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Interference of Ascorbic Acid in the Determination of Hydroquinone Using an Amperometric Enzyme Biosensor Based on Carbon Paste

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Abstract: The topic of this work deals with the interference of ascorbic acid in the determination of polyphenols with an amperometric biosensor containing tyrosinase. The sensor was prepared by covering carbon paste with a Nafion-film containing the enzyme. Hydroquinone (benzene-1,4-diol) is a polyphenolic compound which was used as a model one for amperometric measurements. Factors influencing the amperometric response were studied in detail. Optimized conditions for operating the biosensor in hydrodynamic amperometric mode were evaluated.

Keywords: Ascorbic acid; biosensor; carbon paste electrode; hydrodynamic amperometry; hydroquinone; polyphenols; tyrosinase

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Introduction

The first scientific article dealing with an enzyme biosensor was published by Leland Clark fifty years ago [1]. Research focusing on biosensors including modern biospecific analytical techniques developed very rapidly and played an extremely important role in the field of electrochemical analysis and other analytical techniques.

As regards applications of biosensors, it should be noted that there are analytical devices containing sensitive components of biological origin as receptors, which are part of or in close contact with physico-chemical transducers [2]. The biological entities can be enzymes (biocatalysts), immunoactive substances (antigen-specific antibodies), hormones, parts of cells (cell lysates or isolated organelles) or even whole cells, biomass, microorganisms (bacteria, yeasts) and nucleic acids (DNA or RNA). Usual transducers for biosensors are electrochemical (amperometric, potentiometric, coulometric and conductometric), optical, calorimetric, acoustic and piezoelectric signal converters. In most cases, the output signal is any electric entity such as current, voltage, or similar [3-7].

Applying amperometric biosensors, one is able to measure a current appearing at the working electrode due to an electrochemical conversion of the substrate with electron transfer, while the potential of the working electrode is held constant [8-9]. Fast response (depending on the enzyme kinetics), low detection limit (electroactive products or intermediates are generated directly at the electrode surface), good accuracy and dynamic range of the signal belong to typical characteristics of amperometric biosensors. However, the selectivity of an amperometric biosensor is not controlled by the specificity of the biocomponent only, because the operating potential due to electroactive species present in the sample matrix must be considered [10].

In the sensor studied in this work, the tyrosinase enzyme (from mushroom) was used as a biorecognition element. Tyrosinase is known as a metalloenzyme with two Cu(I) ions at the active site of its deoxygenated form. After interaction of this form with oxygen, a highly reactive chemical intermediate containing Cu(II) is formed being able to oxidize the substrate (see Fig. 1).

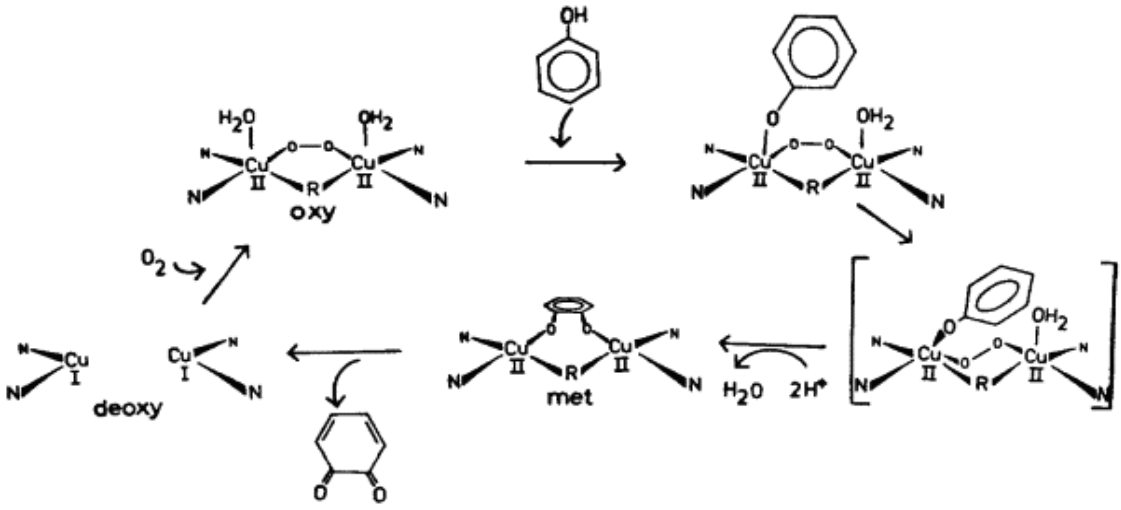

Fig. 1: *Proposed mechanism of phenol hydroxylation and oxidation to form o/quinones by tyrosinase*.

