Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 64 (2004) 452–457
Talanta
www.elsevier.com/locate/talanta

Voltammetric determination of alkaline phosphatase and horseradish peroxidase activity using 3-indoxyl phosphate as substrate Application to enzyme immunoassay

Pablo Fanjul-Bolado, María Begoña González-García, Agustín Costa-García*

Departamento de Química Física y Analítica, Universidad de Oviedo, Quimica Fisica y Analitica, Calle Julian Claveria 8, 33006 Oviedo, Asturias, Spain

Received 3 November 2003; received in revised form 16 January 2004; accepted 3 March 2004

Available online 22 April 2004

Abstract

The use of 3-indoxyl phosphate (3-IP) as an electrochemical substrate for ELISAs with voltammetric detection was investigated. Indirect measurements of alkaline phosphatase (AP) and horseradish peroxidase (HRP) activity in solution were carried out. Picomolar levels of both enzymes can be detected, which enables the design of electrochemical immunoassays using this substrate. The enzymatic turnover of the substrate gives indigo blue, insoluble in aqueous solutions. This product is easily converted into its soluble parent compound, indigo carmine (IC), by addition of fuming sulphuric acid to the reaction media. IC shows a reversible voltammetric peak at the formal potential of −0.15 V (versus Ag pseudo-reference electrode) when a screen-printed carbon electrode (SPCE) is used. The peak current of this process constitutes the analytical signal. Using this approach an ELISA assay to quantify pneumolysin (PLY, a toxin related to respiratory infections) was carried out using AP or HRP as enzymatic label. Calibration plots obtained are reported. 3-IP is demonstrated to be the first suitable substrate for the two most common enzyme labels used in immunoassays.

Keywords: Horseradish peroxidase; Alkaline phosphatase; 3-Indoxyl phosphate; Screen-printed carbon electrodes; ELISA; Cyclic voltammetry

1. Introduction

The enzyme linked immunosorbent assay (ELISA) is a well stablished analytical method which combines the extreme specificity of antigen/antibody recognition with the high amplification capability of enzymatic reactions [1]. The most of ELISAs have been developed for biomedical applications such as diagnosis, but applications in agricultural, environmental and processed food areas are on the rise [2–4].

The enzyme labels most commonly used in ELISAs are alkaline phosphatase (AP) and peroxidase (the cheapest one), both can be detected at femtomole levels [5] and an array of substrates is available for using with either enzyme. 2,2′-Azino-di[3-ethyl-benzotiazolin-(6)-sulfonate] (ABTS), *o*-phenylenediamine (OPD), and 3,3′,5,5′-tetramethyl-benzidine (TMB) [6–8] are the most employed in horseradish peroxidase (HRP)-based ELISAs. The search of sensitive substrates to peroxidase continues and recently examples are available [9]. AP hydrolyzes ortophosphate from a wide variety of phosphate esters under alkaline conditions. Phenylphosphate, *p*-nitrophenylphosphate, and naphtylphosphate [10–12] have been employed. AP and its conjugates are very stable, the main disadvantage is that the purified calf intestine enzyme, which is preferred because of its relatively high specific activity compared with other sources, is expensive.

With regards to the detection system, the formation of the product is generally followed by spectrophotometry, fluorescence or chemiluminiscence. However, last years, immunoassays with electrochemical detection constitute a methodology extensively used [13]. The advantages of this approach include the speed, accuracy, and precision with which many electrochemical measurements can be made.

3-Indoxyl phosphate (3-IP) was proposed as a suitable AP substrate for enzyme immunoassays with voltammetric detection [14] and has been successfully applied to

* Corresponding author. Tel.: +34-985103488; fax: +34-985103125.
E-mail address: costa@sauron.quimica.uniovi.es (A. Costa-García).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2004.03.003

electrochemical immunosensors [15,16]. Development of immunoassays with other indoxyl derivative, 5-bromo-6-chloro-3-indolyl phosphate (BCIP), commercially available, have been also carried out [17].

