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Study of catalase electrode for organic peroxides assays

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Abstract

The catalytic activity of immobilized catalase (EC 1.11.1.6) for two model peroxide compounds (dibenzoyl peroxide and 3-chloroperoxibenzoic acid) in a non-aqueous medium was used to prepare an organic-phase enzyme electrode (OPEE). The enzyme was immobilized within a polymeric film on spectrographic graphite. The amperometric signal of the enzyme electrode in substrate solutions was found to be due to the reduction of oxygen generated in the enzyme layer. The electrode response is proportional to peroxide concentrations up to about 40 μM within the potential range from −450 to −650 mV (vs. Ag/AgCl), and the response time is at most 90 s. The enzyme electrode retains about 35% of its initial activity after a 3-week storage at room temperature.

Keywords: Immobilized catalase; Nafion; Graphite; Organic-phase enzyme electrode

1. Introduction

One of the most important problems concerning food control is the determination of peroxide compounds. As an alternative to conventional techniques used for peroxide analysis, which are time- and labor-consuming, various amperometric biosensors based on immobilized peroxidase [1–3] or catalase [4–6] were developed.

The ability of native catalase to decompose hydrogen peroxide into water and oxygen in an aqueous environment is well known. Moreover, immobilization of the enzyme in various materials increases its stability and thus opens the possibility of repetitive use. Amperometric biosensors based on immobilized catalase have proven to be useful analytical tools for the specific determination of either H_2O_2 [7] or some catalase inhibitors such as cyanides and fluorides [8]. Catalase co-immobilized with H_2O_2-producing or consuming oxidoreductases such as glucose oxidase [9,10], lactate oxidase [11], peroxidase [12], glutamate oxidase [13,14], L-lysine-alpha-oxidase [15] or choline oxidase [16] has also been used for this purpose.

The recently demonstrated ability of catalase to act as a biocatalyst also in non-aqueous media [17] has led to the development of organic-phase enzyme electrodes (OPEEs) for monitoring H_2O_2 in various anhydrous systems [6], or in water-saturated chloroform [5]. Due to the solvent-induced changes in the enzyme substrate specificity occurring under such conditions, it is in principle possible to determine water insoluble organic peroxides [4].

Either H_2O_2 [1,5,6] or other hydroperoxides [1–4,18] were used as model substrates for the majority of OPEEs described for the determination of peroxide compounds based on immobilized peroxidases or catalase. The study of the catalytic activity of immobilized catalase in an organic phase for more complex organic peroxides as model substrates provides the basis for expanding the area of compounds to be analyzed. Dibenzoyl peroxide and 3-chloroperoxibenzoic acid have a more complex molecular structure than H_2O_2. These compounds are not known as typical catalase substrates and their enzyme-catalyzed decomposition in organic media was rarely studied. Our recent investigations [19,20] showed that immobilized catalase is able to decompose these two organic peroxides in aprotic solvents such as acetonitrile and tetrachloromethane. The present paper then deals with the design of an enzyme electrode for the determination of both dibenzoyl peroxide and 3-chloroperoxybenzoic acid in acetonitrile, based on the

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catalytic activity of immobilized catalase for these organic peroxides.

2. Experimental

2.1. Reagents

Catalase (EC 1.11.1.6) was from *Penicillium chrysogenum* 245 (Biovet-Peshtera, Bulgaria). The specific activity of the enzyme was 1000 U/mg protein (1U = 1 μmol of substrate consumed or 1 μmol of product formed per minute). Buffer solutions were prepared with $Na_2HPO_4 \cdot 12H_2O$, KOH, H_3PO_4 and citric acid of analytical grade.

Acetonitrile for UV spectroscopy (Fluka) was used as reaction medium; dibenzoyl peroxide $(C_6H_5CO)O_2$ (BPO) and 3-chloroperoxybenzoic acid (3-CPBA), both of analytical grade, were purchased from Fluka.

The polymer (Nafion™ 117) was obtained from Fluka as a 5% solution in water–alcohols mixture. The polymer solution was neutralized with buffer solution (pH 7.0) and then diluted with bidistilled water before use.

