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IMMOBILIZATION OF ALCOHOL OXIDASE IN SIO_2 MATRIX PREPARED BY SOL-GEL METHOD

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ABSTRACT

The possibility of obtaining a biochemical sensor for colorimetric determination was studied. For this purpose, we have used the redox indicator 2,6- dichlorophenolindophenol (DCIP) and the binary enzymatic system composed by alcohol oxidase (AO) and peroxidase (PER). The binary enzymatic system immobilized in an inert SiO_2 matrix was obtained by modified sol-gel process. The chromogen and binary enzymatic system immobilization into a SiO_2 matrix was confirmed by I.R. spectroscopy. By immobilization of the enzymatic system, both the stability and enzymatic activity increase.

KEYWORDS sol-gel, immobilization, enzymes, alcohol oxidase, 2,6-dichlorophenolindophenol

RESUMO

Foi estudada a possibilidade de obter um sensor para determinações colorimétricas. Para isso foi usado indicador 2,6-diclorofenolindofenol (DCIP) e o sistema enzimático binário consistindo de alcool oxidase (AO) e peroxidase (PER). O sistema binário enzimático foi imobilizado numa matriz inerte de SiO_2 usando um método modificado sol-gel. A imobilização do cromógênio e do sistema enzimático binário foi confirmada através de espectroscopia no infravermelho. A imobilização do sistema enzimático leva a uma aumento de estabilidade e da atividade enzimática.

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1. Introduction

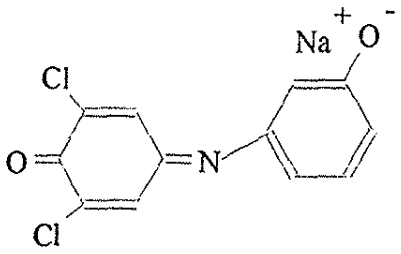
The studies of biochemical sensors based on immobilization of enzymes have lately shown a rapid development The biosensors enjoy a wide application and offer considerable advantages in industry, protection of the environment and medicine [1]. Among the advantages utilization of biosensors ensure in analytical chemistry one should mention the smaller amount of chemicals employed and more facile preparation of samples.

Different methods have been employed to obtain stable operational biosensors to be used in quantitative analysis of some chemical species such as utilization of enzymes originating in thermally stable bacteria or addition of stabilizing compounds to original enzymes. Studies have shown that addition of electrochemical compounds mixed with polyhydroxylic solutions to the enzyme confer them a high thermal stability in a dehydrated state as well as in solution. The mechanism of enzyme stabilization involves the formation of an electrostatic cage around the enzyme, resulting in an enzyme – polyelectrolyte complex which retains the enzyme in a more rigid configuration [2]. The most important characteristic of the biosensors is their selectivity. The preparation of a new biosensor usually aims to radically change the analytical characteristics increasing the ability to determine wider variety of compounds and also the selectivity in the detection of each specific compound. The main factors [3] which determine the selectivity of the biosensors are:

- the nature of the biomaterial;
- the method of biospecific preparation (the type of immobilization and purification, choice of inert matrix for immobilization, biomaterial treatment by specific effectors etc.);
- the characteristics of the analytical detector and the specialties of its combination with the biospecific preparation;
- the conditions of biospecific reactions unfolding (sample composition, temperature).

Operation of electrochemical biosensors requires a conjugation of biochemical and electrochemical reactions. The biological recognition element should be immobilized at the electrode surface. There are two principal ways to do such an immobilization: covalent linking and the entrapment into gel or polymer matrix [4]. Besides the electrochemical biosensors used for amperometric determinations, biosensors used for colorimetric determinations have been also obtained and studied. In both cases, an important feature is the enzyme immobilization in an inert matrix [5].

This contribution aims to obtain biosensors based on alcohol oxidase (AO), using 2,6-dichlorophenolindophenol (DCIP) as redox indicator.



