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Inhibitive detection of benzoic acid using a novel phenols biosensor based on polyaniline–polyacrylonitrile composite matrix

Dan Shan [a,b], Qiaofang Shi [b], Daobin Zhu [b], Huaiguo Xue [a,b,*]

[a] *Key Laboratory of Environmental Materials & Environmental Engineering of Jiangsu Province, Yangzhou 225002, China*
[b] *School of Chemistry & Chemical Engineering, Yangzhou University, Yangzhou 225002, China*

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Abstract

A novel sensitive and stable phenols amperometric biosensor, based on polyaniline–polyacrylonitrile composite matrix, was applied for determination of benzoic acid. The electrochemical biosensor functioning was based on the inhibition effect of benzoic acid on the biocatalytic activity of the polyphenol oxidase (PPO) to its substrate (catechol) in 0.1 M phosphate buffer solution (pH 6.5). A potential value of −50 mV versus SCE, and a constant catechol concentration of 20 μM were selective to carry out the amperometric inhibition measurement. The kinetic parameters Michaelis-Menten constant (K_M^{app}) and maximum current (I_{max}) in the absence and in the presence of benzoic acid were also evaluated and the possible inhibition mechanism was deduced. The inhibiting action of benzoic acid on the polyphenol oxidase electrode was reversible and of the typical competitive type, with an apparent inhibition constant of 38 μM. This proposed biosensor detected levels of benzoic acid as low as 2×10^{-7} M in solution. In addition, the effects of temperature, pH value of solution on the inhibition and the interferences were investigated and discussed herein. Inhibition studies revealed that the proposed electrochemical biosensor was applicable for monitoring benzoic acid in real sample such as milk, yoghurt, sprite and cola.

Keywords: Benzoic acid; Inhibition; Composite biosensor; Polyphenol oxidase

1. Introduction

Benzoic acid is an extensively used preservative. It is generally effective to control mould and inhibit yeast growth, and against a wide range of bacterial attack [1,2]. Although this preservative prevents or delays nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf life, it is harmful at higher than permitted safety levels. The maximum permitted concentrations of benzoic acid in each type of food are controlled by legislation [3]. Therefore, the analytical determination of benzoic acid is not only important for quality assurance purposes but also for consumer interest and protection.

There are various traditional methods for the analysis of benzoic acid in foodstuffs, such as UV spectroscopy, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) [4], gas chromatography (GC), enzyme-linked immunosorbent assay (ELISA) and other immunochemical techniques. Nevertheless, these methods do not allow an easily continuous monitoring, because they are expensive, slow, need well trained operators and in some cases, require steps of extraction or sample pretreatment, increasing the time of analysis.

An alternative to ease the analysis is biosensors development. Biosensors are a sub-group of chemical sensors in which are analytical devices composed of a biological recognition element (such as enzyme, antibody, receptor or micro-organisms) coupled to a chemical or physical transducer (electrochemical, mass, optical and thermal) [5]. The amperometric biosensors measure the current procedued for the chemical reaction of an electroactive species to an applied potential, which is related to the concentration of the species in solution. Compared with optical or piezoelectric biosensors, amperometric biosensors have shown good analytical performance because the enzymatic react with their substrates and the facility to measure associated with high sensitivity.

Polyphenol oxidase (PPO) also called tyrosinase is a binuclear copper monooxygenase enzyme containing metalloprotein, it is widespread in nature throughout the phylogenetic

* Corresponding author at: School of Chemistry & Chemical Engineering, Yangzhou University, Yangzhou 225002, China. Fax: +86 514 7975244.
E-mail address: chhgxue@yzu.edu.cn (H. Xue).

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scale form bacteria to mammals, and takes part in a large number of biological reactions. PPO catalyzes the *o*-hydroxylation of monophenols to form *o*-diphenols (cresolate activity) and the oxidation of *o*-diphenols to *o*-aquinones (cathecolase activity), or both, at the expense of oxygen as an electron-acceptor [6]. PPO has a binding site with affinity of aromatic compounds (the substrate site) and another with affinity for a metal binding agent (oxygen site). Consequently, the activity of PPO is affected by a large variety of inhibitors [7]. Benzoic acid is one of the inhibitors of PPO.