2.2. Preparation of electrodes

A rod of spectrographic graphite RWI (purchased from Ringsdorff-Werke, Bonn-Bad Godesberg, Germany) with a diameter of 0.5–0.6 cm and pressed into a Teflon cylinder with a platinum current lead was used as working electrode. The electrode was first polished on fine emery paper P 400 and then on a filter paper. After polishing, it was thoroughly rinsed with bidistilled water and buffer solution, then dried before enzyme immobilization.

The enzyme electrode was prepared according to a procedure similar to that described by Wang et al. [4]. The electrode surface was coated with a 20-μl drop of a mixture of polymer (1.25% Nafion) and enzyme solution (200 μg catalase). The coating was allowed to dry at room temperature for 3 h. A similar polymeric coating, but without the enzyme, was used for control experiments.

2.3. Apparatus and measurements

The experimental system consisted of a bipotentiostat type BiPAD (TACUSSEL, Villeurbanne, France) and a digital voltmeter type 1AB105 (ZPU, Pravets, Bulgaria). The electrochemical measurements were carried out in potentiostatic regime using a three-electrode cell filled with acetonitrile. A silver–silver chloride electrode was used as a reference electrode and a platinum wire as counter electrode. The solution was purged with argon 20–30 min prior and during the measurements.

In order to obtain the steady state response of the electrode and its dependence on substrate concentration,

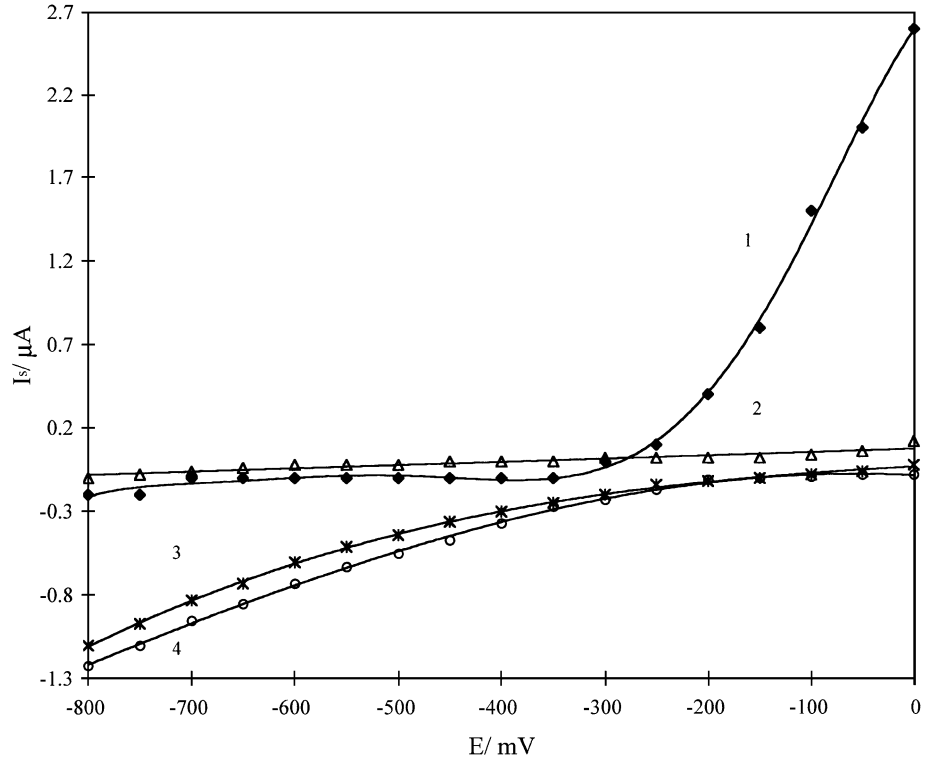

Fig. 1. Steady state current I_s as a function of electrode potential E vs. Ag/AgCl for graphite electrodes with polymer coating without catalase. The content of Nafion in the coating solution was 2.5% (1, ♦), 1.75% (2, △) and 1.25% (3, ∗); curve 4 (○) is for a graphite electrode without polymer coating. Solvent acetonitrile, temperature 25 °C.

an aliquot of a 10^{-3} M stock solution of a substrate in acetonitrile was added to 12 ml acetonitrile in the cell. The current of the electrode was monitored, and when it reached a constant value, the next aliquot of substrate stock solution was added. The time required until the current reached a steady state did not exceed 90 s after any of the additions.

