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Biosensors with Immobilized Penicillin Amidohydrolase and Penicillinase for Determination of β-Lactam Antibiotics*

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Key words: biosensor, pH-membrane electrode, enzyme electrode, β-lactam antibiotics, penicillin amidohydrolase, penicillinase

pH-membrane electrodes with tridodecylamine as a hydrogen-ionophore were used for the construction of β-lactam antibiotics biosensors. The electrodes were enzymaticaly sensitized using penicillin amidohydrolase and penicillinase. Carboxylated poly(vinyl chloride) matrix of pH membranes allowed direct covalent binding of the enzyme. β-lactam antibiotics biosensors with extended life-time (over two months) were obtained. Both types of biosensors allow determination of β-lactam antibiotics within the concentration range from 0.1 to 30 mmol l^{-1}. The steady-state response times were 2.5 min and 1.5 min for the biosensors with immobilized penicillin amidohydrolase and penicillinase, respectively. The selectivity of both types of biosensors with respect to various β-lactam antibiotics is different.

Membranowe elektrody pehametryczne (z tridodecyloaminą jako jonoforem) zostały użyte do konstrukcji bioczujników do oznaczania antybiotyków β-laktamowych. Elektrody uczulano enzymatycznie przy użyciu amidohydrolazy penicylinowej oraz penicylinazy. Matryca membran wrażliwych na pH została wykonana z karboksylowanego poli(chlorku winylu), co umożliwiło kowalencyjne wiązanie cząsteczek enzymów bezpośrednio do jej powierzchni. Otrzymane bioczujniki charakteryzowały się dużą żywotnością (ponad dwa miesiące). Oba rodzaje bioczujników umożliwiają oznaczanie antybiotyków β-laktamowych w zakresie stężeń od 0.1 do 30 mmol l^{-1}. Czas odpowiedzi bioczujników uczulanych amidohydrolazą penicylinową wynosi 2.5 min, zaś uczulanego penicylinazą 1.5 min. Bioczujniki różniły się selektywnością względem wybranych antybiotyków β-laktamowych.

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Many types of potentiometric enzymatic sensors [1] can find applications in clinical and biochemical analysis and in the control of biotechnological processes. The penicillin biosensors [1–5] can serve here as an example. They may be used in analysis of β-lactam antibiotics, *i.e.* penicillins and cephalosporins. The enzymatic methods of determination of these antibiotics can employ two enzymes. Hydrolysis of these antibiotics is catalyzed by penicillin amidohydrolase (EC 3.5.1.11) or by penicillinase (EC 3.5.2.6) according to the scheme in Figure 1. Both enzymatically catalysed reactions produce acids. Therefore, the determination of the substrates of these reactions is possible using enzymatically sensitized pH electrodes.

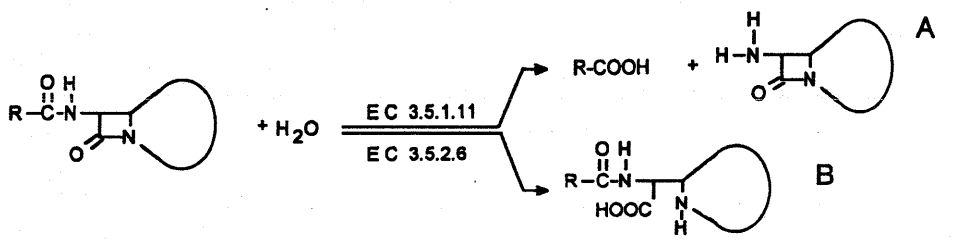

Figure 1. Scheme of the hydrolysis reaction of β-lactam antybiotics catalysed by penicillin amidohydrolase (a), penicillinase (b)

The aim of this work was to obtain β-lactam biosensor based on pH-membrane electrodes with tridodecyloamine (TDDA) as hydrogen ionophore. The enzyme immobilization procedure was the same as thet used for preparation of urea biosensors [6]. Recently we have immobilized penicillinase using this procedure and its modification [5]. In this paper we present a biosensor with penicillin amidohydrolase immobilized using this method. Both kind of investigated penicillin biosensors (sensitized with penicillin amidohydrolase and penicillinase) were compared. The utility of the tested biosensors for determination of some β-lactam antibiotics available on the Polish pharmaceutical market was checked. Both types of biosensors were compared with respect to the β-lactam antibiotics response.

EXPERIMENTAL

Apparatus and reagents

A digital pH meter, Model PHM 85, made by Radiometer, Denmark, was used throughout this work. Ion selective electrodes bodies, Philips, model IS 561, Moller Glasblaserei, Switzerland, were used for the construction of the membrane electrodes. A saturated calomel electrode, model OP 0820P made by Radelkis, Hungary, was used as the reference electrode.

