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A new interference-free lysine biosensor using a non-conducting polymer film[1]

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Abstract

An electrochemical biosensor for the determination of lysine to be used for rapid evaluation of food quality has been developed. Platinum electrodes have been coated by electropolymerisation with 1,2-diaminobenzene (1,2-DAB) using cyclic voltammetry. The reduction in the oxidation of interferents compared with the bare platinum electrode was 100% for ascorbic acid, 99% for acetaminophen and 99% for cysteine. The enzyme L-lysine-α-oxidase was then immobilised onto the polymer layer by passive adsorption and a calibration curve for lysine constructed. This gave a linear range of 1×10^{-5} mol/l to 1×10^{-3} mol/l and a limit of detection of 2×10^{-7} mol/l. © 1998 Elsevier Science S.A. All rights reserved.

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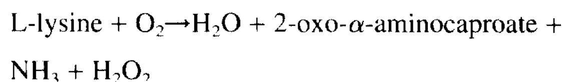
1. Introduction

Lysine is one of eight essential amino acids. It is relatively abundant in animal proteins, unlike plant proteins which are often low in lysine content, making it the limiting amino acid in many foods. It is also easily damaged by heat treatment and storage conditions of food, and therefore may be used in the assessment of food processing techniques as well as an index of the nutritional quality of foods.

In this study we have designed a biosensor suitable for industrial use, which may be used as an on-line monitor of processing and storage conditions of pastas and milk, in order to monitor the effect on the lysine content.

For its determination the enzyme L-lysine-α-oxidase (LyOx) was used. This catalyses the following reaction of lysine with oxygen:



$$\text{L-lysine} + O_2 \rightarrow H_2O + \text{2-oxo-}\alpha\text{-aminocaproate} + NH_3 + H_2O_2$$

This is then followed electrochemically by the oxidation of H_2O_2 at the working electrode, and the current output observed correlated to the concentration of lysine in the sample.

However, at the high working potential required for hydrogen peroxide detection, + 650 mV, other electroactive species present in solution are also oxidised. Several strategies have been previously employed for the purpose of reducing/eliminating the effects of such electrochemical interferents.

The technique used here involves the use of a protective polymer film deposited directly onto the electrode surface which will ideally be permselective to the analyte, but will restrict the passage of larger molecules.

Electropolymerised films may be divided into two categories (Emr et al., 1995): the first type, classed as conducting polymers, are capable of transducing energy arising from certain chemical interactions into electrical signals which are easily monitored.

The three most common monomers used are pyrrole, aniline and thiophene, pyrrole being the most useful of

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the three since it can be electropolymerised from unbuffered aqueous media. Polyaniline on the other hand is only in a conducting form in acidic media, which is not very convenient for work with most biocomponents (Hoa et al., 1992) and polythiophene is only soluble in organic media which may cause problems for biocomponent solubilisation, especially if co-immobilising enzymes.

However, the main disadvantage of these type of films is that they are not very reproducible due to the random nature of the deposition process. Although growth time and charge can control the amount of polymer deposited on the surface, it is not specific for the thickness of the film, which results in many of the films having defects and pinholes. Therefore polymers grown under identical conditions may have different resistances to interferents (Partridge et al., 1996). Also, the anions present in solution can influence the conductivity of the film and, thus, selection of the anion can be useful for generating films with the desired conducting properties: polypyrrole films obtained in presence of Cl^- or ClO_4^- showed the lowest conductivity (Diaz et al., 1986; Vork et al., 1990). The pH of the solution may also affect the conductivity, as with polyaniline.

The other type of polymer films are non-conducting. These monomers form self-regulating films which have a uniform thickness since they only grow thick enough to become insulators, they cover the surface completely with fewer defects and pinholes than are seen with conducting polymers. The most common non-conducting polymers are benzene derivatives, such as phenol (Pravada et al., 1995; Christie et al., 1993), *o*- and *p*-aminophenol, 1.2, 1.3 and 1.4-diaminobenzene (Curulli et al., 1997; Zhang et al., 1996; Madaras et al., 1996; Centonze et al., 1994; Geise et al., 1991; Sasso et al., 1990) and 1.2 and 1.3 dihydroxybenzene (Carelli et al., 1996).