A constant temperature in the cell was achieved by means of a thermostat UH (VEB MLW Prüfgeräte-Werk, Sitz Freital, Germany), and pH of buffer solutions was adjusted using a pH meter OP-208 (Radelkis, Budapest, Hungary).

3. Results and discussion

3.1. Optimization of electrode coating

Control experiments aiming at the optimization of the electrode signal were carried out by varying both the amount of Nafion and the enzyme content in the polymeric coating of the electrode. The polarization curves of graphite electrodes covered with polymeric films devoid of enzyme showed that the electrode background current increases as a function of the amount of Nafion in the coating (Fig. 1). An increase in the polymer concentration

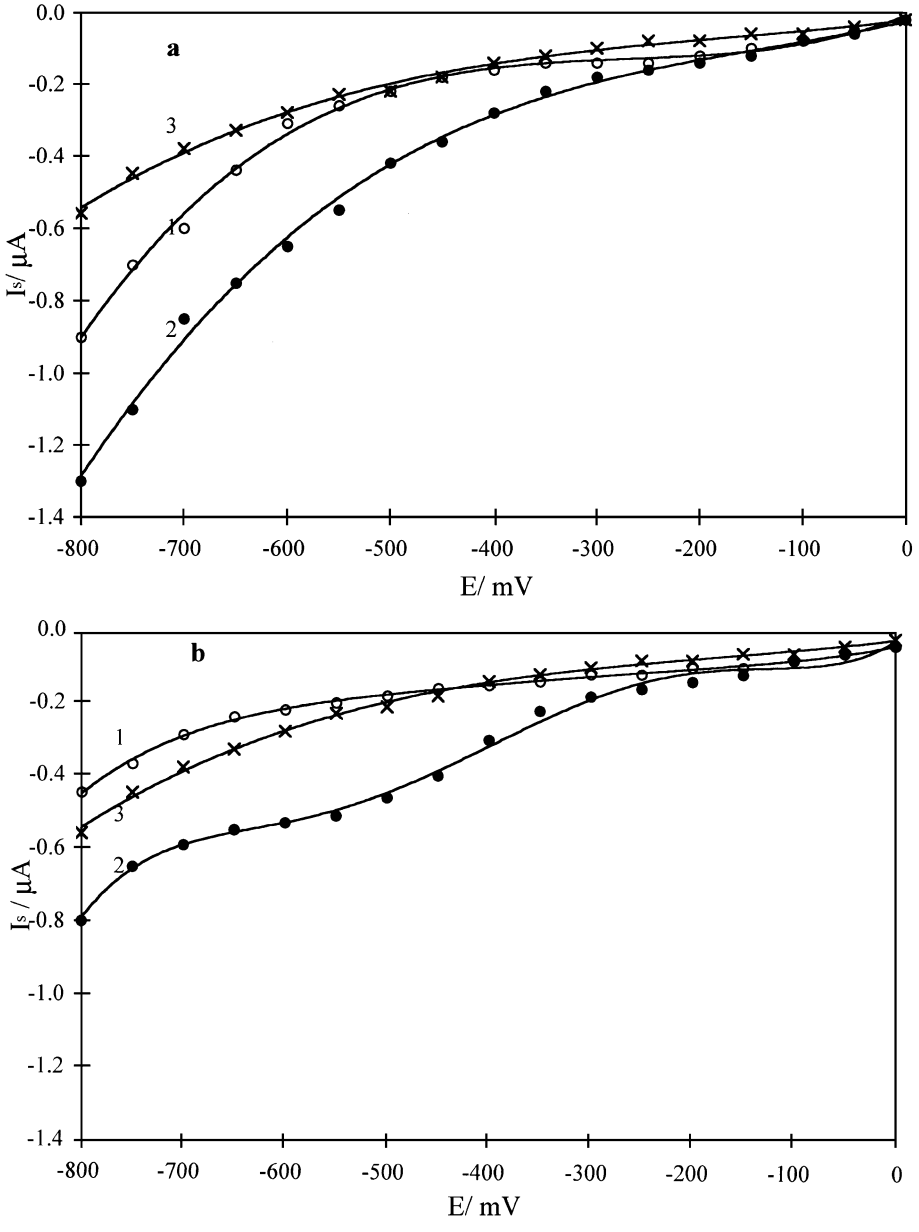

Fig. 2. Steady state current I_s as a function of electrode potential E vs. Ag/AgCl for different electrodes in acetonitrile. An electrode without enzyme (curves 1, ○) and an electrode with immobilized catalase (curves 2, ●) were immersed in solutions with 20 μM BPO (a) or 40 μM 3-CPBA (b). Curves 3 (×) indicate an enzyme electrode in acetonitrile without substrate; temperature 22 °C.