This paper reports on a comparative study using 3-indoxyl phosphate for the determination of HRP or AP activity in solution with voltammetric detection of indigo carmine (IC, disodium 5,5′-indigotindisulfonate), which is a hydrosoluble synthetic dye that has been used in the food, pharmaceutical, and cosmetic industries [18,19]. Although this molecule has been usually determined by a spectrophotometric method [20], carbon paste electrodes have been also employed for the detection of IC. An electrode pretreatment was necessary for obtaining a reproducible methodology [21]. However, in this work inexpensive mass-produced and disposable screen-printed carbon electrodes (SPCEs) are shown as an attractive alternative.

An ELISA assay for pneumolysin (PLY) using both labels is presented and analytical characteristics of the immunoassay system are evaluated. To our knowledge, to date there are no documents related to the use of the same substrate in immunoassays that employs HRP or AP conjugates.

PLY is a multifunctional toxin produced by *Streptococcus pneumoniae*, which possesses cytolitic and complement activation properties. Its structure, activity and relation to respiratory infections have been studied [22,23]. It provokes immune response and enhances the immunogenicity of the *S. pneumoniae* polysaccharide. Pneumolysin has been previously determined by flow cytometry [24].

2. Experimental

2.1. Reagents and solutions

Biotin conjugated to alkaline phosphatase (B–AP, dimmer, four units of B per molecule of AP) and horseradish peroxidase Type VI-A (EC 1.11.1.7, specific activity 987 U mg^{-1}) were obtained from Sigma (Spain). These reagents were supplied as lyophilized powders. Reconstitution and working solutions of B–AP were made in 0.1 M Tris–HCl buffer pH 7.2 containing 1 mM $MgCl_2$ (Panreac, Barcelona, Spain). HRP stock solution (1 mg ml^{-1}) was prepared in ultrapure water.

Tris(hydroxymethyl)amino methane (Tris), 3-indoxyl phosphate disodium salt, and bovine serum albumin (fraction V, BSA) were purchased from Sigma (Spain). 3-Indoxyl phophate solutions were daily prepared in 0.1 M Tris–HCl, pH 9.8, containing 10 mM $MgCl_2$ or in 0.1 M AcOH/AcO, pH 5.0 buffer solution. These substrate solutions were stored refrigerated in an opaque flask.

In the ELISA procedure a 0.05 M carbonate buffer pH 9.6 containing 0.1% NaN_3 (Merck) was employed for the coating with the monoclonal antibody. The blocking was performed with a 10 mM (137 mM in NaCl (Merck), 27 mM in KCl (Merck)) phosphate buffer (PBS) pH 7.4 with 3% of BSA, and 0.1% of NaN_3. Washing buffer was 10 mM PBS pH 7.4 with 0.1% of NaN_3 and 0.1% of Tween 20 (TW, Merck). Dilutions of Pneumolysin were carried out with 10 mM PBS pH 7.4, 0.1% in NaN_3 and 0.05% in Triton X-100 (Sigma). Polyclonal anti-PLY was prepared in 10 mM (150 mM in NaCl) Tris–HCl buffer pH 7.8 with 0.1% of NaN_3, 6% of BSA, and 0.1% of TW. The polyclonal AP conjugated anti-rabbit IgG was diluted with 10 mM TBS with 0.1% NaN_3, 1% of BSA, and 0.1% of TW. The polyclonal HRP conjugated anti-rabbit IgG was diluted in the same buffer solution without NaN_3 (inhibitor of HRP activity). A final washing was made with 0.1 M Tris–HCl buffer pH 9.8 containing 50 mM of $MgCl_2$ and 0.1% of TW in the case of AP and with 0.1 M AcOH/AcO^- buffer pH 5.0, 0.1% of TW in the case of HRP.

The immunological reagents: monoclonal anti-PLY (909.1 μg ml^{-1}), PLY (175 μg ml^{-1}), and rabbit polyclonal anti-PLY were kindly provided by Dr. de los Toyos (Department of Functional Biology, Universidad de Oviedo). The polyclonal AP and HRP conjugated anti-rabbit IgG (whole molecule) was purchased from Sigma.