Amperometric biosensor based on inhibition mechanism of PPO is a promising tool for the task of continuous on-site monitoring of benzoic acid, due to its attractive advantages, such as minimizing the sample pretreatment, reducing time of analysis, allowing for the continuous use of the same enzyme loading and displaying sufficient sensitivity and selectivity [8,9]. In the literature, there are several reports on inhibitive determination of benzoic acid by biosensors [10–14]. The evaluation of benzoic acid was realized by Reviejo et al. via reversed micelle enzyme inhibition biosensor based on tyrosinase [10]. A composite amperometric tyrosinase electrode was designed by Morales et al. for benzoic acid determination in mayonnaise and cola soft drinks [13]. A biosensor based on mushroom (*Agaricus bisporus*) tissue homogenate was developed for the investigation of benzoic acid [14]. However, the biosensor research is ultimately aimed at the development of inexpensive, reliable and simple devices suitable for rapid and sensitive analytical tests.

In our previous work, we had successfully immobilized PPO into the polyaniline–polyacrylonitrile composite matrix [15]. Polyacrylonitrile was synthesized by single rare-earth catalyst $Y(OAr)_3$ and could be easily fabricated into microporous film with the pore size [16]. PPO was in situ eletropolymerized with aniline onto polyacrylonitrile-coated platinum electrode. The resulting biosensor exhibited high operational and storage stability. About 96% initial activity had been retained after 100 measurements. The lifetime is more than 8 months with storage in a phosphate buffer at 4 °C, which is markedly longer than that (in general <3 months) reported in references [17–21]. This phenol biosensor was also satisfactory sensitive with the sensitivity of 2.03 A M^{-1} cm^{-2}. The high sensitivity and stability of biosensor open up new opportunities for purposeful modification of the sensitivity and selectivity of the determination of inhibitors [14].

In this context, we initially explored this highly sensitive and stable biosensor for monitoring benzoic acid. The determination of benzoic acid was carried out through its inhibitory effect on PPO. The influences of pH assay solution, temperature and the kinetic study of inhibition were described in detail.

2. Experimental

2.1. Materials

Polyphenol oxidase (PPO, EC1.14.18.1, from mushroom, 600 units mg^{-1}) was purchased from Amresco. Aniline was distilled before use. Polyacrylonitrile (PAN) (viscosity average molecular weight M_η is 28,000) was synthesized by the polymerization of acrylonitrile with single rare-earth catalyst, $Y(OAr)_3$ [16]. All other chemicals were of analytical grade. Catechol solutions in 0.1 M phosphate buffer solution (PBS) were daily prepared.

2.2. Apparatus

All electrochemical studies were performed with conventional three-electrode system. A saturated calomel electrode (SCE) and a Pt foil electrode were used as reference electrode and counter electrode, respectively. Another Pt foil (3 mm × 3 mm) was used as working electrode. The equipment consisted of a PAR model 173 potentiostat–galvanostat with a model 179 digital coulometer and a PC-I precise potentiostat, a YEW 3036 X-Y recorder and a YEW 3066 pen recorder. All potentials are referred to SCE.

2.3. Procedure

2.3.1. Biosensor preparation

PAN was dissolved in DMF and coated on a platinum electrode, and then was fabricated into porous membrane by phase inversion process. The enzyme electrode was prepared by electropolymerization of aniline in presence of PPO [15]. The electrolysis cell consisted of a PAN-coated Pt working electrode, a Pt counter electrode and a saturated calomel electrode. Polymerization was carried out in 0.1 M PBS (pH 6.5) containing 2 mg mL^{-1} PPO and 0.1 M aniline at a constant potential. During the electrochemical oxidation, aniline was in situ polymerized in PAN matrix and the enzyme co-entrapped into PAn–PAN composite matrix forming a PAn–PAN/PPO electrode. When the potential of the polymerization was controlled at 0.48 V (versus SCE) and the polyaniline content in the composite film was ca. 24%, the resulting biosensor exhibited the highest stability, sensitivity and the largest ratio of signal-to-noise. These parameters were selected for fabricating the enzyme electrode in the following experiments. The resulting enzyme electrodes were washed thoroughly with PBS, and then stored in 0.1 M PBS (pH 6.5) at 4 °C.

