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A multi-enzyme bioelectrode for the rapid determination of total lactate concentration in tomatoes, tomato juice and tomato paste

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This work presents a new multi-enzyme bioelectrode for the combined determination of D(−)-L(+)-lactic acid in samples of whole tomatoes, chopped tomatoes, tomato paste and tomato juice. The principle of the biosensor is based on the catalytic activity of the enzymes L(+)-lactic acid oxidase (LOD), D(−)-lactic acid dehydrogenase (D-LDH) and horseradish peroxidase (HPO). The three enzymes are immobilized on the tip of an amperometric oxygen selective electrode. The total concentration of D(−)-L(+)-lactic acid is proportional to the amount of O_2 consumed. The biosensor has been tested for standard solutions of D(−)-L(+)-lactic acid, as well as for real samples. The latter set of experiments has been carried out in three different laboratories, indicating a good repeatability and reproducibility of the obtained results. The main characteristics of the lactate biosensor on real samples (whole tomatoes, chopped tomatoes, tomato paste and tomato juice) have been compared, in terms of both analytical and practical features, with those of a traditionally employed enzymatic–spectrometric technique, based on the oxidation of D–L-lactic acid, catalysed by D-lactate dehydrogenase and L-lactate dehydrogenase, with production of pyruvate and NADH, and on the subsequent spectrometric determination, at $\lambda = 340$ nm, of the NADH formed.

INTRODUCTION

It is well known that lactic acid exists as the L-(+) and the D-(−) enantiomers. The former is the normal intermediate in mammalian metabolism, while the latter is usually produced by micro-organisms, algae and plants (Soccol *et al.*, 1994; Tsai *et al.*, 1993). In addition, some micro-organisms, especially *lactobacilli*, produce the two enantiomers as a racemic mixture (Gawehn, 1984).

The determination of D(−)- and L(+)-lactic acid is required whenever the quality of some food products has to be assessed. Indeed, D(−)- and L(+)-lactic acid are two reliable indicators of the quality of many alimentary products. Particularly, the D(−)- enantiomer has been proven to be a very good indicator of reduced freshness in vacuum-packed meat products (De Pablo *et al.*, 1989), and the racemic mixture of bacterial contamination of tomatoes, tomato paste and tomato juice. Levels of D–L-lactic acid higher than 0.3 g/kg are usually considered as an index of microbial contamination, associated with an alteration of the organoleptic characteristics (Vicini *et al.*, 1988; Hanewinkel-Meshkini & Hackmann, 1989; Porretta & Vicini, 1993). A rapid and inexpensive method for the monitoring of D–L-lactic acid could therefore prove very useful in evaluating the quality of tomato paste and tomato juice.

The two lactic acid stereoisomers are usually determinated by spectrometry or liquid chromatography (Gawehn, 1984; Robrish *et al.*, 1984; Nielsen *et al.*, 1990; Girotti *et al.*, 1991; Ohmori & Iwamoto, 1988; Shu *et al.*, 1993). Enzymatic methods are based mainly on mono enzymatic systems, such as L(+)-lactic acid-2-mono-oxygenase or L(+)-lactic acid oxidase (Bardelletti *et al.*, 1986; Durliat & Comtat, 1980; Mascini *et al.*, 1987; Adamowicz & Burnstein, 1987; Mizutani *et al.*, 1985), catalysing the oxidation of L(+)-lactic acid. Furthermore, different methods were also set up for the determination of D(−)- and/or L(+)-lactic acid; all

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these methods are based on the determination of the concentration of NADH, associated with the formation of pyruvate from the oxidation of lactate. Such an oxidation reaction is determined by either D(−)-L(+)-lactic acid dehydrogenase (Blaedel & Jenkins, 1976; Durliat *et al.*, 1976; Gorton & Hedlund, 1988; Montagné *et al.*, 1993; Durliat *et al.*, 1990; Blaedel & Engstrom, 1980; Yao & Wasa, 1985; Mizutani *et al.*, 1991). In this latter case, the reaction catalysed by LDH is coupled to a second enzyme-catalysed reaction, usually the NADH oxydation by diaphorase–hexacyanoferrate (III) (Montagné *et al.*, 1993; Durliat *et al.*, 1990; Yao & Wasa, 1985), or by NADH oxidase (Mizutani *et al.*, 1991), both of which give rise to a chemical product that can easily be detected by an adequate electrochemical or optical sensor. Alternatively, NADH detection can also be directly performed by electrochemical oxydation (Blaedel & Engstrom, 1980; Christian, 1976; Moiroux & Elving, 1979).