Polypyrrole may be used in both conducting and non-conducting forms, the non-conducting form being advantageous in that it is not limited by electrostatic binding of anions (Palmisano et al., 1995; Hsueh et al., 1994; Centonze et al., 1992; Witkowski et al., 1992).

Some of the non-conducting polymers have been previously tested in our laboratory using potentiostatic growth, but the films grown using cyclic voltammetry were found to provide a better resistance to interferents. Some of the more commonly used polymers such as overoxidised polypyrrole, poly(pyrrole-2-carboxylic acid), poly(1.2-diaminobenzene), poly(1.3-diaminobenzene), poly(1.4-diaminobenzene and poly(4,4′-dihydroxybenzophenone) were tested against the same interferents. These polymers have been previously tested in the laboratory and found to provide a good degree of resistance to ascorbic acid, uric acid and acetaminophen. The best of these polymers were then chosen, and the enzyme lysine oxidase was crosslinked with glutaraldehyde and left to immobilise by passive adsorption on the polymer film. A calibration curve for lysine oxidase was then constructed for each of the polymers, and the biosensors were again tested against the interferents.

2. Experimental

2.1. Materials and methods

2.1.1. Apparatus

All cyclic voltammetry experiments were performed using an AMEL polarographic analyser, model 433A (AMEL Milan-Italy), controlled by an IBM PS/2 90, and, where stated, a BAS voltammograph model CV-37 (Bioanalytical systems, West Lafayette, USA) was used. The fixed potential studies were performed using an AMEL potentiostat model 559 (AMEL Milan-Italy), and the currents recorded using a LKB 2210 strip chart recorder (Delft, Netherlands). The electrochemical cell consisted of saturated calomel (SCE) or a Ag/AgCl reference electrode (SCE unless stated), platinum (Pt; nominal area 0.071 cm^2) working electrode, and the counter electrode was a platinum wire.

2.1.2. Reagents

1.2-diaminobenzene (1.2-DAB), 1.3-diaminobenzene (1.3-DAB) and 1.4-diaminobenzene (1.4-DAB) were from Sigma Chemical Co. (St Louis, MO, USA). 4,4′-dihydroxybenzophenone (DHB), pyrrole (Py) and pyrrole-2-carboxylic acid (PY-2-COOH) were all purchased from Fluka Chemika–Biochemika (Buchs, Switzerland).

The enzyme L-lysine oxidase (L 6150) (EC 1.4.3.14), phenylalanine (Phe), cysteine (Cys), acetaminophen (APAP), ascorbic (AA), uric (UA) and citric acid (CA) and glutaraldehyde, grade 1:25% were also purchased from Sigma.

Solutions of the interferents (0.1 mol/l) such as phenylalanine, ascorbic acid and citric acid were prepared immediately before use in buffer pH 7.5. L-lysine (0.1 mol/l) was made 24 h prior to use and stored at 4°C. Acetaminophen was not soluble at 0.1 mol/l, therefore 0.05 mol/l stock solutions were used. L-cysteine precipitated out of solution at pH 7.5 and therefore 0.05 mol/l solutions were made up using 50% NaOH. Uric acid was also made up in 50% NaOH at a concentration of 5×10^{-3} mol/l. The buffer used was sodium dihydrogen phosphate dihydrate adjusted to pH 7.5 with sodium hydroxide, also from Fluka.

2.1.3. Electrode pre-treatment

All electrodes were polished with successively finer alumina particles sizes 1 μm, 0.3 μm and 0.05 μm (A12O3) (Buehler, USA) and rinsed with distilled water. Before use the glassy carbon electrodes were held at a fixed potential of 1.2 V in NaOH (1 mol/l) vs Ag/AgCl,

for 5 min, and then scanned between − 0.2 V and 1 V at a scan rate of 50 mV/s for 5 min in the same NaOH solution. The Pt and Au electrodes were pre-treated by potential cycling from − 0.2 V to 1.2 V in 0.5 mol/l H_2SO_4 for 20 min.

2.1.4. Film formation

The films were electrodeposited onto the electrode surface by means of cyclic voltammetry in 0.05 mol/l solutions of the monomers (except Py, which was 4×10^{-4} mol/l in 0.01 mol/l KCl) in sodium phosphate buffer at pH 7.4 or pH 9.1 for DHB. All were deaerated for 15 min with nitrogen before electrodeposition. The electrodes were scanned until the current reached a value which remained constant after further cycling. The scan rate was varied from 20 to 2 mV/s.