2.3.2. Measurement procedure

The inhibitory action of benzoic acid on the enzymatic activity of PPO was followed by consideration of the oxidation of catechol (substrate), catalyzed by PPO, to *o*-quinone, whose electrochemical reduction was monitored amperometrically at −50 mV. The addition of benzoic acid causes inhibition of enzyme, consequently decreasing the amount of liberated *o*-quinone. The procedure for the evaluation of the effect of the inhibition of enzyme activity as a function of benzoic acid concentrations on the biosensor response includes the following steps:

1. The biosensor was soaked in the phosphate buffer solution for 20–30 min until a stable baseline output signal was reached.
2. Determine the initial current response (I_1) of the biosensor to catechol solution of a given concentration.

3. Determine the current response (I_2) of the biosensor to catechol solution of the same concentration in the presence of a certain concentration inhibitor.

The degree of inhibition (Inhi %) is then calculated by comparing the current responses before and after addition of inhibitor according to the relation as generally reported [22]:

$$\text{Inhi}\,\% = \frac{I_1 - I_2}{I_1} \times 100$$

Each experiment was repeated at least three times and a relative standard deviation of the output signal was estimated to be not more than 5%. The control experiment for catechol without the inhibitor was performed after each inhibitor measurement.

3. Results and discussion

3.1. The reversibility of inhibition of benzoic acid to PPO

For an inhibitor biosensor, the amperometric response current of the enzyme electrode to substrate should be controlled by enzyme kinetics because the response is usually proportional to the activity of the immobilized enzyme. When the concentration of substrate is fixed, the detection signal only depends on activity of enzyme. Moreover, the activity of enzyme could be affected by a variety of inhibitors. As a consequence, the response current of enzyme electrode can be relative to the concentration of inhibitor. Benzoic acid as an inhibitor of PPO has long been established [8,9,13]. Thus, the determination of benzoic acid can be realized according to the inhibition degree of benzoic acid to PPO.

The inhibition of benzoic acid to polyphenol oxidase is reversible, and the biosensor can be reused after rinsing with PBS. The reversibility of inhibiting action was tested. When the potential was applied at −50 mV, the current response of the PAn/PAN–PPO bioelectrode to 20 μM catechol in PBS with pH 6.5 at 25 °C was 3.74 μA. The presence of 20 μM benzoic acid induced a strong inhibitory effect on the biosensor response and the response to 20 μM catechol decreased to 2.44 μA. Then, the PAn/PAN–PPO bioelectrode was taken out and rinsed with PBS for 1 min. The response current to catechol with the same concentration was finally investigated. The data indicated the biosensor response to 20 μM catechol retain 98–100% of original response. This behavior suggests that the inhibition of benzoic acid to PPO is reversible, which is in agreement to the previous report [11–13]. This makes it possible to use continuous measurement methodology for benzoic acid assay.

3.2. Effect of pH on the inhibition of benzoic acid

Enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined empirically. Therefore, the influence of pH on the response current of the sensor was carried out in this work. Curve b in Fig. 1A displays the influence of pH value of assay solution upon the amperometric response of PAn/PAN–PPO bioelectrode to 20 μM catechol in the presence of 20 μM benzoic acid. Over the pH range of 5–8, the maximum response current appears at pH 6.7. It is in a good concordance with the optimum pH value (6.5) of the free enzyme in solution without addition of inhibitor [23]. This indicates that inhibitor benzoic acid does not alter the optimum pH of PPO significantly. Compared with curve a in Fig. 1A (in the absence of inhibitor), the shape of curve b is similar to that of curve a in high pH range. While in low pH range, there is a great difference. We also calculated the inhibition degree of benzoic acid to PPO at the different pH values. The result was shown in curve c. With increment of pH value, the percentage of inhibition decreased. It implies that at acid condition benzoic acid exhibits higher inhibitive ability to PPO than that at base condition. According to the suggestion of Amine et al., it is best to choose a plateau region so that the pH should not have any effect on enzyme activity and will not interfere with the results obtained relative to the inhibition of the enzyme by the inhibitor [24]. Therefore, PBS at pH 6.5 was selected as the electrolyte in subsequent experiments.