leads to a thickening of the membrane layer and probably causes surface non-uniformity, which results in an increased background current. On the other hand, electrode coatings with Nafion concentrations less than about 1% did not yield contiguous layers and hence are not suitable. Electrodes were therefore prepared with 1.25% Nafion, which yields the lowest background current with a still intact film.

As a rule, the electrode sensitivity is affected by the enzyme load [4]. It was found that the electrode response for both substrates increases as a function of the amount of immobilized catalase from 50 to 200 μg within the potential range from −200 to −700 mV. Amounts of enzyme larger than 200 μg did not yield higher electrode sensitivities but enhanced the noise level. A double-layer electrode coating (each layer containing 100 μg catalase) decreased the electrode sensitivity, which is probably due to a higher resistance for diffusion. Based on these results, an electrode coating with 200 μg of catalase and 1.25% Nafion was considered as optimal conditions and was used for the subsequent experiments.

3.2. Dependence of the electrode response on substrate concentration

Polarization curves of different electrodes in acetonitrile in the absence or presence of BPO and 3-CPBA are shown in Fig. 2a and b, respectively. In both cases, the background polarization curves of electrodes without enzyme in the presence of substrates (curves 1) are very similar to those of electrodes with enzyme in the absence of substrates (curves 3) in the potential range from −400 to −600 mV. This indicates that in this potential range, no electrochemical process occurs on the electrode covered with a polymeric film without enzyme despite of substrates being present. Considerably higher values of cathodic currents are detected for the electrodes with immobilized catalase in the presence of substrates (curves 2), and a biocatalytic effect is clearly demonstrated at potentials more negative than −400 mV. The oxygen liberated in the enzyme layer due to the biocatalytic activity of immobilized catalase is reduced electrochemically at the graphite electrode. Wang et al. [4] described a similar amperometric biosensing of *t*-butyl hydroperoxide in acetonitrile or acetone by means of oxygen reduction in the potential range from −350 to −500 mV.

In the range of working potentials from −450 to −650 mV, an extended range of proportionality between electrode response and concentration was found for both peroxides. With I_s and I_0 denoting the steady state current in the presence of substrate and the background current, respectively, these proportionalities can be expressed by the average relations $I_s - I_0 = (0.0116 \pm 0.0014)$ μA μM^{-1} C_{BPO} ($r^2 = 0.9954$) for BPO and $I_s - I_0 = (0.0115 \pm 0.0008)$ μA μM^{-1} $C_{3\text{-CPBA}}$ ($r^2 = 0.9957$) for 3-CPBA. Using a signal to noise ratio of 3:1, a detection limit of ~4 μM for dibenzoyl peroxide and of ~8 μM for 3-chloroperoxibenzoic acid can be determined at these working potentials. Applying working potentials more negative than −650 mV led to increased background currents and noise levels for both substrates.

An example of the dependence of electrode response on substrate concentration at a working potential of −600 mV is shown in Fig. 3a for BPO and in Fig. 3b for 3-CPBA. In both cases, the proportionality turns into a plateau with a sharp break occurring at about 40 μM for BPO and 45 μM for 3-CPBA. Such a behavior cannot be explained by a saturation of the enzyme but is most likely due to the fact that the solubility limit of the substrates in the polymeric film is reached. It thus appears that the rate of the multistep

