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The Enzyme Source Effect on the Performance of a Catalase Organic Phase Enzyme Electrode

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Summary. The role of the enzyme source was studied in the reaction of hydrogen peroxide decomposition by immobilised catalase in acetonitrile. Enzymes isolated from bacterial and mammalian sources were conveniently immobilised on a spectroscopic graphite to obtain an organic-phase enzyme electrode (OPEE). Amperometry at constant potential was employed as basic analytical approach in this study.

Keywords. Enzymes; Heterogeneous catalysis; Biosensors; Immobilised catalase; Aprotic solvent.

Introduction

During the past decade non-aqueous enzymology attracted lots of current interest due to the importance of this phenomenon for the fine organic synthesis and for related fields of industrial chemistry. Despite the numerous studies of the effect of the organic solvent properties on the enzyme catalytic activity [1–5], other principal factors affecting the kinetic regularities of the enzyme-catalysed reactions under non-aqueous medium still remain unexplored.

It should be pointed out that each micro- or macro-organism produces the enzyme according to its own requirements, thus affecting important parameters as the molecular weight of biopolymer, its amino acids sequence, an enhanced or suppressed activity toward the substrate [6]. That is one of the main reasons for the differences noted in the kinetic parameters of one reaction catalysed by diverse biocatalysts, *e.g.* by microbial or by mammalian enzymes.

Since the impact of the source for enzyme purification on its catalytic activity is a problem rather poorly discussed in current literature, the present work deals with

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the effect of the enzyme source on the performance of a catalase electrode at hydrogen peroxide detection in acetonitrile.

Results and Discussions

The enzyme catalysed disproportionation of hydrogen peroxide leads to the formation of water and oxygen according to: $2HOOH \rightarrow 2HOH + O_2$.

Our earlier investigations [7] showed that the oxygen produced in analogous reactions with organic peroxides could be detected amperometrically in actonitrile and the resulting current is directly proportional to the concentration of the peroxide analyzed. It should be pointed out, however, that a similar determination in buffer solutions is not applicable due to the instability of the dioxide (1-) ion (O_2^- generated upon oxygen electrochemical reduction) in aqueous medium [8]. The removal of oxygen dissolved in acetonitrile prior to measurements was found to considerably increase the sensitivity of its amperometric detection.

The control experiments performed with an electrode without enzyme in the cover layer showed that an increasing hydrogen peroxide concentration does not cause electrochemical response on the electrode, *i.e.* the substance of interest, H_2O_2, was not reduced electrochemically in acetonitrile at potentials up to -400 mV (Fig. 1, curve 4′).

The steady-state current of a catalase enzyme electrode as a function of hydrogen peroxide concentration (Fig. 1) was explored within the range of potentials from -100 to -400 mV (*vs.* Ag | AgCl). The concentration dependence is linear up to H_2O_2 concentrations of $\sim 600\ \mu mol \cdot dm^{-3}$ and reaches a constant

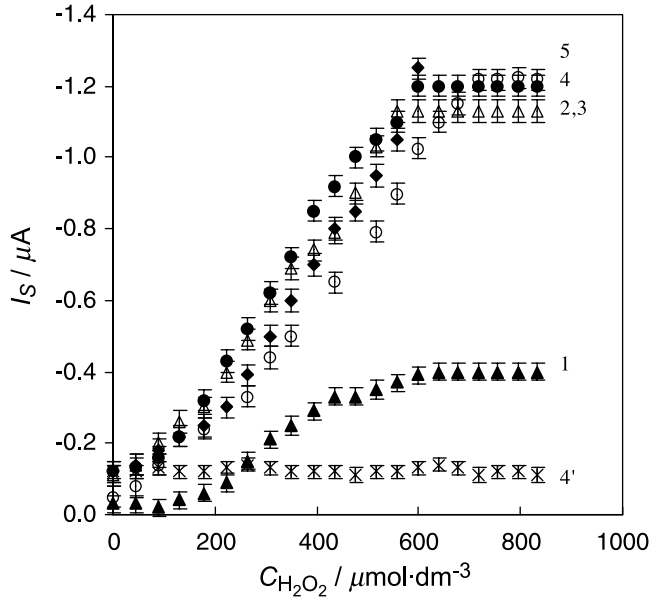

Fig. 1. Dependence of the enzyme electrode steady-state current on the hydrogen peroxide concentration at various applied potentials (*vs.* Ag | AgCl): 1) -100, 2) -200, 3) -300, 4) and 4′) -400, 5) -450 mV; curve 4′ was obtained with a polymer film without enzyme covered electrode; the symbols indicate the experimental results

Table 1. Electrode sensitivity $\{\frac{\partial(I_S-I_0)}{\partial C}/\mathrm{A}\cdot\mathrm{dm}^3\cdot\mathrm{mol}^{-1}\}$ and the correlation coefficient R^2 of the linear portion of the response dependence upon hydrogen peroxide concentration, determined with catalase OPEEs containing enzymes obtained from microbial and mammalian sources; acetonitrile was the background electrolyte, temperature $(22\pm2)^\circ$C