The present work describes the realization and the application of a new multi-enzyme bioelectrode for the simultaneous determination of D(−)- and L(+)-lactic acid in tomato products (whole tomatoes, chopped tomatoes, tomato paste and tomato juice). The biosensor is based on the combined catalytic activity of L(+)-lactic acid oxidase (LOD), D(−)-lactic acid dehydrogenase (D-LDH) and horseradish peroxidase (HPO), suitably assembled with a commercially available O_2-sensitive Clark-like amperometric electrode. According to the following reactions, (1)–(3),

$$\text{L(+)-lactic acid} + O_2 \xrightarrow{\text{LOD}} \text{pyruvate} + H_2O_2 \quad (1)$$

$$\text{D(−)-lactic acid} + NAD^+ \xrightarrow[\text{D-LDH}]{} \text{pyruvate} + NADH + H^+ \quad (2)$$

$$NADH + O_2 + 2H^+ \xrightarrow{\text{HPO}} 2NAD^+ + 2H_2O \quad (3)$$

the total concentration of D(−)-L(+)-lactic acid is directly correlated to the oxygen consumption, which is promptly detected by the inner amperometric O_2-electrode.

The proposed biosensor has first been characterised in standard solutions of D(−)-L(+)-lactic acid and tested accordingly on tomato juice and tomato paste samples. Results of this last set of experiments have been compared with those obtained by a traditionally employed enzymatic–spectrometric method (Gawehn, 1984), based on the spectrometric determination, at $\lambda = 340$ nm, of the NADH formed in the oxidation reaction of D–L-lactic acid, catalysed by D-lactate dehydrogenase and L-lactate dehydrogenase, respectively:

$$\text{D(−)-lactic acid} + NAD^+ \xrightarrow[\text{D-LDH}]{} \text{pyruvate} + NADH + H^+ \quad (4)$$

$$\text{L(+)-lactic acid} + NAD^+ \xrightarrow[\text{L-LDH}]{} \text{pyruvate} + NADH + H^+. \quad (5)$$

To further displace the equilibria of reactions (4) and (5) in favour of NADH by removing pyruvate, L-glutamate and glutamate–pyruvate transaminase (GPT) were also employed:

$$\text{pyruvate} + \text{L-glutamate} \xrightarrow[\text{GPT}]{} \text{L-alanine} + \text{2-oxoglutarate}. \quad (6)$$

MATERIALS AND METHODS

Materials

L(+)-lactic acid oxidase (from *Pediococcus* sp., 20 U/mg solid), D(−)-lactic dehydrogenase (from *Staphylococcus epidermidis* 10 U/mg solid), peroxidase (from Horseradish, 300 U/mg solid), D(−)-lactic acid lithium salt, L(+)-lactic acid lithium salt and NAD^+ were supplied by Sigma Chemical Co. (St. Louis, Missouri, USA).

Biodyne Transfer membrane (nylon 6.6 membrane, porosity 0.45 μlm), with carboxylic groups on the surface, were supplied by Pall Italia s.r.l. (Milano, Italy). Cellulose acetate dialysis membrane (0.001 inch. thick; molecular weight cut-off = 12 000 Da) were supplied by Sigma Chemical Co. (St. Louis, Missouri, USA). Prior to the immobilization of the three enzymes, the membranes were stored dried at room temperature.

The polyazetidine prepolymer (PAP) solution (Hercules Polycup 172, 12% solids in water), used for the physico-chemical immobilization of the enzymes, was obtained from Hercules Inc. (Wilmington, Delaware, USA) and stored at 4°C.

Reagents for the enzymatic–spectrometric determination of D–L-lactic acid according to the method described by Gawehn (1984), i.e. glycylglycine buffer, pH 10.0. L-Glutamic acid, GPT, NAD, D-LDH, L-LDH, D-lactate and L-lactate, were purchased, in the form of a complete kit, from Boehringer Mannheim, Germany (Cat. No. 1112821).