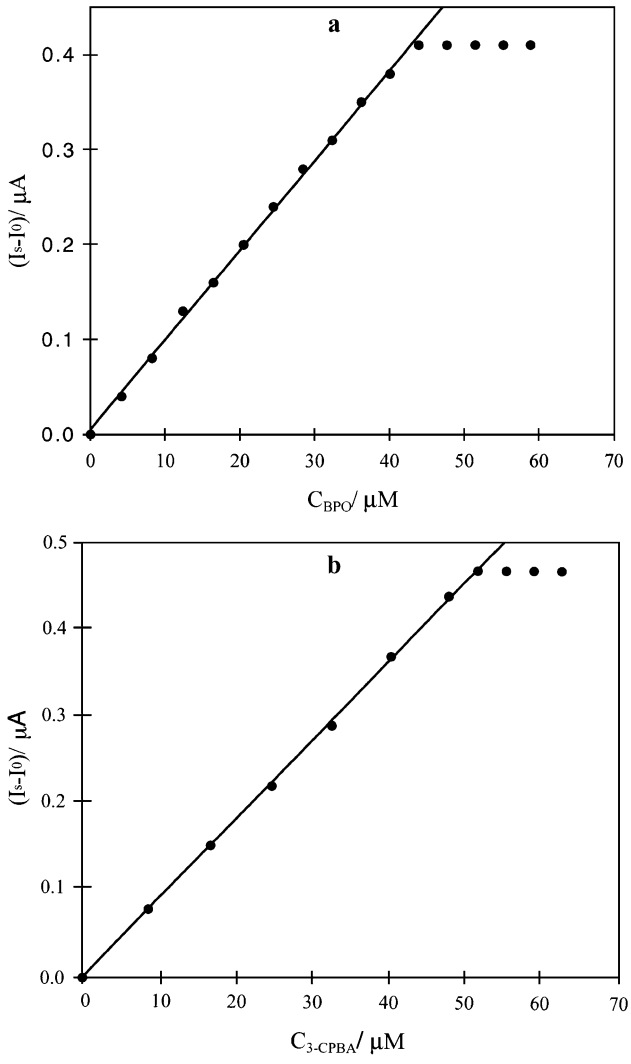

Fig. 3. Dependence of the response of a catalase electrode on substrate concentration. The difference between the steady state current I_s at a given substrate concentration and the background current I_0 in the absence of the substrate is plotted vs. the concentration of BPO (a) and 3-CPBA (b) in acetonitrile. The working potential E in (a) and (b) was −600 and −500 mV, respectively, measured vs. a Ag/AgCl reference electrode; temperature 22 °C.

enzymatic electrochemical process is probably controlled by substrate diffusion.

3.3. Dependence of electrode response on temperature

The effect of temperature on the electrode response was investigated for both substrates at a working potential of − 500 mV in the temperature range from 15 to 32 °C. The dependence of $I_s - I_0$ on substrate concentration remains proportional for the three temperatures studied, but the electrode sensitivity $\partial(I_s - I_0)/\partial C$ increases with increasing temperature (Fig. 4). This dependence was analyzed using the Arrhenius approach, i.e. $(I_s - I_0) \propto \exp[\Delta G_{Ar}^*/(RT)]$, where ΔG_{Ar}^* denotes the apparent activation energy, R