Though the exact way of action of the enzyme is still unknown, analogies between tyrosinase and other type 3 copper enzymes (a pair of copper ions each coordinated by 3 histidines) may be supposed. The activity of tyrosinase is similar to catechol oxidase, a related class of copper oxidases, laccase, and ascorbate oxidase (a suggested mechanism for monophenol oxidation/hydroxylation is shown in Fig. 2) [11-14].

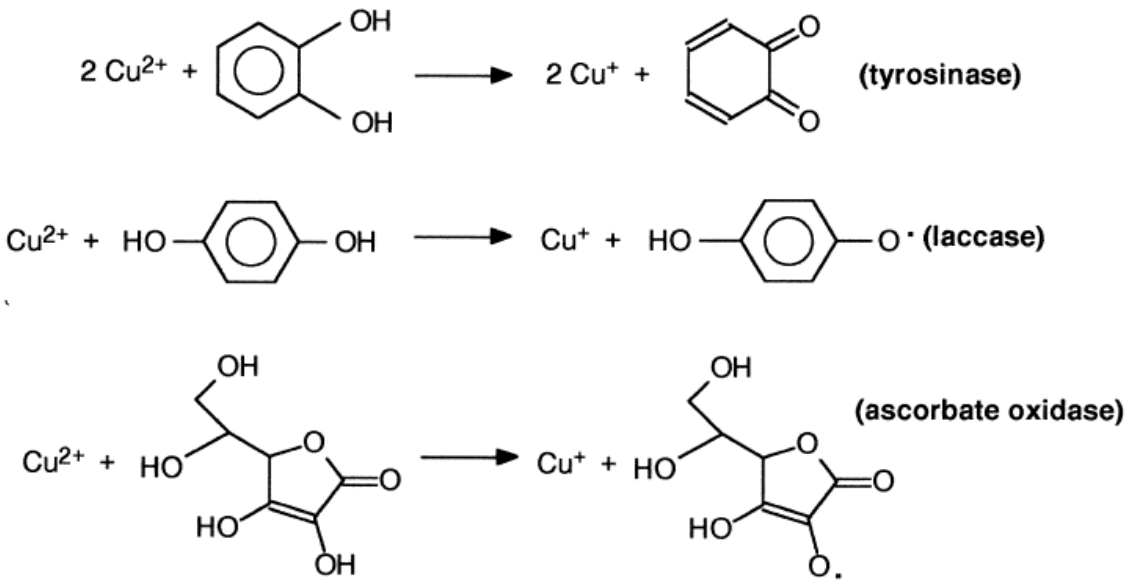

Fig. 2: *Direct oxidation by copper enzymes*.

Oxidations proceed over several steps with the involvement of Cu(II) being converted to Cu(I); in fact, oxygen is the final electron acceptor, which is in consequence reduced to water. Substrates of phenol oxidases can be mono- or polyphenols, where the former are primarily oxidized to *o*- or *p*-diphenols with ensuing further oxidation to the corresponding quinones. The latter are oxidized to quinones directly if they possess phenol groups in *ortho*- or *para*-positions, but undergo similar fate as the former if this position is free or blocked with other groups. Polymerization may follow up which is typical in plant and mushroom tissues with typical browning effects after lesion of the cells.

An amperometric biosensor used in this work is based on the carbon paste electrode [15] whose surface is covered by a Nafion®-membrane containing the tyrosinase enzyme. Hydroquinone (benzene-1,4-diol) was chosen as one of the simplest polyphenolic template substrate in the study presented here. The analyte is biocatalytically oxidized in the presence of oxygen to *p*-quinone, which is electrochemically active and can therefore be reduced back to hydroquinone.

L-ascorbic acid (vitamin C, AA) has also an antioxidant activity and can often be found in fruits, herbs, vegetables and products thereof [16]. It occurs commonly together with polyphenols in foodstuffs. In this respect, ascorbic acid can be understood as a part of the sample matrix and therefore, the study of its interference on the determination of phenolic antioxidant capacity is important. Table I brings usual contents of ascorbic acid and phenols in selected food samples, where concentrations of phenols are represented as mass equivalents of gallic acid (EGA), whereas equivalents of catechin (CAT) for flavonoids are given [17].