E/mV *vs.* Ag | AgCl	Mammalian enzyme		Microbial enzyme	
	$\frac{\partial(I_S-I_0)}{\partial C}\cdot10^4/\mathrm{A}\cdot\mathrm{dm}^3\cdot\mathrm{mol}^{-1}$	$R^2_{\text{aver.}}$	$\frac{\partial(I_S-I_0)}{\partial C}\cdot10^4/\mathrm{A}\cdot\mathrm{dm}^3\cdot\mathrm{mol}^{-1}$	$R^2_{\text{aver.}}$
−100	3.5 ± 1.5	0.98_9	7.0 ± 1.5	0.96_9
−200	6.3 ± 1.5	0.99_6	19.0 ± 1.5	0.99_7
−300	7.0 ± 2.5	0.99_4	18.0 ± 1.5	0.98_4
−400	9.0 ± 1.6	0.99_4	16.2 ± 3.2	0.97_9

value at above $700\,\mu\mathrm{mol}\cdot\mathrm{dm}^{-3}$, which is typically observed at enzyme saturation with substrate molecules. Within the range of working potentials from -200 to -400 mV (Fig. 1, curves 2–4) the detection minimum (determined at a signal to noise ratio 3:1) is $\geq 45\,\mu\mathrm{mol}\cdot\mathrm{dm}^{-3}$, while at a potential of $E=-100$ mV and under the same experimental conditions the detection limit to substrate concentrations exceeds $100\,\mu\mathrm{mol}\cdot\mathrm{dm}^{-3}$. The most probable explanation of this result is a much lower electrode sensitivity at -100 mV. For the range of working potentials from -200 to -400 mV the sensitivity was found practically constant. The electrode sensitivity can be defined as the slope $(\mathrm{A}\cdot\mathrm{dm}^3\cdot\mathrm{mol}^{-1})$ of the linear part of the electrode response upon substrate concentration, $\frac{\partial(I_S-I_0)}{\partial C}=const.\neq 0$.

When higher potentials, $E=-500$ mV or $E=-600$ mV, were applied the noise levels increased considerably and background currents were registered. Thus, the optimal range of working potentials of the so obtained enzyme electrode is between -200 and -400 mV.

The electrode sensitivity and the average coefficient of correlation of two identically prepared electrodes, containing mammalian and bacterial catalases, within the specified range of potentials are compared in Table 1.

The sensitivity of the electrode based on microbial catalase does not change with decreasing the working potential from -200 to -400 mV, while for the mammalian catalase based electrode the sensitivity fairly increases within the specified range of potentials. For both types of catalase electrodes $E=-100$ mV was considered inappropriate for work due to the low electrode sensitivity and relatively high detection minimum.

The temperature studies within the temperature interval 20–30°C showed practically constant electrode sensitivity for both catalase electrodes. At higher temperatures the dispersion of experimental points increases.

Changes of enzyme electrode response dependent on the storage time of the start solution for enzyme immobilization were noted during the present study. Partial denaturation of enzyme protein or co-factor removal from the apo-enzyme are considered as the most probable reasons for this phenomenon.

The effect of the start enzyme solution aging on the electrode response was noted for both catalases. It is depicted in Fig. 2 and the following common regularities were noted.