The Na_2CO_3, $NaHCO_3$, H_3PO_4 (85%), NaOH, HCl (37%), and H_2SO_4 (95–97%) employed were provided by Sigma.

Ultrapure water obtained with a Milli-R 3 plus/Milli-Q plus 185 purification system from Millipore Ibérica S.A. was used throughout this work.

2.2. Apparatus

Staircase cyclic voltammetric measurements were performed with an Autolab PGSTAT 12 (Eco Chimie, The Netherlands) potentiostat interfaced to an AMD K-6 266 MHz computer system and controlled by Autolab GPES software version 4.8 for Windows 98.

Screen-printed carbon electrode strips were purchased from AndCare Inc. (Durham, NC, USA, http://www.andcare.com) together with an edge connector. These sensors consisted of working (a 4 mm diameter disk) and counter electrodes both printed using heat curing carbon composite inks, and a silver pseudo-reference electrode. They are printed onto a polycarbonate substrate (4.5 cm × 1.5 cm). An UV-cured insulating layer serves to delimit the working area and electric contacts. A specific connector supplied by the company (Sensor Connector Model SC-01P) allows their connection to the potentiostat as is shown in Fig. 1. Cyclic voltammetric experiments were performed, at room temperature, in 40 μl drops pipetted over the strip that was attached to the connector.

Microtiter plates (flat bottom, high binding) were purchased from Costar (Cambridge, MA 02140, USA). Eight channels micropipette and 1.5 ml tubes obtained from Eppendorf (22331 Hamburg, Germany), a MS1 minishaker (IKA-Werke GmbH & Co. KG, Germany) with a microtiter adapter and a Sanyo incubator were also employed.

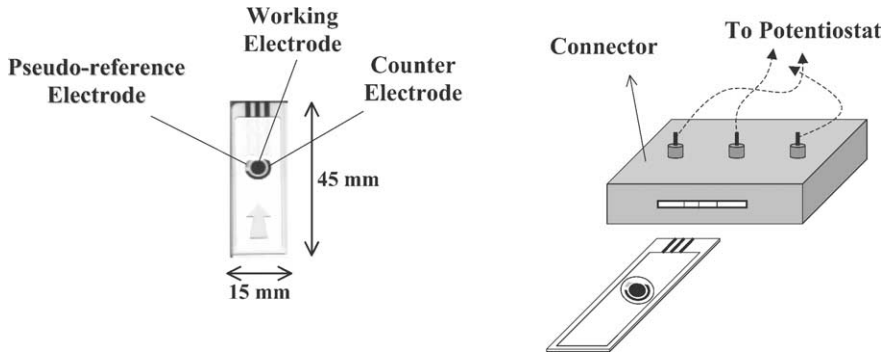

Fig. 1. Diagram of the screen-printed carbon electrode and connector.

2.3. Analytical procedures

2.3.1. Enzyme determination

The enzymatic reactions were carried out in a microtiter plate in which a 200 μl of the blocking buffer were added to each well and incubated overnight at 4 °C. After washing four times with adequate buffer and drying, the measurement of the enzyme activity was performed as follows.

- *AP determination*: Hundred microliters of 3-IP (in 0.1 M Tris–HCl buffer, 10 mM $MgCl_2$, pH 9.8, 0.4% BSA) were added to each well and mixed with 8 μl of an AP solution. They were then incubated at 37 °C under constant shaking.
- *HRP determination*: An amount of 50 μl of 3-IP (in 0.1 M AcOH/AcO^- buffer, pH 5.0, 0.4% BSA) and 50 μl of H_2O_2 (2×10^{-4} M) were mixed with 8 μl of a HRP solution. Reacting for 30 min at 37 °C.

The reactions were stopped in both cases by adding 100 μl of fuming sulphuric acid to each well. Stopping solution causes a drastic increase in the acidity, enough for breaking the reaction off. This addition also pursues another objective: to dissolve the enzymatic product, indigo blue.