The following enzyme were used: penicillin amidohydrolase (EC 3.5.1.11) from *Escherichia coli*, and penicillinase (penicillin amido-β-lactam hydrolase, EC 3.5.2.6), type I, from *Bacillus cereus*. The antibiotics: penicillin G (potassium salt), penicillinV (potassium salt), carbenicillin (sodium salt), ampicillin (sodium salt), cephatoxime (sodium salt), and cephradine (sodium salt) were obtained from Polfa, Poland. Amoxicilline (sodium salt) was purchesed from Belmac, France, cephapirin (sodium salt) from Bristol, France, and cefazolin (sodium salt) from Allarol, France. The materials used in membrane preparations were: bis-(2-ethylhexyl)sebacate (DOS) from Fluka, tridodecylamine (TDDA) (Fluka),

PVC-COOH (Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma). Buffer solutions were prepared from analytical grade reagents. Double distilled water was used for all experiments. Tetrahydrofuran (THF) was purified by distillation. Solutions of antibiotics were prepared directly before use.

Preparation of biosensors

The membranes of pH electrodes were prepared according to the procedure reported earlier [5,6]. The composition of pH membranes was as follows: 1% TDDA, 33% PVC-COOH, and 66% DOS. Both enzyme, penicillin amidohydrolase and penicillinase, were immobilized on pH membranes in the same manner. 50 μl of the solution containing 4 mg of carbodiimide (EDC) and penicillinase or penicillin amidohydrolase (activity 1.5 U/μl) was placed on the surface of the PVC-COOH membrane electrode and left for 12 h. Before use the biosensors were washed in vigorously stirred phosphate buffer solution (pH 7.00, C_B = 5 mmol l^{-1}, I = 0.1 mol l^{-1} NaCl) for 2 h to remove an excess of unbound enzyme.

Measurements

The standard procedure of calibration (the method of multiple addition of analyte) was described before [5,6]. The calibrations were preformed in various buffer solutions to investigate the influence of pH and buffer concentration on the response of the biosensors, and were repeated every day to examine the life-time of the biosensors.

RESULTS AND DISCUSSION

Analytical parameters of the pH electrode made of carboxylated polyvinyl chloride with tridodecylamine appear to be useful in the construction of biosensors [5,6]. The electrodes show linear response with nearly Nernstian slope (55 mV/pH) in pH range from 4 to 10 (this range covers pH changes caused by the enzymatic reactions). There is no interference from the alkaline cations and the response time is short (30 s).

The presence of carboxylic groups on the surface of pH membranes allows covalent binding of the enzyme molecules directly to the surface of the pH sensor. Carbodiimide activates the carboxylic groups and in such a way allows to form amide bonds between these groups and the amine groups of the enzymes. This method of immobilization of enzymes is very effective. The life time of biosensors was more than two months. After this period the initial signal decreased about 30% for both types of biosensors. The stable steady-state signal was obtained after 2.5 min for the biosensor with immobilized penicillin amidohydrolase, and after 1.5 min in the case of biosensor sensitized with penicilinase. The response times were short since the enzymatic layer was thin.

Typical calibration curves for both types of investigated biosensors are presented in Figure 2. The basic characteristics for both types of biosensors are similar. The sigmoidal shape of the calibration curves, and the influence of buffer pH, concentration, and the stirring rate on the shape of these curves are in a good agreement with the theoretical predictions derived from the kinetic model of the pH-based enzymatic sensors [7]. All these effects were described in detail earlier on example of the penicillinase sensitized biosensor [5].

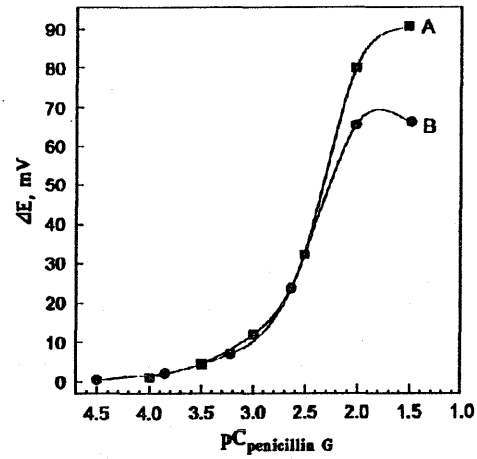

Figure 2. Calibration graphs of biosensors with penicillin amidohydrolase (a) and penicillinase (b). Measurements were performed in 5.0 mmol l^{-1} phosphate buffer, pH=7.00

The fundamental differences in the performance of both types of investigated biosensors are connected with different substrate and reaction selectivity of the two enzymes used. The results of the primary studies of the selectivity of both kinds of biosensors in respect to some selected β-lactam antibiotics are illustrated in Figure 3. Part (a) of this figure presents the data obtained for five different penicillins, whereas part (b) shows the data for four cephalosporins. For all cases the obtained analytical signals are presented as the precentage of the signal obtained for penicillin G at the same concentration (30 mmol l^{-1}).

