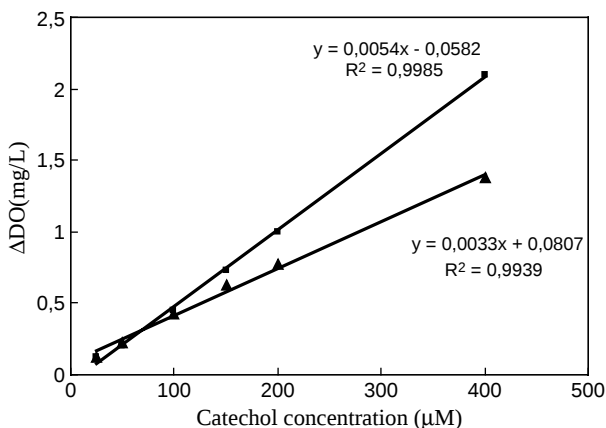


Figure 3. Calibration graphs of the biosensors for catechol [(■) imprinted PPO biosensor; (▲) PPO biosensor. Working conditions: Sodium phosphate buffer pH 8.5, 50 mM and 35°C].

Table 3. Repeatabilities of biosensors

	Average value(μ M)	n	S.D.(μ M)	C.V.(%)
Biosensor 1 ^a	192.5	10	± 9.42	4.9
Biosensor 2 ^b	198	10	± 5.76	2.9

The concentration of catechol used in the experiments was 200 μ M.

^aThe biosensor with unmodified.

^bThe biosensor modified with thiourea.

and variation coefficients (C.V.) are shown in Table 3. It can be concluded that the complexation of the polyphenol oxidase with thiourea was very affected by the biosensors in terms of their repeatabilities and analytical characteristics such as S.D., C.V., etc.

Operational Stabilities

In these studies several measurements were carried out by two biosensors. Figure 4 showed that the bio-imprinted biosensor was more stable than the other one through the long operational period.

At the 20th measurement, the bio-imprinted polyphenol oxidase biosensor kept 80% of its initial activity; however, the biosensor unmodified was carrying about 67% of its initial activity. This result also should support the effect explained in the thermal and pH stability section.

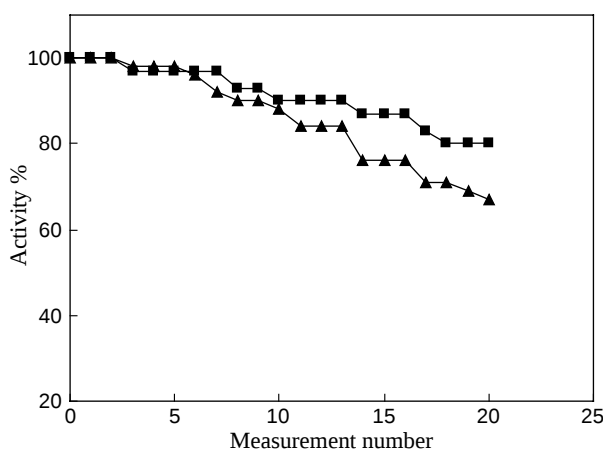


Figure 4. Operational stabilities [(■) imprinted PPO biosensor; (▲) PPO biosensor. Working conditions: Catechol concentration was injected to a final concentration of 200 μ M. Sodium phosphate buffer pH 8.5, 50 mM and 35°C].

Storage Stabilities of the Biosensors

The long-term performance of the biosensor was evaluated intermittently over a period of 15 days by monitoring its response to catechol standard solution. Both biosensors were stored in a flask containing some distilled water at 4°C when not in use. Water in the flask prevented drying of bioactive layer of biosensors. However, they were not in contact with distilled water. In this condition, after 3 days storage period, the modified biosensor just lost 2% of its initial activity; however, the biosensor unmodified lost about 19% of its initial activity. Besides, at the end of the 15th day, the remaining activities of the modified and unmodified biosensors were 70 and 56%, respectively. These results showed that storage stability of the unimprinted biosensor was not so good. The results also revealed that the bio-imprinted technique used in the presented study overcame the insufficient storage stability of the biosensor.

Substrate Specificities of the Biosensors

The final studies of this investigation were substrate specificity tests. The biosensors were applied to eight different phenolic compounds. The results are summarized in Table 4.

As seen in Table 4, both of the biosensors showed the highest activity for catechol. The pyrogallol activity of the imprinted biosensor was 7.7% higher than activity of the unimprinted biosensor. Likewise, pyrogallol activity was

Table 4. Substrate specificities of the biosensors

Phenolic compound	Activity(%)	
	Biosensor1 ^a	Biosensor2 ^b
Catechol	100	100
Pyrogallol	63.7	71.4
Caffeic acid	18.2	23.8
Phenol	9.1	14.3
Sinapinic acid	11.4	9.5
Ellagic acid	11.4	4.8
Naphtol	18.2	ND*
Ferulic acid	11.4	ND

Concentrations of substrates tested by biosensors were 200 µM.

Working conditions: sodium phosphate buffer 50 mM, pH 8.5, and $T = 35^{\circ}\text{C}$.

^aThe biosensor with unmodified.

^bThe biosensor modified with thiourea.

*Not determined.

followed by caffeic acid and phenol as 5.6% and 5.2%, respectively. Signals obtained for Sinapinic acid and ellagic acid of the unimprinted biosensor were higher than those of the bio-imprinted biosensor. Interestingly, the bio-imprinted biosensor showed no activity for naphthol and ferulic acid.

CONCLUSION

In this study, very successful application of the bio-imprinted technique for Bay leaf tissue homogenate for construction of a bio-imprinted biosensor was demonstrated. The biosensor based on Bay leaf tissue homogenate had the advantage of being simple, rapid to use, and inexpensive. The most important goal of the study was improvement of very important parameters of the biosensor. Detailed stability studies showed that the molecular bio-imprinted technique was a perfect way to enhance the stabilities of the biosensors. In conclusion, the technique should provide a significant, easy procedure for the preparation of a biosensor system with the desired stabilities for many classes of biologic substances.

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