2.3.2. ELISA procedure

The optimization of this procedure is reported elsewhere [25] and schematized in Fig. 2. One microgram of PLY monoclonal antibody per 100 μl solution in coating buffer are added to each well. After incubation for 6 h at 37 °C the plate is emptied and 200 μl of the blocking buffer are added and incubated overnight at 4 °C. After washing four times with the washing buffer and drying, 100 μl of PLY solution were placed in each well and incubated for 1 h at 37 °C under shaking. Once they have been washed six times and dried, 100 μl of 1:500 diluted polyclonal rabbit anti-PLY

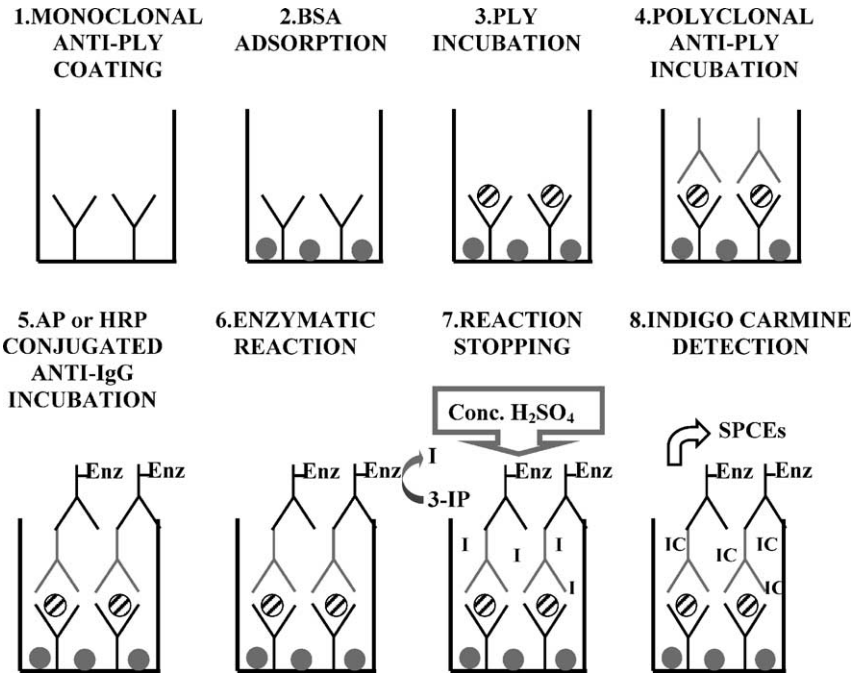

Fig. 2. Schematic diagram of the PLY ELISA procedure.

are added and maintained at 37 °C for 30 min under stirring. The step following the washing (for six times) and drying is the incubation at 37 °C with the AP or HRP conjugated anti-rabbit IgG (100 μl, 1:5000 diluted) for 30 min under shaking. Afterwards, the washing is made for six times with the corresponding final washing buffer, the substrate solution (3-IP or 3-IP/H_2O_2) is added to each well and the enzymatic reaction takes place at 37 °C until it is stopped by addition of fuming sulphuric acid (100 μl). Then the generated indigo carmine is detected by cyclic voltammetry.

2.3.3. Voltammetric detection

IC generated after dissolving the enzymatic product, indigo blue, is electroactive and can be easily detected by cyclic voltammetry (CV). A 15 μl drop of each reaction well was diluted with 25 μl of H_2O and transferred to the electrochemical cell of the SPCEs (Fig. 1). A potential scan was applied to the electrode from −0.4 to +0.1 V with a scan rate of 50 mV s^{-1} and the corresponding voltammogram was recorded. The starting potential, −0.4 V is applied for 45 s before the potential is scanned. All measurements were carried out at room temperature.