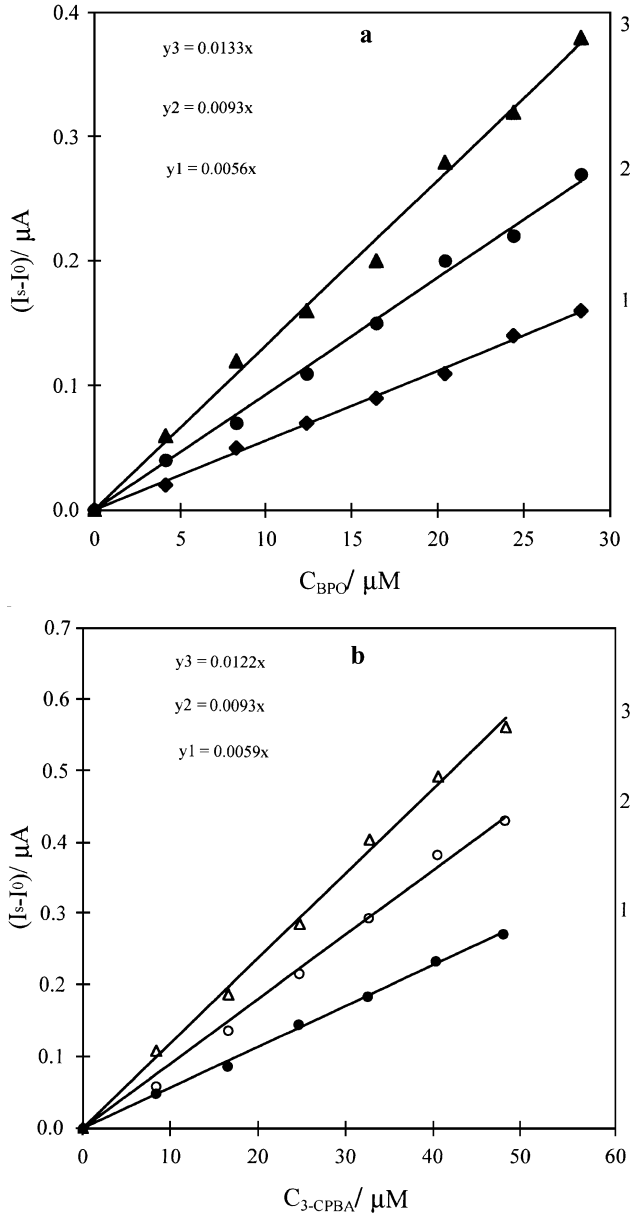

Fig. 4. Effect of temperature on the linear relation between electrode response and substrate concentration for BPO (a) and 3-CPBA (b). Conditions as given in the legend of Fig. 3 except for the temperature, which was 15 °C (1), 25 °C (2), 32 °C (3), and the working potential E, which was − 500 mV in both cases. Parameter values for the fitted straight lines are given in the insets.

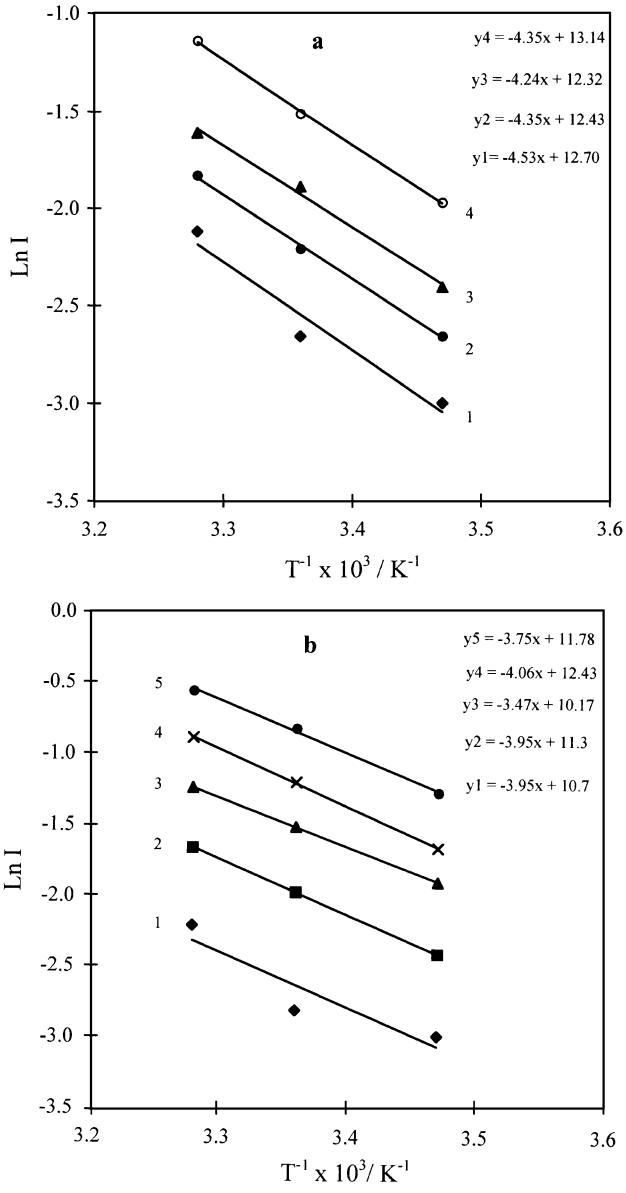

Fig. 5. Arrhenius plots, i.e. logarithm of electrode response vs. reciprocal absolute temperature for BPO (a) and 3-CPBA (b) at the following micromolar concentrations: 8.3 (1), 16.4 (2), 24.4 (3), 32.3 (4), and 47.6 (5). Working potential $E = -500$ mV vs. Ag/AgCl electrode. Parameter values for the fitted straight lines are given in the insets.