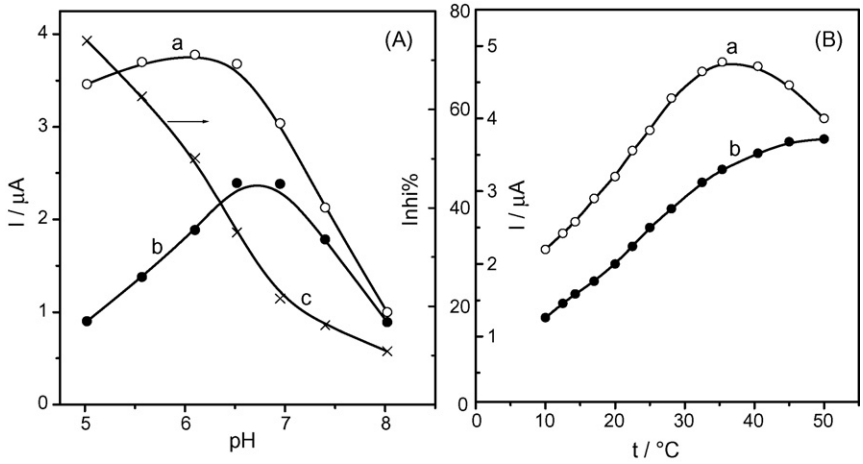


Fig. 1. (A) The effect of pH on the inhibition of PAn/PAN–PPO electrode. Response to 0.1 M PBS containing 20 μM catechol (a) and 20 μM catechol +20 μM benzoic acid (b); Inhi% (c). Applied potential: −50 mV, 25 °C. (B) The effect of temperature on the inhibition of PAn/PAN–PPO electrode. Response to 0.1 M PBS (pH 6.5) containing 20 μM catechol (a) and 20 μM catechol +20 μM benzoic acid; applied potential: −50 mV.

Table 1
The kinetic constants of PAn/PAN–PPO electrode in various concentration of inhibitor (benzoic acid)

[Benzoic acid] (μM)	K_M^{app} (μM)	I_{max} (μA)
0	219	45.7
20	339	45.8
40	462	45.6
60	577	46.3
100	851	46.0

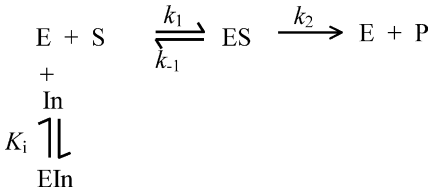
3.3. Effect of temperature on the inhibition of benzoic acid

The effect of temperature on the biosensor was one of the most important parameter that affected analytical performance of the biosensor. At the applied potential of −50 mV, the influence of temperature on the response currents of the PAn/PAN–PPO to 20 μM catechol solution containing 20 μM benzoic acid is shown in Fig. 1B (curve b). In the temperature range 10–50 °C, the response current of biosensor increased with the increment of temperature, no maximum current was obtained. While in the absence of benzoic acid (curve a in Fig. 1B), the maximum response current appears at about 35 °C. According to the Arrhenius equation, the $\ln i$ versus T^{-1} relationships in the temperature range of 10–28.1 °C, the apparent activation energies (E_a) are 30.5 and 26.2 kJ mol^{-1}, for the determination of catechol with and without inhibitor benzoic acid, respectively. In the presence of benzoic acid, the apparent activation energy of the biosensor is slight higher and the activity of immobilized PPO was inhibited, which might result in no maximum response in the temperature range of 10–50 °C. Although the difference response between curve a and curve b (Fig. 1B) obtained at 35 °C was about 15% larger than that obtained at 25 °C, to prolong the life of the biosensor and operate conventionally, a temperature of 25 °C was used throughout the experimental work.