The potential was continuously cycled until a minimum value of current, which remained constant after further cycling was observed: this indicated that the electrode surface was completely covered by the polymer.

The response to the interferents was monitored weekly for about 2 months and normalized to the initial response. The efficiency of the polymeric film was investigated by the evaluation of the decrease of the oxidation current of the interferents tested.

2.1.5. Enzyme immobilisation

The enzyme was immobilised as follows, 10 μl of buffer containing 4.1 units of enzyme, was mixed with 10 μl of a 0.25% solution of glutaraldehyde (to give a 0.125% solution) and this was placed directly on the polymer film on the electrode in 5 μl injections, each being left to dry before the subsequent addition.

2.1.6. Lysine analysis

The electrodes at + 0.6 V, applied potential, were equilibrated in phosphate buffer solution until a constant current baseline was reached. Then aliquots of lysine were injected and the current variations recorded.

3. Results and discussion

3.1. Electropolymerisation studies

In previous works carried out in our laboratory on 1.2-DAB, 1.3-DAB, 1.4-DAB, and PPY-2-COOH (Curulli et al., 1997) the electropolymerisation conditions were optimised giving a scan rate of 2.0 mV/s, concentration of 5×10^{-3} mol/l and pH 7.4.

PDHB was electrosynthesised using the same scan rate and concentration, but at pH 9.1.

Py (4×10^{-4} mol/l) was electropolymerised from a solution of 10 mM KCl at a scan rate of 2 mV/s over the ranges shown in Table 1.

Table 1
Electropolymerization scan range

Monomers	Scan range (V)
1.2-DAB	0.0–0.8
1.3-DAB	0.0–0.8
1.4-DAB	0.0–0.8
PY	0.2–1.0
4,4-DHB	0.3–0.8
PY-2-COOH	0.3–0.8

The polymers were again tested for their resistance to the electrochemical interferents; ascorbic acid, acetaminophen, cysteine, citric acid and uric acid.

These interferents are present in physiological solutions in concentrations of 5×10^{-5} mol/l for uric acid and 1.1×10^{-4} mol/l for ascorbic acid (Madaras et al., 1996). However, this sensor was designed with the objective of testing the lysine concentration in foods, mainly milk and pasta, where the concentrations ranges from 36 to 660 mg/100 g food (Official Gazette EC, 1995).

The concentration of interferents used was 1×10^{-4} mol/l; since uric acid was only sparingly soluble, it was tested at the lower concentrations of 1×10^{-5} mol/l. The results are presented in Table 2.

1.2-DAB appeared to give the best overall coverage of the electrode, giving a decrease in the interferent response of 100% for ascorbic acid, 99% for acetaminophen and 99% for cysteine. As can be seen from the table, PPY-2-COOH was not very efficient in the reduction of the interferents and therefore no further work has been carried out.

The comparison between the poly(1.2-DAB) and PPy showed that 1.2-DAB provided a better coverage of the electrode, this could be due to the electrodeposition process as pyrrole is deposited as a conducting film and only afterward converted to its non-conducting form. This deposition feature of pyrrole could in part be the cause of more defects in the polymer film, as conducting films have been found to form less uniform films, as they are less likely to form monolayers on the electrode surface (Hsueh et al., 1994). Another method of depositing pyrrole is to apply a high constant potential after the deposition of a small amount of pyrrole, in order to form an insulating layer on the surface (Hsueh et al., 1994). The polymer obtained in this way showed a permselectivity not suitable for our purpose.

3.2. Enzyme immobilisation

The five best polymers were chosen for enzyme immobilisation, poly(1.2-DAB), poly(1.3-DAB), poly(1.4-DAB), PPy and PDHB.

The fabrication of the sensitive layer was performed by cross-linking the enzyme with a bifunctional reagent

Table 2
Response of interferents on polymer modified Pt electrodes (results are expressed as a percentage of the bare Pt electrode)

Polymer	AA (%)	CA (%)	UA (%)	Ph (%)	Cy (%)	Act (%)	RSD % (10 measurements)
1.2-DAB	0	0.1	0.4	0.2	4.0	1.2	2
1.3-DAB	0.7	0.5	0.3	0.4	0.9	1.5	3
1.4-DAB	0.2	0.4	0.5	0.6	0.3	0.1	2
PPy	0.7	0.4	0.5	1.2	10.8	0.1	4
PDHBP	0.2	0.3	0.1	1.4	9.6	0.4	2
PPY-2-COOH	80.5	1.2	0.05	3.2	24.9	0.3	3

(glutaraldehyde). It is important to make the additions of the crosslinking solution using a small volume, 5 μl or less, to obtain an enzyme layer with a good adhesion on the polymer film. The electrode, assembled in such way, was stored in a buffer solution pH 7.5 at 4°C. A dry storage at 4°C resulted in a detachment of the enzymatic layer, from the electrode surface.