All other chemicals were analytical grade. Twice distilled water was used for the preparation of all solutions, including the 0.1 M phosphate buffer, pH 7.0 (305 ml 0.2 M Na_2HPO_4 + 195 ml 0.2 M KH_2PO_4, diluted to 1000 ml with H_2O) and the glycine buffer, pH 8.6 (500 ml 0.2 M glycine, corrected to pH 8.6 with 0.2 M NaOH, and diluted to 1000 ml with H_2O).

Preparation of lactic acid biosensors

Immobilization of the three enzymes (LOD, D-LDH and HPO)

The immobilization was carried out by chemical bonding, based on an available nylon 6.6 membrane with carboxyl groups on the surface, and of a prepolymer, polyazetidine. L(+)-lactic acid oxidase, D(−)-lactic acid dehydrogenase, HPO and PAP (0.25 mg LOD + 0.5 mg D-LDH + 0.25 mg HPO/20 μl PAP) were spread uniformly on a disk of the membrane (0.8 cm diameter; total density of the enzymes 0.200 mg/cm^2). The enzyme

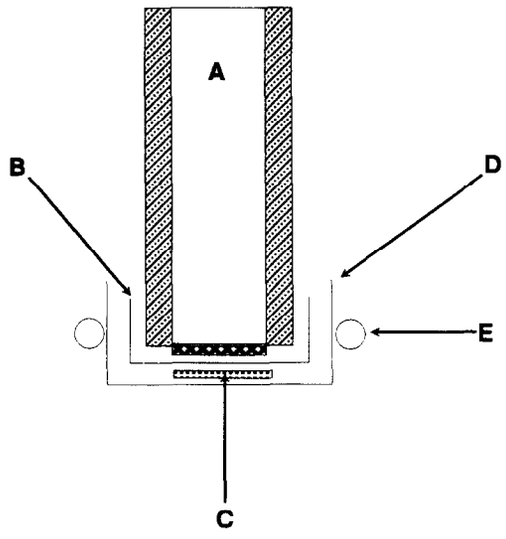

Fig. 1. Schematic representation of the D(−)-L(+)-lactic acid biosensor: (A) internal amperometric O_2-sensing electrode; (B) O_2-permeable Teflon membrane; (C) biocatalytic membrane; (D) dialysis membrane; and (E) rubber O-ring.

membrane was left for 24 h at room temperature, washed out with 0.1 M phosphate buffer, pH 7.0, and then fixed on the tip of the outer body of the O_2-sensitive Clark-like amperometric electrode (Universal Sensors Inc., New Orleans, Louisiana, USA). Although this trienzymatic membrane can be stored at −15°C, without any detectable loss of enzyme activity, for a period between 2 and 5 months, depending on the total number of assays performed in the same period by the membrane itself, a new membrane was always freshly prepared every time it was necessary to assemble a new lactate biosensor.

Assembly of the sensors

The D(−)-L(+)-lactic acid sensor, whose section is schematically represented in Fig. 1, was assembled by placing the tip of the outer body of the O_2 sensing electrode (A in Fig. 1) on a sequence of three different membranes, in the following order: (i) a Teflon gas permeable membrane (B in Fig. 1), to eliminate interferences from other electroactive substances, possibly present in the sample; (ii) the trienzymatic membrane (C in Fig. 1), prepared as described above; and (iii) a dialysis membrane (D in Fig. 1), to prevent any possible microbial attack of the enzymes and/or their leaking from the membrane. A rubber O-ring (E in Fig. 1) was used to fix the three layers on the tip of the oxygen sensor.

Between measurements, and for short-term storage (up to 24 h), the biosensor was kept into a buffer solution at pH 7.0. For longer term storage the biosensor was disassembled and stored dried, while the enzymatic membrane was kept at −15°C.

Amperometric experiments

Calibration of the sensor

Amperometric measurements were carried out by connecting the trienzymatic bioelectrode to an amperometric detector (ABD, Universal Sensors Inc., New Orleans, Louisiana, USA). A constant potential difference of −650 mV was applied between the platinum cathode and the Ag/AgCl anode of the oxygen electrode. The electrode jacket was filled with an internal filling solution of KH_2PO_4 and KCl both 0.1 M, pH 7.4. Experiments were carried out in 5 ml of 0.1 M glycine buffer, pH 8.6, in a glass cell, kept thermostated, by forced water circulation, at 37°C, which is the optimum temperature of operation for the combined catalytic activity of the three enzymes. Uniform magnetic stirring, at a constant rate, was used during the operation. $MnCl_2$—the co-factor of HPO for the oxydation of NADH—and NAD^+ (both to a final concentration 1 mM) were added to 5.0 ml of buffer.