3.4. Kinetic study and mechanism of the inhibitory

It is well known that the preparation procedure of a sensor affects the substrate as well as inhibitor kinetics of an enzyme. Thus, in this present work, the kinetic study of the inhibitory for benzoic acid to the PPO-based biosensor was also carried out.

The inhibition mechanism can be studied by examining the relationship between the response of the sensor to the inhibitor and to the substrate concentration. The calibration curves to catechol for the PPO-based bioelectrode, recorded in the absence and in the presence of the investigated inhibitors (Fig. 2A), were interpreted using the Linweaver-Burk plots (Fig. 2B). Finally, the parameters describing the inhibition process were estimated using I_{max} values and K_M^{app} values (Table 1). The maximum current has practically the same value (I_{max} = 45.8 μA), irrespective of inhibitors presence or absence, while the K_M^{app} value increased with increase in inhibitor concentration. It was concluded that benzoic acid exerted a competitive inhibition, located at PPO cresolase active site [25]. The possible mechanism of inhibitory effect could be deduced as follows:



Here, E, S, In, P, represent PPO enzyme, substrate catechol, inhibitor benzoic acid, and product *o*-quinone, respectively. Inhibitor structurally related to the substrate may be bound to the enzyme active center and compete with the substrate. In the works of Deng and Dong [11] and Morales et al. [13], the inhibitory effect of benzoic acid was also a competitive inhibition process.

Using the Lineweaver–Burk equation adapted for the response of an amperometric biosensor in the case of a competitive inhibition [26].

$$\frac{1}{I} = \frac{K_M^{app}}{I_{max}}\left(1 + \frac{[In]}{K_i}\right)\frac{1}{[S]} + \frac{1}{I_{max}}$$

where (1/*I*), stands for the reciprocal value of the steady-state response developed for substrate concentration [S] and inhibitor concentration [In]. According to the above equation, an apparent inhibitor binding constant (K_i = 38 μM) was determined for the biosensor by the Dixon plots from different catechol concentrations (Fig. 2C).

3.5. Analytical performance of the biosensor for benzoic acid

In the case of competitive inhibition, at high substrate concentrations, the inhibition effect is not observed since the substrate competes with the inhibitor [24]. Thus, in our work, the concentration of the substrate (catechol) was fixed at 20 μM. A typical inhibition calibration curve for the determination of benzoic acid is shown in Fig. 3. A maximum inhibition of about 70% was obtained for 100 μM benzoic acid. The linear range of benzoic acid concentration is obtained up to 20 μM with a slope of 1.67 (% μM^{-1}) and a correlation coefficient of 0.994 (n = 5). The detection limit is 0.2 μM (0.0244 mg L^{-1}), which is much lower that those obtained by the traditional determination method of HPLC (0.5 mg L^{-1}) [27] and GC (1 mg L^{-1}) [28]. $I_{0.5}$, i.e. the concentration of the inhibitor corresponding to 50% of the inhibition signal was calculated to be 40 μM from the data of Fig. 2. This value was much lower than the value determined for PPO in solution [29] and that obtained via screen-printed tyrosinase-containing electrode [30].

The reproducibility of the biosensor fabrication was evaluated via the comparison of the sensitivity of different electrodes. Seven different enzyme electrodes were tested independently for the determination of benzoic acid, providing a R.S.D. value of 6.2%. This indicates, in particular, an efficient and reproducible immobilization process of polyphenol oxidase in PAn/PAN composite matrix. Good reproducibility can be ascribed to the controllable electrochemical process of the sensor construction.