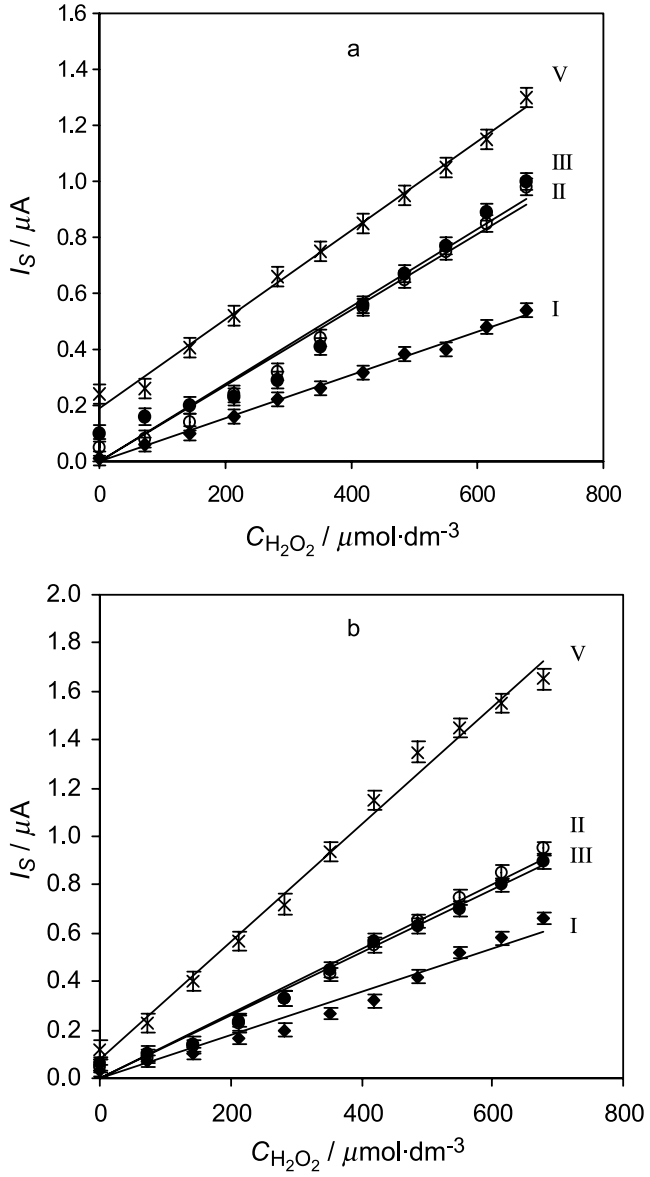

Fig. 2. Dependence of the steady-state response of the catalase electrode on hydrogen peroxide concentration: a) electrode prepared with the enzyme purified from bacterial source, b) electrode prepared with immobilized mammalian catalase; the storage time of the enzyme solutions for electrode preparation (in weeks) is indicated by Roman numbers close to each plot, as follows: I – freshly prepared solution, II – stored two weeks, III – stored three weeks, V – stored five weeks

1) With increasing the storage time both the background current and the steady-state response increase.
2) The electrode sensitivity practically is not affected by the aging of enzyme solution up to the third week of its storage.
3) A relatively higher electrode sensitivity was observed when using enzyme solutions for immobilization which were stored for 5 or more weeks.

The last finding is due to the removal of haemine from the enzyme active site. The catalatic activity of the prosthetic group itself must exceed considerably those of microbial or mammalian enzymes, due to negligible diffusional restrictions in the first case.

In support of this hypothesis are the UV-VIS spectra recorded of freshly prepared and long time stored enzyme solutions (not shown). The following changes in the *Soret* region were observed.

For the microbial enzyme the *Soret* band, registered for the freshly prepared enzyme solution at a characteristic wavelength $\lambda_{max} = 411\,nm$, appears as a broad shoulder around the specified wavelength at the spectrum of the aged enzyme solution.

For the mammalian enzyme the *Soret* band of the freshly prepared enzyme solution appears at $\lambda_{max} = 412\,nm$, while the characteristic wavelength of the corresponding region for the continuously stored catalase solution is shifted 5 nm to the red region (*i.e.* $\lambda_{max} = 417\,nm$).

Another difference is noted between the microbial and mammalian enzyme spectra. Whereas in both spectra of microbial enzyme solutions the absorbance registered at *Soret* band was practically the same, the extinctions in this region for the mammalian catalase solutions were found rather different.

Experimental

Reagents

Two types of catalase (EC 1.11.1.6) were used within the present study: i) microbial enzyme, purified from *Penicillium chrysogenum* 245 (Biovet-Peshtera, Bulgaria), and ii) mammalian enzyme purified from bovine liver (Fluka) with specific homogeneous activities of the native enzyme of $1000\,U/mg$ protein and $2114\,U/mg$ protein, respectively ($1\,U = 1\,\mu mol$ of substrate consumed or $1\,\mu mol$ of product formed per minute at 25°C and optimum *pH*).

Buffer solutions were prepared with $Na_2HPO_4 \cdot 12H_2O$, KOH, H_3PO_4, and citric acid all of analytical grade.

Acetonitrile for UV spectroscopy (Fluka) was used as reaction medium, 30% solution of hydrogen peroxide, analytical grade, was purchased from Fluka and used without further purification. The polymer (Nafion^{TM} 117) was obtained from Fluka as a 5% solution in water-alcohol mixture. Prior to use, the polymer solution was neutralized with buffer (*pH* 7.0) and then diluted with bi-distilled water.

Electrode Preparation

The enzyme electrode was prepared according to previously reported procedure [7]. The freshly polished on emery paper (P400), then smoothed on a filter paper to mirror-like lustre and thoroughly rinsed with bi-distilled water electrode surface was coated with a $20\,mm^3$ drop of a mixture of polymer (1.0% Nafion) and enzyme solution ($200\,\mu g$ 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.

Electrochemical Measurements and Apparatus

The electrochemical workstation was equipped with a bipotentiostat type BiPAD (TACUSSEL, Villeurbanne, France) and a digital voltmeter type 1AB105 (ZPU, Pravets, Bulgaria). The electrochemical measurements were carried out in the 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 to 30 min prior to and during the measurements.

In order to obtain the steady state response of the electrode and its dependence on substrate concentration an aliquot of a 1 mmol · dm^{-3} stock solution of the substrate (hydrogen peroxide) in acetonitrile was added to 12 cm^3 of 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.

All the data reported were obtained as average of at least 3 independent measurements.

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

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