We used 4.1U of enzyme. When we used less enzyme (1.5U–3.0U) to obtain a thinner layer, the sensitivity decreased. When we used more enzyme (up to 10U) the enzyme layer detached and the adhesion on the electrode surface was unsatisfactory.

3.3. Characteristics of the biosensor

The electrodes were once again tested for the same interferents, and a calibration curve was then constructed for each of the biosensors, as reported in Table 3 and showed in Figs. 1 and 2. The detection limit was defined as a signal-to-noise ratio of 3.

The response time of the sensor in diffusion mode of operation is not dependent on the concentration of L-lysine: it was 12–16 s.

PDHB/LysOx/Pt and poly(1.2-DAB)/LysOx/Pt showed the best analytical performances, as reported in Table 3.

PPy/LysOx/Pt and PDHB/LysOx/Pt showed a quite good rejection vs AA, CA, UA, Cys and APAP, but a very poor vs Phe, while poly(1.2-DAB)/LysOx/Pt showed the best rejection vs all the interferences tested.

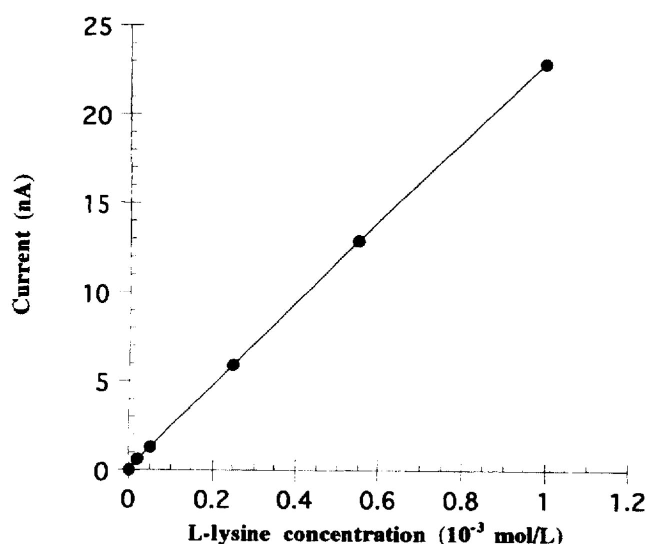

Fig. 1. Calibration curve at poly (1.2-DAB)/LyOx/Pt electrode in phosphate buffer pH 7.4; E = + 0.6 V vs Ag/AgCl.

3.4. pH effect on sensor response

pH effect was then tested over the pH range 6–9.

As shown in the Fig. 3, we observed a rapid increase in the sensor response at pH 9. However, at this pH we observed a rapid degradation of the polymeric layer which resulted in a poor sensor stability. The response at pH 6 and 7 was quite similar and the probe stability was very good. A good compromise for those two effects was to work at pH 7.5.

Table 3
Properties of lysine electrodes probes. PBS buffer, pH 7.5

Probe	Concentration range of lysine (mol/l)	Response time (s)	Reproducibility rsd % (6 measurments)	Detection limit (mol/l)
PPY/LysOx/Pt	3×10^{-5}–5×10^{-4}	16	4	2×10^{-5}
PDHB/LysOx/Pt	4×10^{-6}–1×10^{-3}	12	4	2×10^{-7}
poly(1.2-DAB)/LysOx/Pt	2×10^{-6}–2×10^{-3}	13	4	1×10^{-7}
poly(1.3-DAB)/LysOx/Pt	1.4×10^{-5}–5×10^{-4}	15	6	1×10^{-5}
poly(1.4-DAB)/LysOx/Pt	2×10^{-5}–4×10^{-4}	15	5	1×10^{-5}