3. Results and discussion

3.1. Voltammetric studies

Horseradish peroxidase catalyses the following redox general reaction:

$$3\text{−IP} \xrightarrow{H_2O_2/\text{pH}\,5} \text{indigo blue}$$

Indigo carmine is generated after dissolving indigo blue with fuming sulphuric acid as is shown in Fig. 3. This sulphonated derivative is employed since it possesses similar electrochemical behavior and better solubility in aqueous solution than indigo blue. The electrochemistry of 3-indoxyl phosphate and indigo carmine on screen-printed carbon electrodes has already been studied showing electrodic processes at different potentials [26,27]. The electrochemical behavior of IC is characterized by a well-defined reversible electrodic signal with $E_{1/2} = -0.15$ V versus Ag, and a more positive oxidation peak a $E_p = +0.53$ V versus Ag. A linear dependence of the square root of the potential scan rate on the peak current recorded was found. Such dependence revealed that both are diffusion-controlled processes. The current measured by cyclic voltammetry due to IC can be therefore directly related to the enzyme concentration.

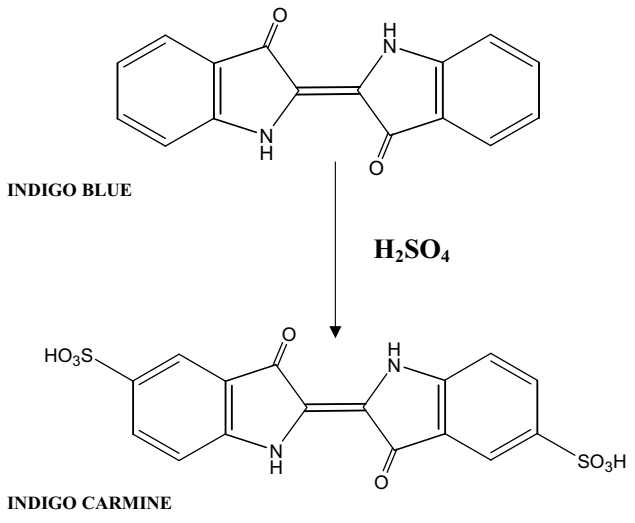

Fig. 3. Solubilization of indigo blue.

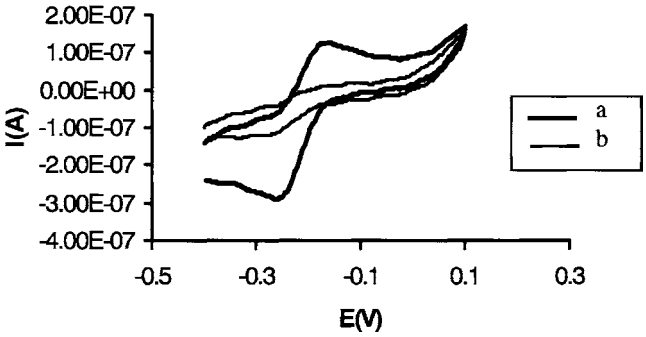

Fig. 4. CV voltammograms. (a) Reaction in the presence of HRP: 1.5×10^{-10} M. Reacting for 30 min at 37 °C and (b) reaction in absence of HRP: pH 5.0 AcOH/AcO^- buffer, 6 mM of 3-IP, 0.4% BSA, and 1×10^{-4} M of hydrogen peroxide. Potential sweep rate 50 mV s^{-1}.

Fig. 4 shows the cyclic voltammogram of IC (enzymatically generated, curve a) that exhibit a reversible diffusion controlled signal at a formal potential ($E_{1/2}$) of −0.15 V versus Ag pseudo-reference electrode. Curve b) represents the voltammogram of the background signal. There is a blank peak due to the spontaneous oxidation of 3-IP in acid media (pH 5.0) and in presence of H_2O_2 (blank after 30 min: 30 nA). Oxidation of HRP substrates by hydrogen peroxide has already been observed by other authors [8,28,29].

Alkaline phophatase catalyses the hydrolysis of 3-IP to produce indigo blue in basic media (pH 9.8) and in absence of H_2O_2. 3-IP appears to be more suitable for use as substrate for AP determination because of its limited spontaneous hydrolysis (blank after 60 min: 10 nA). The enzymatic reaction that takes place comprises the hydrolysis of the phosphate moiety of the substrate by AP, the formation of the unstable enol product and its subsequent oxidation in air to give indigo blue.