The biosensor was employed to determine substrate concentration, by adding the sample, appropriately diluted from a stock solution, to the glycine buffer solution. More specifically, known amounts of D(−)-L(+)-lactic acid from a stock, 0.01 M standard solution, were added stepwise every 2 min, and the corresponding current values, as read by the biosensor, were recorded.

Determination of total lactate levels in tomato samples

All samples (whole tomatoes, chopped tomatoes, tomato paste and tomato juice) were analysed both by the biosensor and by the reference spectrometric method. Samples of tomato juice were filtered through a

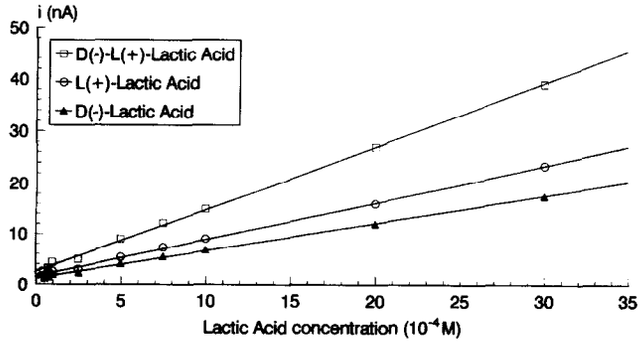

Fig. 2. Calibration curves obtained by the multi-enzyme D(−)-L(+)-lactic acid biosensor in standard solutions of D(−)-lactic acid (triangles), L(+)-lactic acid (circles) and D(−)-L(+)-lactic acid (squares).

paper filter; portions of the clear solution were assayed both according to the procedure described for the spectrometric method (Gawehn, 1984) and by the biosensor-based amperometric method. In the latter case, a known volume of the clear solution, usually of between 50 and 200 μl, according to the estimated total lactate concentration, was added directly to 5.0 ml of glycine buffer solution containing $MnCl_2$ and NAD^+ (see calibration experiments). Samples of tomato paste were homogenized by a rotating blade (1000 rpm for 15–30 s), at room temperature, in a dark vessel, before being filtered and assayed as described above. Whole and chopped tomatoes were minced in small pieces before being homogenized, filtered and assayed by the same procedure.

RESULTS AND DISCUSSION

Figure 2 shows the calibration curves (current intensity vs lactic acid concentration), obtained by the multi-enzyme D(−)-L(+)-lactic acid biosensor in standard solutions of D(−)-lactic acid (triangles), L(+)-lactic acid (circles) and D(−)-L(+)-lactic acid (squares). As it can be seen, the biosensor reponse is linear in a wide range of lactate concentrations.

Table 1. Analytical characterization of the trienzymatic, lactic acid-selective biosensor, in D(−)-L(+)-lactic acid standard solutions

Temperature of analysis:	37°C
pH:	8.6
Buffer:	0.1 M glycine
Response time:	2 min
Lifetime of operation (as total number of assays):	180–200
Maximum lifetime of operation:	5 months
Equation of the calibration graph: $Y=\Delta i$ (nA); $X=$[lactic acid] (M)	$Y=2.6+12217X$
Linearity range:	5.0×10^{-5}–3.0×10^{-3} M
Correlation coefficient:	0.9990
Lower detection limit:	2.5×10^{-5} M
Repeatibility of the measurements (as pooled standard deviation in the linearity range):	2.8%

Table 2. Comparison of total lactic acid concentration values obtained by the spectrometric and by the biosensor methods, in D(−)-L(+)-lactic acid standard solutions. Each value is the mean of 10 determinations

Sample No.	Nominal value (in mM) *a*	Values found by the biosensor method *b*	Values found by the spectrometric method *c*	$(b-a)/a$(%)	$(c-a)/a$(%)	$(b-c)/c$(%)
1	0.25	0.25 (RSD% = 1.8)	0.24 (RSD% = 1.3)	—	−4.0	+4.2
2	0.50	0.49 (RSD% = 2.0)	0.48 (RSD% = 1.2)	−2.0	−4.0	+2.1
3	0.75	0.76 (RSD% = 2.1)	0.74 (RSD% = 1.9)	+1.3	−1.3	+2.7
4	1.00	1.02 (RSD% = 1.5)	1.02 (RSD% = 1.7)	+1.9	+2.0	—
5	1.50	1.48 (RSD% = 1.7)	1.50 (RSD% = 1.4)	−1.3	—	−1.3
6	2.00	2.04 (RSD% = 1.4)	2.02 (RSD% = 1.7)	+2.0	+1.0	+1.0