Chemical structure of DCIP (2,6- dichlorophenolindophenol)

This investigation intends to determine the optimal conditions required for immobilization and stabilization of AO+PER type binary system, of redox indicator(DCIP) and finally the immobilization of the whole enzymatic system (alcohol oxidase (AO)+peroxidase (PER) + redox indicator (DCIP)) in a SiO_2 inert matrix prepared by the sol-gel method. The influence of the alkoxide, solvent, catalysis type and hydrolysis ratio upon the catalytic activity of the selected enzymatic system are also studied.

2.Experimental

The (AO + PER + DCIP) enzymatic system was selected to obtain a biosensor based on colorimetric determination to be employed in detecting alcohol traces.

The unfolding of the process involves the following steps:

- the redox indicator (DCIP) immobilization in a SiO_2 matrix prepared by a sol-gel method;
- immobilization of alcohol oxidase (AO) in inert SiO_2 matrix;
- immobilization of the whole (AO + PER + DCIP) enzymatic system in SiO_2 matrix prepared by the sol-gel method.

The sol was obtained from the following components: tetraethoxysilane alkoxide; 2-methyl – 1 – propanol (1-isobutanol) as a solvent. The basic catalysis employed NH_4OH as catalyst.

The conditions of inclusion and immobilization of the enzymatic system in the silica gel are listed in Tables 1 and 2. All reactions were carried out in alkaline catalyzed sols, at pH ranging between 8 and 9.

Incorporation and immobilization of the selected enzymatic system into the silica gel were monitored by IR spectroscopy.

The qualitative test employed to determine the activity of alcohol oxidase immobilized in SiO_2 gel was carried out as follows : 0.1 mL of concentrated peroxidase and chromogenic reagent (DCIP) and 0.03 mL of substrate (95%

ethanol), over 0.5 cm^2 surface area of SiO_2-containing AO was poured, as uniformly as possible.

In case when AO, as well as PER and DCIP were immobilized, the qualitative determination of the enzymatic activity was done by uniform drop wise addition of the substrate only on gel surface. In both cases, the enzymatic reaction was evaluated by visualizing the changes of the color DCIP redox indicator.

3. Results and Discussions

Immobilization of DCIP redox indicator

The preparation of a colorimetric biosensor requires utilization of a chromogen in the enzymatic system which has to fulfill two basic conditions: (i) colorimetric difference between the reduced and oxidized forms;(ii) low electrochemical potential which should be correlated with that of the enzymatic reaction.

In this contribution, we have used DCIP as chromogen indicator , since its redox potential (+ 0.23) is adequate for the alcohol oxidase--peroxidase binary enzymatic system. At the same time it is characterized by a net difference between the colors which correspond to both oxidation states: $DCIP_{red}$ (leucoform) and $DCIP_{ox}$ (blue form). This redox indicator may be employed in the enzymatic reaction in the reduced as well as in the oxidized state; in the latter the DCIP decomposition ($DCIP_{decom}$) reaction occurs(pale mauve form).

The low electrochemical potential of the redox indicator employed in the enzymatic reaction represents a draw back from the point of view of indicator stability. Thus, regardless of the chromogen form which is accessible in the enzymatic reaction, it is susceptible to chemical oxidation produced by the action of oxygen in solution or in the atmosphere this influences the biosensor's activity. Under these circumstances, a first step in obtaining an alcohol oxidase--based biochemical sensor with colorimetric determination requires the testing of the stability of DCIP immobilized in the inert silica matrix .

Comparison of the reduced stability of the both DCIP forms in aqueous solution at room temperature (maximum two days),shows that their immobilization in SiO_2 matrix increases significantly the stability to 60-70 days (Table 1).