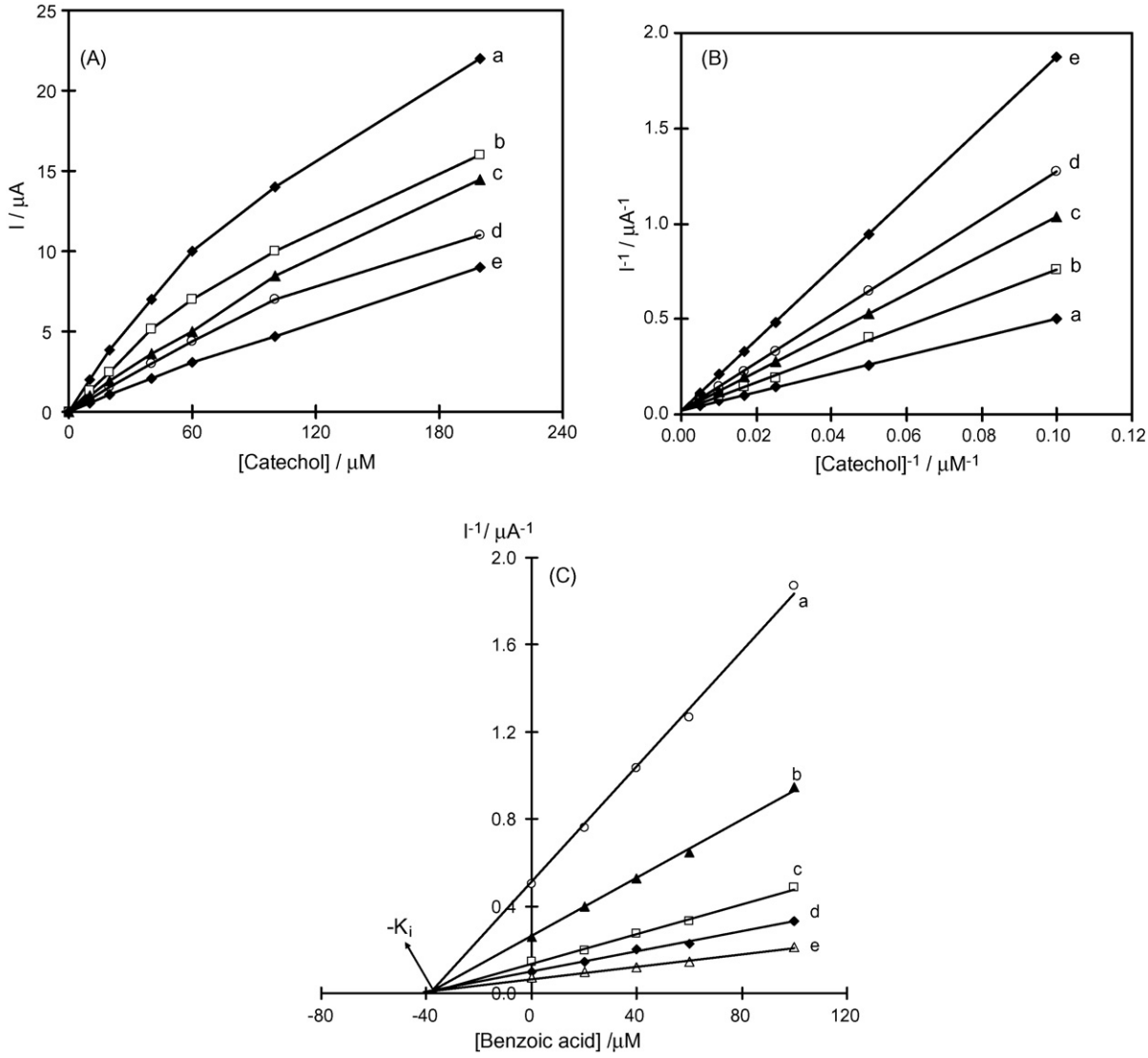


Fig. 2. (A) Calibration curves of PAn/PAN–PPO electrode for catechol in 0.1 M PBS (pH 6.5) containing 0 μM (a), 20 μM (b), 40 μM (c), 60 μM (d) and 100 μM benzoic acid (e). Conditions are as in Fig. 1. (B) Lineweaver–Burk plots for the catechol without (a) and with 20 μM (b), 40 μM (c), 60 μM (d) and 100 μM benzoic acid (e). (C) Dixon plots of the benzoic acid inhibition at PAn/PAN–PPO bioelectrode in the presence 10 μM (a), 20 μM (b), 40 μM (c), 60 μM (d) and 100 μM catechol.