The electrodic process measured for IC corresponds to the oxidation of the conjugated enol groups of the leuco-indigo carmine specie (generated by means of the application of the initial potential for 45 s) to yield indigo and its subsequent reduction (Fig. 5).

3.2. Enzymatic determination

The highest sensitivity for the measurement of HRP activity was attained by performing the enzymatic reaction (with

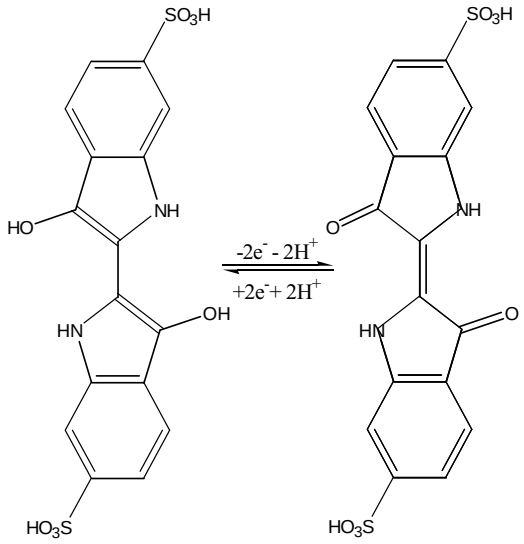

Fig. 5. Mechanistic scheme of the electrodic process.

6 mM of 3-IP, 0.4% BSA, 1×10^{-4} M of H_2O_2) at pH 5.0. Cyclic voltammetry was used to measure the height of the anodic peak of IC showing a good linear relationship with the concentration of free HRP in the range from 1.7×10^{-11} to 4.5×10^{-10} M. The corresponding equation is:

$$\text{Ip (nA)} = 3 \times 10^{11}\,[\text{HRP}]\,(\text{M}) + 39.84$$

$$r^2 = 0.9984 \quad n = 4$$

The relative standard deviation (R.S.D.) for six parallel determinations to 8.7×10^{-11} M of HRP was 4.8% with 75 nA of average peak current. The detection limit is 6.8×10^{-12} M (calculated as the concentration corresponding to three times the standard deviation of the estimate).

Under the selected AP-catalyzed reaction conditions (6 mM of 3-IP and 0.4% BSA at pH 9.8) the calibration curve for AP was obtained for a 20 min incubation of the enzyme with the substrate mixture at 37 °C. A linear dynamic range was achieved from 4×10^{-12} to 7.7×10^{-11} M of AP with a correlation coefficient of $r^2 = 0.9985$, according to the following equation:

$$\text{Ip (nA)} = 1 \times 10^{13}\,[\text{AP}]\,(\text{M}) - 0.78$$

The detection limit is 2×10^{-12} M and the R.S.D. is 4.1% for six consecutive measurements (with a mean oxidation peak current of 188 nA) of 1.5×10^{-11} M of AP. The sensitivity of this assay is much better than that of the HRP due to the very steep slope of the calibration curve.

Better detection limits could be obtained with the use of sensitive electrochemical techniques such as alternating current voltammetry or by increasing the incubation time of the enzymatic reaction. Voltammetric detection after 1 h incubation increase the detectability of AP, thus a linear dynamic range is obtained from 1.5×10^{-13} to 1.5×10^{-12} M. In the case of the HRP determination, an increase in the incubation time supposes an increase in the blank signal (due to the spontaneous oxidation of 3-IP) therefore, it is not possible to improve the limit of detection of HRP making greater this time.