Table 1. Experimental conditions of *2,6- dichlorophenolindophenol* (DCIP) immobilization in the sol-gel silica

No. Sol.	i-BuOH/TEOS	H_2O/TEOS	$DCIP_{OX}$/TEOS	$DCIP_{RED}$/TEOS	Time Activity (days)
1	2.5	6.25	$5.7 \cdot 10^{-6}$	$5.7 \cdot 10^{-6}$	60
2	2.5	6.25	$2.9 \cdot 10^{-6}$	$2.9 \cdot 10^{-6}$	60
3	7.5	1.25	$5.7\ 10^{-6}$	$5.7\ 10^{-6}$	70
4	7.5	1.25	$2.9\ 10^{-5}$	$2.9\ 10^{-5}$	70
5	5.0	2.5	$5.7\ 10^{-6}$	$5.7\ 10^{-6}$	65
6	6.2	3.12	$2.8\ 10^{-6}$	$2.8\ 10^{-6}$	65
7	-	1.53	$3.4\ 10^{-7}$	$3.4\ 10^{-7}$	70

*The ratios in the table are molar ratios

DCIP - 2,6- dichlorophenolindophenol
TEOS - tetraethoxysilane
i-BuOH – iso-butanol

Immobilization of Alcohol Oxidase

The obtention of the matrix combination by the sol-gel requires two important steps: transition from sol to gel and transition from gel to finite product (film, powder or ceramic).

Schematically, such a process consists in the following important steps:

$$\text{Precursor} \xrightarrow{\text{Hidrolysis}} \text{Reactive monomer} \xrightarrow{\text{Condensation}} \text{Sol} \xrightarrow{\text{Gelation}} \text{Gel}$$

Because sol-gel transition takes a relatively long time (10-12 days), compared to the lifetime of enzyme in aqueous solution at the room temperature, one resorts to utilization of a small quantity of water and to the delayed introduction of alcohol oxidase in the silica sol, such that the inactivation process is reduced or even eliminated.

The AO was added 10-11days after the start of the hydrolysis-polycondensation reaction and the gelation occurred in 25-30 minutes. If the enzyme is maintained for the shortest time in aqueous solution one avoids the denaturation and the loss of catalytic activity due to the operational instability of the enzyme in aqueous medium..

Methanol is the main substrate of the alcohol oxidase oxidation reaction, but the enzyme does not possess an absolute substrate specificity. It can also catalyze oxidation reactions of lower alcohols with a reduced number of carbon atoms and a linear chain, such as ethanol. Therefore utilization of this solvent to obtain SiO_2 sols was avoided ; ethanol was replaced by iso-butanol , a four carbon atom alcohol with branched chain, less accessible for the enzyme, but equally efficient from the viewpoint of silica sol preparation.

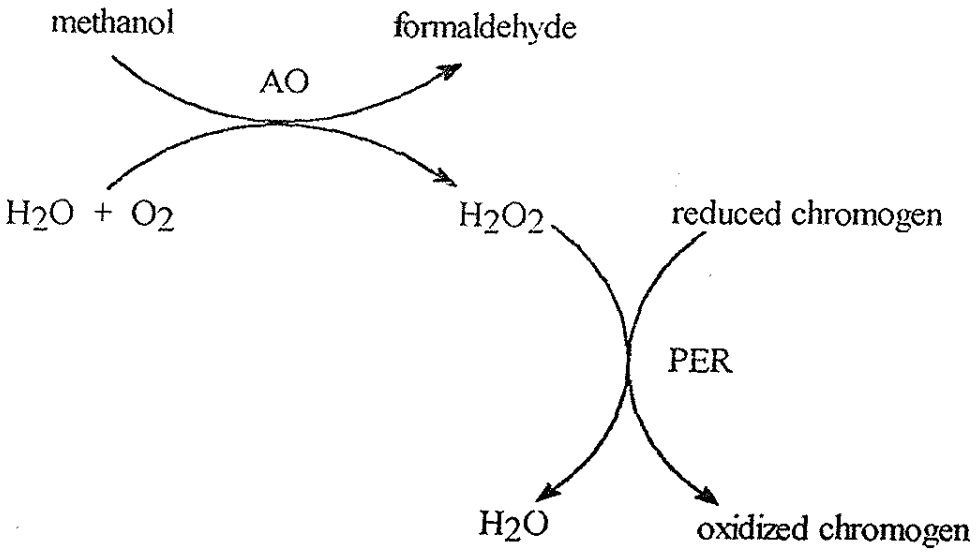
However, small amounts of ethanol resulted from the basic reaction used to obtain the silica matrix by hydrolysis reaction. The slowing down of the hydrolysis reaction by employing a small hydrolysis ratio, modification of the solvent nature and delaying of the introduction of the enzyme in the system offer the advantage of eliminating ethyl alcohol traces by evaporation.