Table 3. Comparison of total (D + L) lactic acid concentration values obtained by the spectrometric and by the biosensor methods, for 16 samples of whole tomatoes (1–3), chopped tomatoes (4–6) tomato paste (7–11) and tomato juice (12–16). Each value is the mean of five determinations. Values in mM

Sample No.	Values found by the biosensor method *a*	Values found by the spectrometric method *b*	$(a-b)/b$(%)
1	0.74 (RSD% = 1.6)	0.72 (RSD% = 1.3)	+2.7
2	0.58 (RSD% = 1.8)	0.59 (RSD% = 1.0)	−1.7
3	1.20 (RSD% = 1.2)	1.17 (RSD% = 1.4)	+2.5
4	4.62 (RSD% = 0.9)	4.47 (RSD% = 1.8)	+3.2
5	3.24 (RSD% = 1.6)	3.17 (RSD% = 1.4)	+2.2
6	2.12 (RSD% = 2.1)	2.16 (RSD% = 0.8)	−1.9
7	0.65 (RSD% = 2.0)	0.66 (RSD% = 1.2)	−1.5
8	1.48 (RSD% = 1.5)	1.44 (RSD% = 1.0)	+2.8
9	2.10 (RSD% = 2.2)	1.71 (RSD% = 1.7)	+2.3
10	5.22 (RSD% = 1.8)	5.03 (RSD% = 1.4)	+3.8
11	2.12 (RSD% = 1.1)	2.17 (RSD% = 1.1)	−2.3
12	2.90 (RSD% = 1.6)	2.99 (RSD% = 2.2)	−3.0
13	3.25 (RSD% = 2.3)	3.19 (RSD% = 1.5)	+1.9
14	1.95 (RSD% = 1.8)	2.00 (RSD% = 1.6)	−2.5
15	3.70 (RSD% = 1.2)	3.60 (RSD% = 0.9)	+2.8
16	7.20 (RSD% = 1.5)	7.50 (RSD% = 1.9)	−4.0

Table 1 reports the main physico-chemical and analytical features of the multi-enzyme biosensor, characterized by D(−)-L(+)-lactic acid standard solutions. As it can be seen, the bioelectrode is suitable for on site determinations of D(−)-L(+)-lactic acid in real samples, thanks to its short response time, good repeatability of the results and extended range of linearity. The sensitivity limits of the biosensor, which lie well below the contamination level (4 mM), do not limit the application of the biosensor. Indeed, in addition, samples containing high levels of lactate can be easily assayed, since the dilution factor of the dilution step, which always follows the filtration of the sample, can be made more or less pronounced according to the estimated total concentration of lactate.

The results obtained by the multi-enzyme bioelectrode and the enzymatic–spectrometric assays are compared in Table 2, again in D(−)-L(+)-lactic acid standard solutions. The results show a good agreement between the spectrophotometric and the biosensor results, with differences not greater than 4.0%.

Table 3 reports the results obtained by the bioelectrode and the enzymatic spectrophotometric method in the determination of D(−)-L(+)-lactic acid on 16 samples of commercially avalilable whole or chopped canned tomatoes, and of tomato paste and tomato juice. There is a very good agreement between the two methods, with differences never exceeding 4.0%, and usually oscillating between 1.5 and 3.0%.

On the basis of the obtained results, the biosensor described here appears very suitable for the direct determination of D(−)-L(+)-lactic acid in samples of tomato paste and tomato juices. The biosensor is easy to use, especially in the case of inexperienced personnel. Furthermore, the assays performed by the biosensor do not require any particular sample pretreatment (apart from a crude filtration or homogenization/filtration step in the case of liquid or solid and semi-solid samples, respectively), thus drastically reducing the time required for each analysis. Finally, the prolonged lifetime of operation for the biosensor (up to 200 analyses performed by a single multienzymatic membrane, whose lifetime of operation can reach 5 months) markedly reduces the overall analytical costs of operation of the biosensor itself, when compared to the traditionally employed techniques.

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