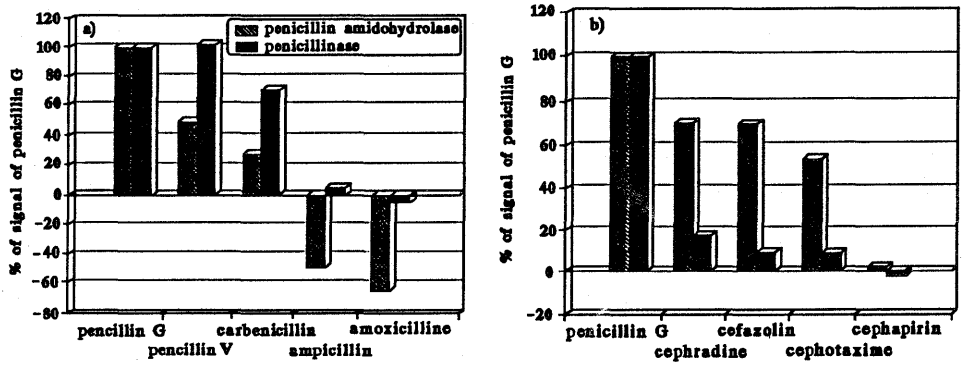

Figure 3. Selectivity of the biosensors in respect to various penicillins (a) and cephalosporins (b). The analytical signals were presented in relation to the signal for penicillin G (30.0 mmol l^{-1} penicillin in 5.0 mmol l^{-1} phosphate buffer, pH=8.0)

The response of biosensors in the presence of different antibiotics depends on three factors: the efficiency of the enzymatic reaction, the strength of the acid formed in the enzymatic reaction and pH changes in the analyzed solution caused by the addition of antibiotic.

In the case of penicillin G, penicillin V, and carbenicillin (Fig. 3a) no changes of pH of the test solution was observed (pH of the solution was controlled in the course of calibration procedure). It means that the difference in the measured analytical signals for these antibiotics are due to the first and the second factors mentioned above. No changes in pH of the test solution was also observed for cephapirin. Taking this into account, and on the basis of the results presented in Figure 3b, we can conclude that no hydrolysis of cephapirin in the presence of penicillinase and penicillin amidohydrolase take place. In the course of the calibration procedure with the use of other cephalosporins, a slight acidification of the test solution was observed. Due to this acidification the same changes in the potential for pH sensors with and without immobillized penicilinase were observed. It can be said that penicillinase does not catalyse hydrolysis of these cephalosporins. However, these cephalosporins are enzymatically hydrolized when penicillin amidohydralase is used. The analytical signal of the biosensor in that case is result of two effects: the effect of the enzymatic reaction within enzymatic layer of biosensor, and the effect of pH changes in the bulk solution. Seemingly surprising results were obtained for ampicillin and amoxicilline (Figure 3a). The "negative value" of the analytical signal indicates that on addition of antibiotics to the test solution caused an increase in pH. This was confirmed by control of pH of the analyzed solution in the course of the calibration procedure. It is difficult to say whether hydrolysis takes place in the presence of penicillin amidohydrolase. But it is sure that these antibiotics are hydrolized enzymatically when penicillinase is used.

Conclusions

The biosensors described in this paper can be used for determination of some penicillins and cephalosporins present at the milimolar range. The good analytical parameters of the biosensors result from the used method of enzyme immobilization. The obtained monomolecular enzyme layers which sense pH electrodes are active, stable and extremely thin. Good sensitivity, high stability and short response time make the described biosensors useful in FIA systems. Their utility for determination of penicillins in natural samples is now under investigations.

The primary studies of their selectivity indicates that the biosensor with immobilized penicilinase may be used for determination of penicillins, and is not sensitive to cephalosporins, whereas the biosensor with penicillin amidohydrolase responds to both of them.

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REFERENCES

1. Koncki R. and Głąb S., *Chem. Anal. (Warsaw)*, **36**, 423,1991
2. Taguchi H., Ishihara N., Okumura K. and Shimabayashi Y., *Anal. Chim. Acta*, **228**, 159, 1990.
3. Meier H., Kumaran S., Danna A. M. and Tran-Mihn C., *Anal. Chim. Acta*, **249**, 405, 1991.
4. Carlsen M., Johansen C., Min R. W., Nielsen J., Meier H. and Lantreibecq F., *Anal. Chim. Acta*, **297**, 51, 1993.
5. Koncki R., Leszczyńska E., Cybulska A. and Głąb S., *Anal. Chim. Acta*, **321**, 27 (1996).
6. Głąb S., Koncki R., Kopczewska E., Wałcerz I. and Hulanicki A., *Talanta*, **41**, 1201, 1994.
7. Głąb S., Koncki R. and Hulanicki A., *Analyst*, **116**, 453, 1991.

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