Immobilization of AO + PER + DCIP enzymatic system

A biosensor was developed, which is utilized in colorimetric measurements employing alcohol oxidase as biochemical product. The colorimetric dosage of alcohol oxidase activity in aqueous medium is based on determining one of the products of methanol oxidation reaction, i.e. hydrogen peroxide, by coupling the first enzymatic reaction with the second enzymatic system: peroxidase-chromogen (DCIP). The hydrogen peroxide resulting from the main reaction forms a strongly oxidizing complex with peroxidase, which will oxidize the chromogen according to Scheme 1.

The immobilization and stabilization of the complex enzyme system consisting of the mixing all compounds that are the alcohol oxidase, the peroxidase and the redox indicator (2,6 dichlorophenolindophenol) was obtained under similar conditions with inclusion of simple alcohol oxidase into the silica matrix. The experimental conditions are listed in Table 2.

The immobilization of AO+PER enzymatic system and DCIP redox indicator in the silica matrix was evidenced by IR spectroscopy. Therefore the IR spectra of the pure SiO_2 gel and of samples SiO_2+PER and SiO_2+AO+DCIP were recorded in the 4000-400 cm^{-1} range.



Scheme 1. Diagram of alcohol oxidase activity determination

AO – alcohol oxidase; PER - peroxidase

Table 2. The experimental conditions for immobilization of alcohol oxidase (AO) + peroxidase (PER) + 2,6- dichlorophenolindophenol (DCIP) enzymatic system in the silica matrix obtained by sol-gel method.

No. sol	pH	AO (mU)	PER (U)	DCIP (μmoli)	Gelation time (minutes)	Time activity (days)
1	8	1	8	0,25	30	70
2	9	5	40	0,25	25	65
3	8	10	80	0,25	20	60

The bands assigned to Si-O vibration bonds in SiO_2 are present in all spectra within the wavelength number 400-500, 800-900 and 1000-1100 cm^{-1} as can be seen in Figure 1 [6].

The redox indicator DCIP presence is illustrated by the existence of the specific bands in the range 600-800 cm^{-1} (Figure 1c). The inclusion of the enzymes in the silica matrix is suggested by the splitting of the bands around 1600 cm^{-1} which are assigned to C=O and N-H enzymes vibrations . On the other hand, the strong band at 1370-1380 cm^{-1} noted in the spectra shown in Figures 1b and 1c was assigned to the enzyme carboxylate ion symmetric vibration of υ_{symCOO^-}.