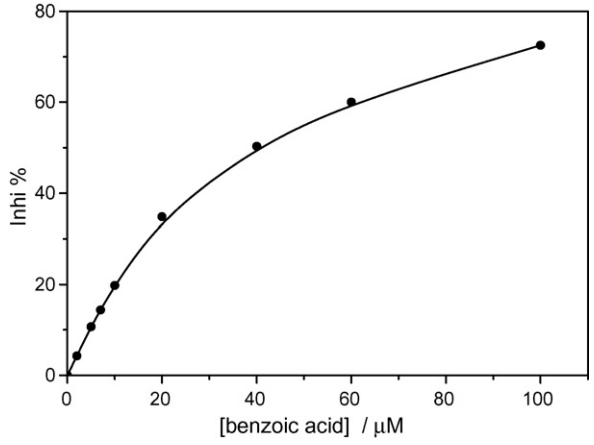


Fig. 3. Relationship between the inhibition percentage and the concentration of benzoic acid; determined in PBS (pH 6.5) containing 20 μM catechol at −50 mV, 25 °C.

Both operational stability and long-term storage stability are very important from the practical application point of view, so these two parameters were examined. The operational stability of the PAn–PAN/PPO electrode was investigated by consecutive measurements of its response to the mixture of 20 μM catechol and 20 μM benzoic acid. The activity of the PAn–PAN/PPO bioelectrode was hardly changed after 30 measurements. The long-term stability of the biosensor in storage was investigated by recording periodically its current response to the mixture of 20 μM catechol and 20 μM benzoic acid. When the enzyme electrode was stored in 0.1 M PBS (pH 6.52) at 4 °C and measured intermittently, the response to the mixture of 20 μM catechol and 20 μM benzoic acid remained unchanged for about 12 weeks. Good stability is probably due to that the enzyme was entrapped into the polymer and was protected by the polymer matrix, instead of adsorbed simply on the surface of PAn/PAN composite film [15].

Table 2
Determination of benzoic acid in some drinks by the proposed biosensor and comparison with HPLC method

Sample	HPLC method (mg/L)	Biosensor (mg/L)	Relative error (%)
Milk	12.1 ± 0.4	12.8 ± 0.1	+5.8
Yoghurt	14.6 ± 0.3	15.4 ± 0.2	+5.5
Sprite	109.4 ± 2.3	106.0 ± 0.7	−3.1
Cola	108.3 ± 2.7	110.8 ± 0.8	+2.3

3.6. Interferences

In order to demonstrate the selectivity of the biosensor, the potential interference from other substances which can be found in drinks on the determination of benzoic acid was checked. The effect of the interferents on the detection signal was checked using 20 μM catechol, 20 μM benzoic acid and 100 μM interferents. Under the experimental conditions, the influence of lactic acid, sorbic acid, citric acid, saccharin sodium, caffeine to the inhibition response of benzoic acid were acceptable, namely 1–4%, only ascorbic acid influenced on the benzoic acid response producing an increase in the signal of 11%.

3.7. Real sample detection

Four kinds of samples were assayed in order to demonstrate the practical usage of the biosensor. Milk and yoghurt was first centrifuged to remove insoluble residue. Two soft drinks sprite and cola were used without any treatment. The sample (0.5 mL) was added into 49.5 mL of PBS (pH 6.5) containing 20 μM catechol, and the inhibition response was obtained by performing the experiment procedure described in Section 2. The contents of benzoic acid can then be calculated from the calibration curve and were listed in Table 2. The results agreed well with those measured by an HPLC official method [31].

4. Conclusions

An inhibition biosensor for determination of benzoic acid, based on immobilized PPO into polyaniline–polyacrylonitrile composite matrix, has been developed. The kinetic interpretation of the amperometric response to catechol for the PPO-based bioelectrode, recorded in the absence and in the presence of benzoic acid, allowed identification of an inhibition process of a competitive type. The inhibition constant is 38 μM. The rapidity, sensitivity and easy handling operability of the method make it as a good alternative to traditional methods for benzoic acid analysis.