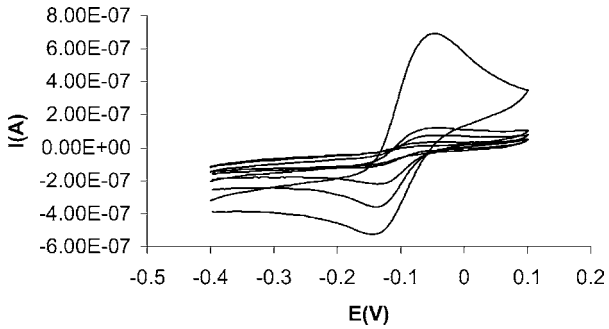

Fig. 6. CV voltammograms obtained for AP-based ELISA corresponding to 50, 10, 5, 2.5, and 1.25 ng ml^{-1} of PLY after adding fuming sulfuric acid to the reaction media. Potential sweep rate 50 mV s^{-1}.

The 3-IP concentration used in AP assay is >10 K_m [27] enough to ensure that the enzymatic reaction proceeds under saturating substrate conditions and, therefore, the analytical signal only depends on the AP concentration. In the case of HRP assay the 3-IP concentration used is ~3 K_m. No higher concentrations were employed in order to avoid high background signals.

3.3. Electrochemical PLY ELISA

The PLY immunoassay was carried out following the sandwich procedure reported in the Section 2. Electrochemical detection of PLY using HRP or AP as label was compared through the calibration curves obtained, whose values are given in Table 1. Slopes of the equations are shown in nanoampere per concentration unit and n is the number of points of the calibration curve. The results of HRP-based assay fitted on a linear $\log y - \log x$ while the curve of the AP-based assay was linearly fitted with a $y - x$ scale. The choice was made in order to get the widest linear working range and better regression coefficient. The slope was higher using AP and a better limit of detection was achieved in this case. Cyclic voltammograms corresponding to the dynamic linear range obtained for AP-based ELISA are shown in the Fig. 6. If calibration curve for this immunoassay was made measuring the cathodic currents a linear relationship between intensity and concentration of antigen is obtained with a dynamic range of 1.25–10 ng ml^{-1} ($-I$(nA) = 28.15 [PLY] − 3.22; r^2 = 0.995). The sensitivity is higher but the linear working range is shorter than that achieved with anodic currents.

Immunoassays were performed with an incubation time of 30 min for the enzymatic reaction. A relative standard deviation lower than 7% was always found.

The IC measured, which is generated after the addition of fume sulfuric acid is stable for at least 2 h without a decrease

Table 1
Analytical characteristics of the determination of PLY using secondary antibodies HRP or AP conjugated

Enzyme	LDR (ng ml^{-1})	LD (ng ml^{-1})	Equation	n	r^2
HRP	2.5–50	2.1	log I (nA) = 0.19 log [PLY] (ng ml^{-1}) +2.22	4	0.9951
AP	1.25–50	0.6	I (nA) = 12.25 [PLY] (ng ml^{-1}) +11.35	5	0.9996

LDR: linear dynamic range; LD: limit of detection; r: correlation coefficient.

in the analytical signal in contrast to other enzymatic products [8]. Furthermore, other advantage of detecting IC in ELISAs, is its reversible electrochemical behaviour shown at low potentials ($E_{1/2} = -0.15$ V).

4. Conclusions

The indirect electrochemical determination of alkaline phosphatase and horseradish peroxidase can be achieved at a disposable screen-printed carbon electrode using the enzyme substrate 3-indoxyl phosphate. Single use SPCEs solve problems related to contamination between samples and fouling of the sensing surface. The feasibility of using voltammetric detection of indigo carmine to ELISAs has been demonstrated through the quantitation of pneumolysin. Therefore, to our knowledge 3-IP is shown to be the first substrate successfully used in AP and HRP-based immunoassays with electrochemical detection. Better performance was achieved (lower limit of detection and higher sensitivity) with AP than that obtained using the other common enzymatic label, HRP. Work is in progress to remove the fuming sulfuric acid of the procedure and to develop an automated detection system in combination with the use of sensitive electrochemical techniques such as alternating current voltammetry.

Acknowledgements

This work has been supported by the project BIO2000-0588 from the Spanish Ministry of Science and Technology.