Table I: Reported contents of vitamin C, phenols and flavonoids in selected food samples

Food	**Vitamin C** (mg/100 g)	**Phenols** (mg EGA/100 g)	**Flavonoids** (mg CAT/100 g)
red wine (Moravian "Frankovka")	0.4	60.8 ± 3.8	4.7 ± 1.1
red wine (Moravian "Merlot")	2.6	72.0 ± 2.4	32.8 ± 0.9
apple	10.5	155.5 ± 6.8	61.3 ± 0.3
pear	2.5	83.0 ± 6.8	11.1 ± 0.6
plum	5.4	122.0 ± 8.0	25.1 ± 0.1
kiwi	99.0	101.0 ± 4.0	5.7 ± 0.1
lemon	41.4	19.7 ± 0.5	7.4 ± 0.9
orange	56.0	113.2 ± 2.3	15.5 ± 1.0
garlic	2.6	31.4 ± 1.7	5.6 ± 2.0
carrots	3.4	33.0 ± 3.0	11.5 ± 0.6
spinach	9.6	103.0 ± 1.3	61.0 ± 2.2
white cabbage	24.9	47.4 ± 1.3	3.2 ± 0.7

In this work, the influence of ascorbic acid on the determination of polyphenols (i.e., on the template compound *p*-hydroquinone) using a tyrosinase-based biosensor is studied and the principal results and observations reported.

Experimental

Chemicals and Reagents

Hydroquinone (benzene-1,4-diol), L-ascorbic acid, Nafion (5 % m:m solution in lower alcohols) and mushroom tyrosinase (E.C. 1.14.18.1, 4276 units / mg solid) were purchased from Sigma-Aldrich (Vienna, Austria). Carbon powder CR-2 (2 μm particle average) was obtained from Maziva Týn nad Vltavou (Czech Republic).

Paraffin oil (Uvasol) was from Merck (Darmstadt, Germany). Other chemicals and reagents were of analytical grade (Sigma-Aldrich). Highly pure water (18 MΩ resistivity) was prepared using a purification system Milli-Q (Millipore) and used throughout. A phosphate buffer solution (PBS, 0.1 mol/L, pH 7.0) was used as the supporting electrolyte.

Preparation of Biosensor

Carbon powder (0.5 g) and mineral oil (0.13 mL) were intensively mixed in a porcelain mortar for 30 min. The resultant carbon paste was pressed into a piston-like electrode holder [18], and the surface of the CPE was polished with a wet filter paper. Nafion® solution was dropwise neutralized with ammonia (1 mol/L) against indicator paper. Membrane solution was prepared by mixing neutralized Nafion solution (40 µL), aqueous enzyme solution (140 µL; 400 mg/L) and ethanol (60 µL, 50 %). An aliquot (10 µL) was applied to the electrode surface and left for one hour under laboratory conditions for drying. If not in use, the biosensors were stored dry in a refrigerator at a temperature of 5 °C.

Instrumentation

A conventional three-electrode cell was used for all experiments. The working compartment consisted of the carbon paste electrode (diameter 3 mm) with immobilized tyrosinase, a reference electrode (Ag/AgCl/3.0 M KCl) and an auxiliary electrode (platinum metal sheet, 32 mm^2). A miniature potentiostat (PalmSens, Ivium Technologies, The Netherlands) operated by the corresponding software (PSTrace, version 2.4.2.0) was used for electro-chemical measurements. All potentials discussed in this work are referred to the above mentioned reference electrode.

Methods

A phosphate buffer solution (10 mL, not de-aerated) was put into the electrochemical cell and the potential -0.2 V was applied. Optimizing the operating potential, a study was varied between -0.15 and -0.30 V. After attaining a constant base, line aliquots (usually 10 µL) of the hydroquinone solution (60 mmol/L in PBS) were added. During measurements, the solutions were stirred at 400 rpm. Differences between the sample response and the base line were evaluated.

Results and Discussion

p-Hydroquinone (HQ) is oxidized by tyrosinase to *p*-quinone (Fig.3). Unlike many other oxidases, tyrosinase does not generate hydrogen peroxide as an electroactive intermediate but the reduction of oxygen proceeds to water. Thus, detection of quinone, which is electroactive and can be reduced to HQ again, seems to be the most convenient way to monitor the enzymatic process.