Table 1
Apparent activation energies ΔG_{Ar}^* of electrode response at various working potentials E and substrates (BPO or 3-CPBA) in acetonitrile

E (mV) vs. Ag/AgCl	ΔG_{Ar}^* (kJ mol^{-1})	
	BPO	3-CPBA
− 500	36.3 ± 1.5	32.0 ± 2.5
− 550	34.4 ± 1.5	34.5 ± 2.5
− 600	32.7 ± 1.5	33.5 ± 2.5

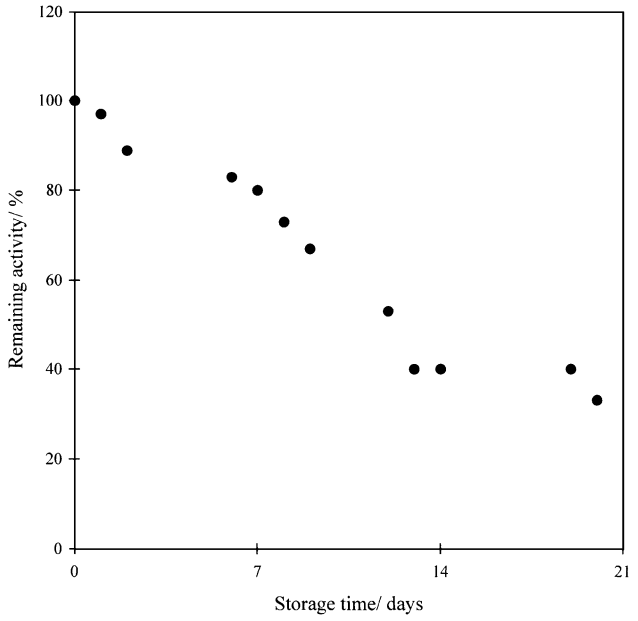

Fig. 6. Dependence of activity of catalase enzyme electrode on storage time. An electrode was stored at room temperature (dry state). The remaining activity was calculated as the ratio of $(I_s - I_0)$ determined with a constant substrate concentration of 25 μM (dibenzoyl peroxide dissolved in acetonitrile) after a given storage time and at the first day of life of the electrode.

the gas constant and T the absolute temperature. The corresponding Arrhenius plots for different substrate concentrations are shown in Fig. 5, and the average values for ΔG_{Ar}* calculated from the slopes of these plots are 36.3 ± 1.5 kJ mol^{-1} for BPO and 32.0 ± 2.5 kJ mol^{-1} for 3-CPBA, both in acetonitrile. These activation energies were found to be essentially independent of the electrode potential (Table 1). According to Berezin et al. [21], the activation energy of enzymatic heterogeneous reactions controlled by diffusion of substrates in solution (outer diffusion) does not exceed 15–20 kJ mol^{-1} but is usually characterized by higher values up to about 40 kJ mol^{-1} if the reactions are controlled by substrate diffusion in the electrode (inner diffusion). The values of ΔG_{Ar}* found for our electrode ($\sim$ 35 kJ mol^{-1}), as well as the fact that they do not depend on the electrode potential, support the above conclusion of a control by substrate diffusion and show that the diffusion of both substrates in the polymeric film is most likely rate-determining.

3.4. Stability of the enzyme electrode

Fig. 6 presents data on the activity of our enzyme electrode as a function of storage time. It is seen that after 7 days (approximately 10 working hours) and 3 weeks (about 24 working hours) of storage at room temperature, the electrode retains more than 80% and about 35% of its initial activity, respectively.

4. Conclusions

By means of two model substrates (dibenzoyl peroxide and 3-chloroperoxibnenzoic acid) dissolved in acetonitrile, it could be shown that an organic-phase enzyme electrode based on catalase immobilized in a polymeric film prepared with Nafion on spectrographic graphite is feasible. The electrode response, which arises from the electroreduction of oxygen generated in the enzyme layer due to peroxide decomposition, is proportional to substrate concentrations up to about 40 μM. The rate of the electrochemical process is most likely controlled by substrate diffusion in the polymeric film. The electrode is reasonably stable, retaining more than 80% and about 35% of its initial activity after 1 and 3 weeks of storage, respectively. It allows the determination of organic peroxides in aprotic solvents and thus may have relevance to the development of organic-phase amperometric biosensors for peroxide control in food samples.

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