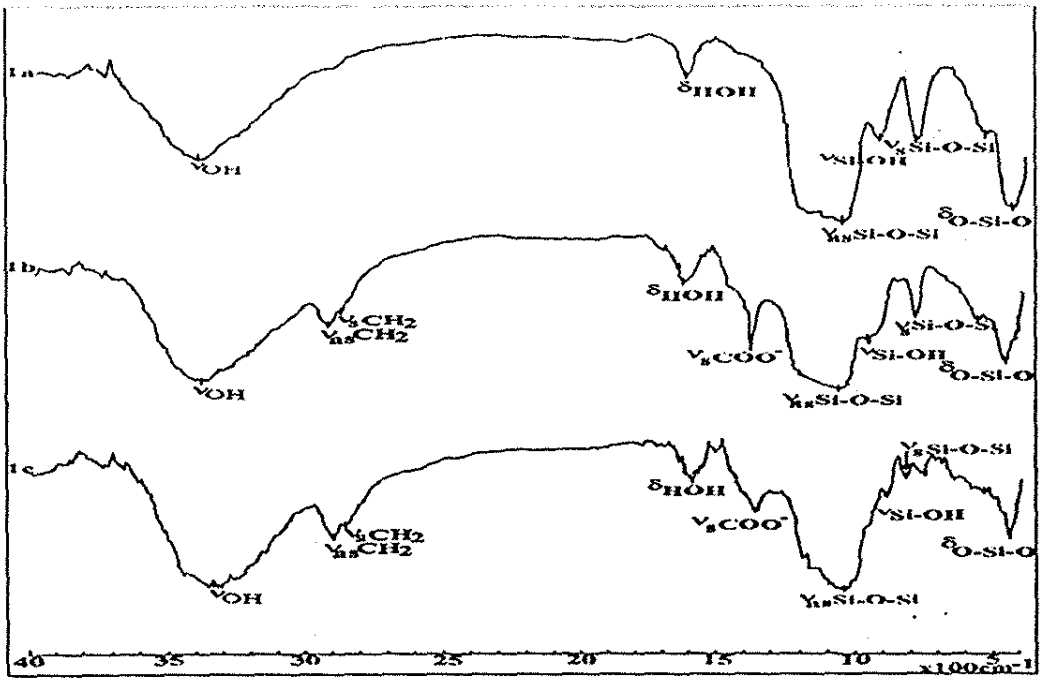


Figure 1. Infrared spectra of the pure SiO_2(a), SiO_2 doped with peroxidase(b) and SiO_2 doped with alcohol oxidase and 2,6- dichlorophenolindophenol (c)

The general aspect of higher splitting of the bands in the spectrum was assigned to the larger volume of the alcohol oxidase which induced perturbation in the molecular symmetry (Figure 1c).
The structure of SiO_2 gel is favored by peroxidase , a bulkier enzyme (Figure 1b). The band at 800 cm^{-1},assigned to symmetric vibration $\upsilon_{symSi\text{-}O\text{-}Si}$ is present in the spectrum 1b and it miss from the spectrum 1c.

The evaluation of IR spectra suggests that the enzymes (AO and PER) and the redox indicator (DCIP) were successfully immobilized in silica gel. Besides one may conclude that the inclusion and immobilization of peroxidase affects to a lesser extent the structure of the SiO_2 matrix.

Catalytic activity of the AO+PER+DCIP enzymatic system

Besides immobilization and stabilization of the selected enzymatic system, in an inert silica matrix prepared by sol-gel method, we attempted to uncover the conditions which preserve the catalytic activity of the enzyme. This is conditioned by the retaining of enzyme molecule quaternary structure. The activity of alcohol oxidase molecule, whose size is 12x12x12 nm, is ensured by the eight identical subunits (molecular weight amounts to 80000 Dalton per monomer); the subunits are bonded by 2-3 FAD (flavine adenine dinucleotide) molecules [7].

Immobilization and stabilization of alcohol oxidase in silica matrix prepared by sol-gel method was optimal from the viewpoint of catalytic activity which suggest that the oligomer structure of the enzyme remained unchange. The monomers unbounded to FAD are inactive from the enzymatic viewpoint. Retaining the catalytic activity of the alcohol oxidase was carried out by separate immobilizing of the alcohol oxidase as well as immobilizing the whole(AO+PER+DCIP). enzymatic system.

4. Conclusions

Immobilization has significantly increased the redox indicator, DCIP, time stability in reduced as well as in oxidized form compared to aqueous medium.

The sol-gel method of preparing the inert inorganic SiO_2 matrix was adapted to alcohol oxidase characteristics (stability in aqueous solution, substrate specificity) by establishing the hydrolysis ratio, the change of solvent and establishing the adequate time when the enzymatic system is introduced.

The immobilization of chromogen DCIP and of AO+PER enzymatic system in silica matrix was confirmed.

The possibility of immobilizing a AO+PER binary system into silica gel, retaining the catalytic activity of the enzyme was outlined;

The possibility to obtain, for the first time in our country, a biochemical sensor for colorimetric determination was studied. Use has been made of DCIP redox indicator and of the enzymatic binary AO+PER system immobilized in a SiO_2 inert matrix prepared by sol-gel matrix to detect traces of lower alcohol (methanol or ethanol).

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