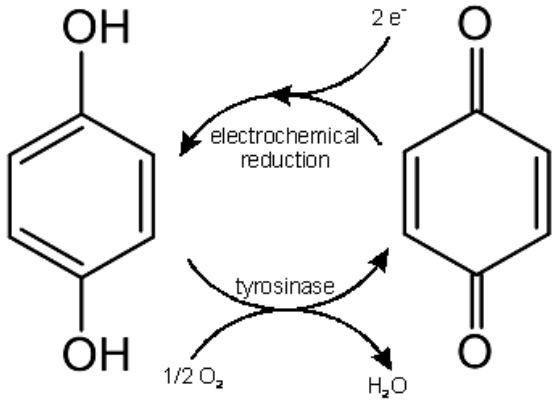

Fig. 3: *Detection of hydroquinone with the tyrosinase-modified CPE*

From cyclic voltammetric studies (not shown here), it was found that HQ gives a quasi-reversible redox signal at CPEs with the reduction occurring at more negative potentials yielding a maximum at around -0.2 V.

To optimize the experiment under hydrodynamic amperometric batch conditions, the potential was varied from -0.15 to -0.3 V; an optimum value of -0.24 V was found with respect to the signal height. At potentials below -0.2 V, however, the base line is deformed by the dissolved oxygen which is reduced to hydrogen peroxide (at -0.2 V, no interference of oxygen was observed). In fact, oxygen is an essential co-substrate for the enzymatic reaction.

Cyclic voltammetric investigations of ascorbic acid (not shown) revealed that under these experimental conditions, vitamin C is directly irreversibly oxidized to dehydroascorbic acid at more positive potentials, but does not cause a current in the negative potential range. For all these reasons an operation potential of -0.2 V was chosen as the best compromise. The influence of ascorbic acid on the amperometric determination of hydroquinone is shown below, in Fig. 4, overleaf.

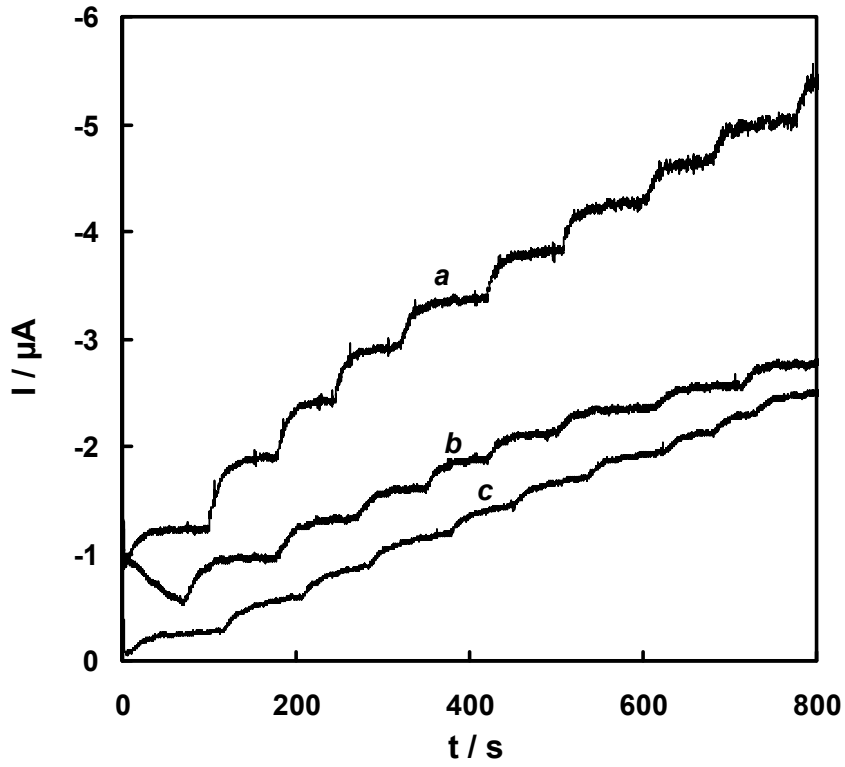

Fig. 4. *Influence of ascorbic acid on the determination of hydroquinone with a tyrosinase-modified CPE*; ascorbic acid concentration, (a) 0 µM, (b) 580 µM and (c) 1130 µM; additions, 60 µM hydroquinone per step; supporting electrolyte, PBS (0.1 M, pH 7.0); operating potential, -0.2 V, speed of stirring, 400 rpm; temperature, 25°C.