Acknowledgments

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References

[1] F.J.M. Mota, I.M.P.L.V.O. Ferreira, S.C. Cunha, M. Beatriz, P.P. Oliveira, Food Chem. 82 (2003) 469.
[2] S.A.V. Trouni, M.C.F. Toledo, Food Control 13 (2002) 117.
[3] European Parliament and Council Directive No. 95/2/EC, February 1995.
[4] C. Dong, Y. Mei, L. Chen, J. Chromatogr. A 1117 (2006) 109.
[5] L.D. Mello, L.T. Kubota, Food Chem. 77 (2002) 237.
[6] H.W. Duckworth, J.E. Coleman, J. Biol. Chem. 245 (1970) 1613.
[7] J.C. Vidal, S. Esteban, J. Gil, J.R. Castillo, Talanta 68 (2006) 791.
[8] S.E. Stanca, I.C. Popescu, J. Mol. Catal. B: Enzym. 27 (2004) 221.
[9] S.E. Stanca, I.C. Popescu, Bioelectrochemistry 64 (2004) 47.
[10] A.J. Reviejo, C. Fernandez, F. Liu, J.M. Pingarron, J. Wang, Anal. Chim. Acta 315 (1995) 93.
[11] Q. Deng, S.J. Dong, Analyst 121 (1996) 1979.
[12] K. Streffer, H. Kaatz, C.G. Bauer, A. Makower, T. Schulmeister, F.W. Scheller, M.G. Peter, U. Wollenberger, Anal. Chim. Acta 362 (1998) 81.
[13] M.D. Morales, S. Morante, A. Escarpa, M.C. Gonzalez, A.J. Reviejo, J.M. Pingarron, Talanta 57 (2002) 1189.
[14] S. Topçu, M.K. Sezgintürk, E. Dinçkaya, Biosens. Bioelectron. 20 (2004) 592.
[15] H. Xue, Z. Shen, Talanta 57 (2002) 289.
[16] H. Zheng, H. Xue, Y. Zhang, Z. Shen, Biosens. Bioelectron. 17 (2002) 541.
[17] H. Kotte, B. Gruendig, K.D. Vorlop, B. Strehlitz, U. Stottmeister, Anal. Chem. 67 (1995) 65.
[18] J. Li, L.S. Chia, N.K. Goh, S.N. Tan, Anal. Chim. Acta 362 (1998) 203.
[19] D. Shan, C. Mousty, S. Cosnier, S.L. Mu, J. Electroanal. Chem. 537 (2002) 103.
[20] D. Shan, S. Cosnier, C. Mousty, Anal. Chem. 75 (2003) 3872.
[21] D. Shan, C. Mousty, S. Cosnier, S.L. Mu, Electroanalysis 15 (2003) 1506.
[22] A.M. Tencaliec, S. Laschi, V. Magearu, M. Mascini, Talanta 69 (2006) 365.
[23] T.E. Barman, Enzyme Handbook, vol. 1, Springer-Verlag, New York, 1985, pp. 226.
[24] A. Amine, H. Mohammadi, L. Bourais, G. Palleschi, Biosens. Bioelectron. 21 (2006) 1405.
[25] S.E. Stanca, I.C. Popescu, L. Oniciu, Talanta 61 (2003) 501.
[26] R. Eisenthal, M.J. Danson, Enzyme Assays A Practical Approach, Oxford, New York, Tornoto, 1992, pp. 306.
[27] B. Saab, M.F. Bari, M.I. Saleh, K. Ahmad, M.K.M. Talib, J. Chromatogr. A 1073 (2005) 393.
[28] Z. Pan, L. Wang, W. Mo, C. Wang, W. Hu, J. Zhang, Anal. Chim. Acta 545 (2005) 218.
[29] V. Kahn, A. Andrawis, Phytochemistry 24 (1985) 905.
[30] J. Wang, V.B. Nascimento, S.A. Kane, K. Rogers, M.R. Smyth, L. Angnes, Talanta 43 (1996) 1903.
[31] National Standard of China, GB/T5009.29-2003, The Detection Method of Sorbic Acid and Benzoic Acid in Foodstuffs, National Standard Press, Beijing, 2003.