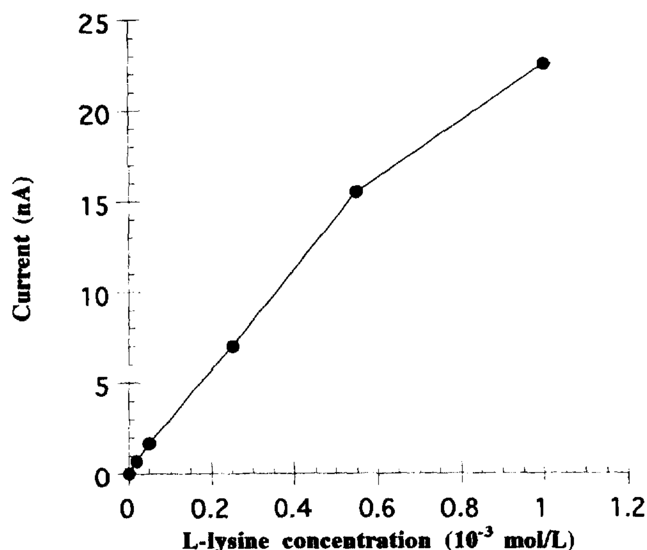

Fig. 2. Calibration curve at PPy/LyOx/Pt electrode in phosphate buffer pH 7.4; $E = +0.6$ V vs Ag/AgCl.

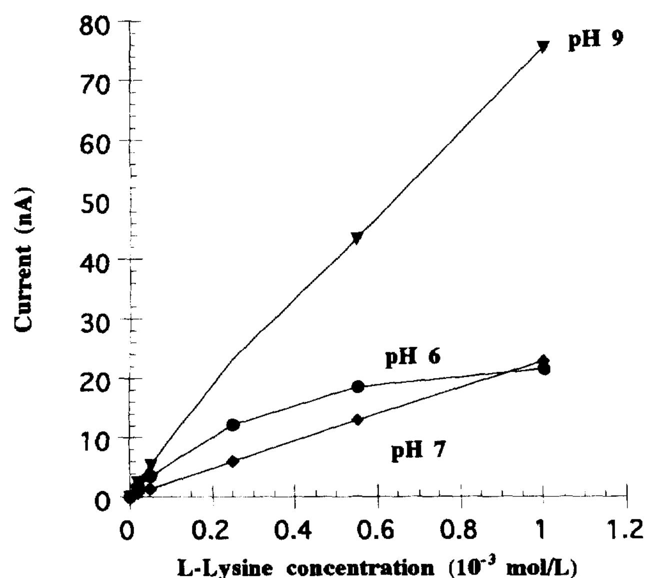

Fig. 3. Calibration curve at poly (1.2-DAB)/LyOx/Pt electrode in phosphate buffer pH 6–9; $E = +0.6$ V vs Ag/AgCl.

3.5. Optimisation

Further work has been carried out to obtain an optimisation of our system. First, we have considered the electropolymerisation conditions. The time for electropolymerisation was investigated to see if a better response vs the interferents could be obtained. The electrodes were left to cycle, at scan rate of 5 mV/s, for 5, 10 and 20 cycles and tested against the interferents. The best coverage was obtained for 20 cycles. This whole process, however, takes nearly 5 h. If we compared the data obtained at different numbers of cycles, we observed a decrease of the interferent (e.g. Cy, Ph and UA) response with the increase of the cycles. This trend indicates that a slower electropolymerisation could improve the sensor performance.

Probably, the best response to the substrate and the best coverage is represented by an arrangement between a scan rate slow enough to obtain a quite good permselectivity and a scan rate fast enough to carry out electropolymerisation for more than one electrode simultaneously.

4. Conclusions

The purpose of this paper was to find the best polymer which would prevent the interferents from reaching the electrode surface. As can be seen from the data, all of the polymers provide some degree of coverage of the electrode surface and the best polymer chosen was 1.2-DAB. This polymer reduced the interferent response as follows: AA by 100%, UA by 99.97% and Cy by 99.9%, also the lysine calibration curve showed good linearity, with a regression coefficient of 0.99 and a limit of detection of 2×10^{-7} mol/l. This biosensor will be tested further in order to optimise the polymerisation time, since after five scans the CV does not appear to change.

We have assembled a new, promising lysine biosensor, but further work is necessary to improve the stability, the reproducibility and the optimisation of the sensor operative conditions.

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