If the ascorbic acid concentration was less than that of hydroquinone, no interference could be observed (Fig. 4, curve a). With increasing concentration of ascorbic acid, the amperometric response of HQ decreased (curves b c). The influence of vitamin C expressed as its molar concentration ratio to the HQ analyte is summarized in Fig. 5. With increasing AA:HQ ratio, an almost linear decrease of the current response up to 80 % was observed. With higher ratios, the decrease remained somehow constant.

At a molar ratio of 1, the 3% current decrease was found (this corresponds to the experimental error). Thus, it may be concluded that the molar concentration ratio AA:HQ should not exceed 1 in samples which are analyzed with the tyrosinase-modified CPE.

The reason for the interference of AA with the determination of HQ seems obvious: vitamin C in higher concentrations reduces chemically the enzymatically generated quinone which is a concurring counter current reaction to the electrochemical detection process; as a consequence the reduction current is diminished.

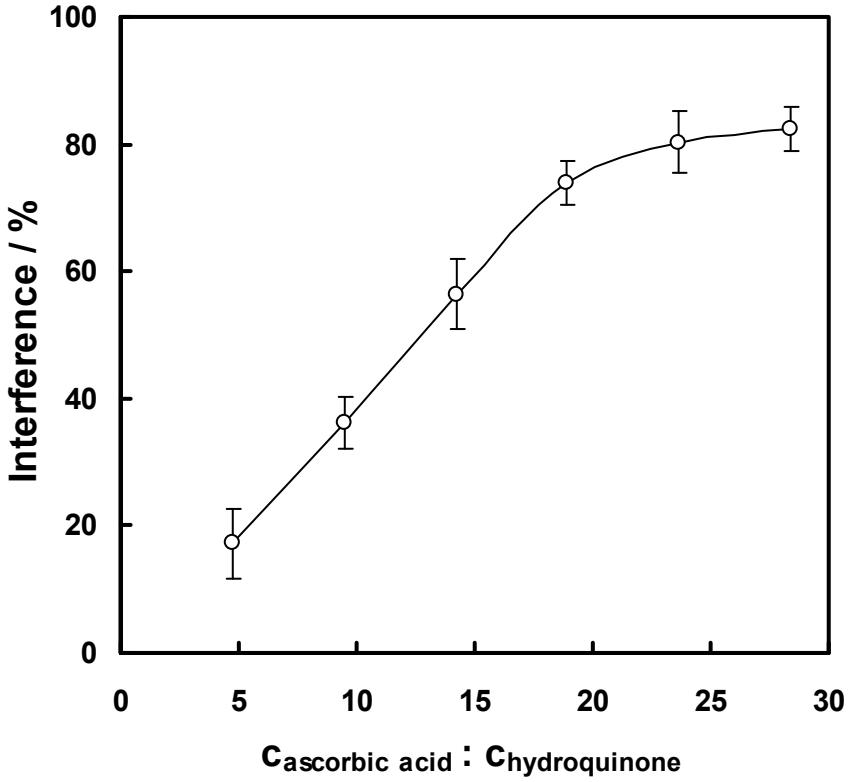

Fig. 5: *Influence of ascorbic acid on the amperometric determination of hydroquinone at various AA:HQ molar ratios*; hydroquinone concentration, 60 μM; supporting electrolyte, PBS (0.1 M, pH 7.0); operating potential, -0.2 V; speed of stirring, 400 rpm; temperature, 25°C.

Thus, the biosensor can be useful for analyzing samples such as red wine, coffee, chocolate, teas, or even extracts of fruits and herbs, where — as known — the content of vitamin C is quite low.

Comparisons of the amperometric tyrosinase biosensor with a photometric reference method (determination of Trolox equivalents) [19] revealed that the bioelectrochemical mode is more selective than the optical method (not shown).

Conclusions

As observed, interfering effect of ascorbic acid on the determination of hydroquinone applying the amperometric enzyme biosensor based on a tyrosinase-modified carbon paste electrode was practically negligible in cases when the molar concentration ratio of ascorbic acid to hydroquinone in model samples was equal to 1 or less.

Hence, the biosensor should be useful for the determination of the antioxidant capacity in samples in which the total antioxidant capacity (TAC) is mainly caused by phenols and where the concentration of ascorbic acid is not too high.

Acknowledgement

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