References

[1] C. Blake, B.J. Gould, Analyst 109 (1984) 533.
[2] U. Bilitewski, Anal. Chem. 72 A (2000) 692.
[3] E. Mallat, D. Barceló, C. Barcen, G. Gauglitz, R. Abuknesha, TrAC 20 (2001) 124.
[4] R.J. Schneider, Anal. Bioanal. Chem. 375 (2003) 44.
[5] C.P. Price, D.J. Newman, Principles and Practice of Immunoassay, Stockton Press, New York, 1991.
[6] S.M. Brook, T.M. Bliss, L.R. Franklin, R.M. Sapolsky, Neurosci. Lett. 267 (1) (1999) 21.
[7] S. Morais, P. Casino, M.L. Marín, R. Puchades, A. Maquieira, Anal. Bioanal. Chem. 374 (2002) 262.
[8] G. Volpe, D. Compagnone, R. Draisci, G. Palleschi, Analyst 123 (1998) 1303.
[9] A. Manaka, M. Sakai, S. Igarashi, Anal. Chim. Acta 478 (2003) 37.
[10] K.R. Wehmeyer, H.B. Halsall, W.R. Heineman, C.P. Volle, I-Wen. Chen, Anal. Chem. 58 (1986) 135.
[11] E.M. Abad-Villar, M.T. Fernández-Abedul, A. Costa-García, Anal. Chim. Acta 409 (2000) 149.
[12] O. Niwa, Y. Xu, H.B. Halsall, W.R. Heineman, Anal. Chem. 65 (1993) 1559.
[13] N.J. Ronkainen-Matsuno, J.H. Thomas, H.B. Halsall, W.R. Heineman, TrAC 21 (2002) 213.
[14] C. Fernández-Sánchez, A. Costa-García, Electroanalysis 10 (1998) 249.
[15] C. Fernández-Sánchez, A. Costa-García, Anal. Chim. Acta 402 (1999) 119.
[16] C. Fernández-Sánchez, M.B. González-García, A. Costa-García, Biosens. Bioelectron. 14 (2000) 917.
[17] R.C. Ebersole, M.D. Ward, J. Am. Chem. Soc. 110 (1988) 8623.
[18] The Merck Index, Ralway Inc, New York, 1983.
[19] J.J. Berzas, J. Rodríguez-Flores, M.J. Villaseñor-Llerena, N. Rodríguez-Fariñas, Anal. Chim. Acta 391 (1999) 353.
[20] E.L. Roberts, S. Burguieres, I.M. Warner, Appl. Spectrosc. 52 (1998) 1305.
[21] C. Fernández-Bobes, M.T. Fernández-Abedul, A. Costa–García, Electroanalysis 13 (2001) 559.
[22] C.J. Boulnois, J.C. Paton, T.J. Mitchell, P.W. Andrew, Mol. Microbiol. 5 (1991) 2611.
[23] J. Rossjohn, R.J. Gilbert, D. Crane, P.J. Morgan, T.J. Mitchell, A.J. Row, P.W. Andrew, J.C. Paton, R.K. Tweten, M.W. Parker, J. Mol. Biol. 284 (1998) 449.
[24] J.R. De los Toyos, F.J. Méndez, J.F. Aparicio, F. Vázquez, M.M. García-Suarez, A. Fleites, C. Hardisson, P.J. Morgan, P.W. Andrew, T.J. Mitchell, Infect. Immun. 64 (1996) 480.
[25] M.D. Cima-Cabal, F.J. Méndez, F. Vázquez, M.M. García-Suárez, J.R. de los Toyos, J. Immunoassay Immunochem. 22 (2) (2001) 99.
[26] M. Díaz-González, C. Fernández-Sánchez, A. Costa-García, Electroanalysis 14 (2002) 665.
[27] M. Díaz-González, C. Fernández-Sánchez, A. Costa-García, Anal. Sci. 18 (2002) 1209.
[28] M. Del Carlo, M. Mascini, Anal. Chim. Acta 336 (1996) 167.
[29] Y.-N. He, H.-Y. Chen, J.-J. Zheng, G.-Y. Zhang, Z.-L. Chen, Talanta 44 (1997) 823.