3,3′,5,5′-Tetramethylbenzidine as electrochemical substrate for horseradish peroxidase based enzyme immunoassays. A comparative study

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The use of 3,3′,5,5′-tetramethylbenzidine (TMB) as an electrochemical substrate for horseradish peroxidase (HRP) was investigated. HRP activity has been detected using flow injection analysis at a glassy carbon working electrode polarised at +100 mV *versus* Ag/AgCl in 0.1 mol l^{-1} citrate–phosphate buffer (pH 5.0). The optimum concentrations were 2×10^{-4} mol l^{-1} TMB and 10^{-3} mol l^{-1} H_2O_2. The detection limit obtained after 15 min of incubation was 8.5×10^{-14} mol l^{-1} HRP with the amperometric method. This limit was lower than that obtained using hydroquinone as HRP substrate and comparable to that with the *p*-aminophenyl phosphate–alkaline phosphatase system. Better performance was achieved with amperometric than spectrophotometric detection using TMB in a competitive ELISA for rabbit immunoglobulin G as a model analyte.

Keywords: *3,3′5,5′-Tetramethylbenzidine; horseradish peroxidase; amperometry; enzyme-linked immunosorbent assay; flow injection analysis*

Enzyme immunoassays are based on selective antigen–antibody binding and a label enzyme. Depending on the assay format, the antigen or antibody is labelled and an enzyme activity measurement is performed as a final step of the assay. Fluorimetric,[1–6] luminometric[7–11] and colorimetric[12–14] detection are widely used. The excellent sensitivity and wide linear range typical of electrochemical (particularly amperometric) detection have attracted attention in recent years, with the development of electrochemical enzyme immunoassays.[15] This technique can be coupled with flow injection analysis (FIA), giving high reproducibility, partial automation and high sample throughput.[16–20] Moreover, electrochemical detection is the first step for the future development of immunosensors in which the antigen–antibody reaction takes place on the surface of the transducer.[21–27]

Alkaline phosphatase (AP) is the most commonly used enzyme label for enzyme linked immunosorbent assay (ELISA). This enzyme catalyses the hydrolysis of phosphate esters to give inorganic phosphate and a phenolic group. The formation of this phenolic moiety is generally followed by spectrophotometry using 4-nitrophenyl phosphate,[28] by fluorescence with fluorescein phosphate,[29] by chemiluminescence using dioxetane phosphate[30] and by amperometry with 1-naphthyl phosphate[31–33] or *p*-aminophenyl phosphate (PAPP). So far, PAPP has been defined as the best substrate for ELISA with amperometric detection[34,35] and electrochemical immunoassays using this substrate have been reported.[17,18,20,36–38] Horseradish peroxidase (HRP) is another enzyme label widely used in immunoassays. The enzyme activity can be determined by measuring the absorbance in the visible region,[13,14] by fluorescence[4–6,39] or by electrochemistry.[19,24,25]

This paper reports on the use and analytical optimisation of 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate for the determination of HRP activity using FIA coupled with amperometric detection. This substrate has been reported to be suitable for use in ELISA with spectrophotometric detection[40] and has already been used as an electrochemical mediator for cholesterol and H_2O_2 detection.[41,42] Recently, He and co-workers[43,44] reported the use of TMB in a horseradish peroxidase based immunoassay with differential-pulse voltammetric detection. The electrochemical behaviours of TMB, hydroquinone (for HRP) and PAPP (for AP) were compared. TMB was found to be a good substrate for electrochemical detection of low levels of HRP. An ELISA competition assay using rabbit IgG as a model system and TMB as HRP substrate was also developed and the results are discussed.

Experimental

Reagents and materials

3,3′,5,5′-Tetramethylbenzidine dihydrochloride, hydroquinone, benzoquinone, *p*-aminophenol, horseradish peroxidase (EC 1.11.1.7) type VI-A, 1310 U mg^{-1}, alkaline phosphatase from bovine intestinal mucosa (EC 3.1.3.1) type VII-L, 1100 U mg^{-1}, rabbit immunoglobulin G (IgG), monoclonal anti-rabbit IgG (γ-chain specific) peroxidase conjugate and all other reagents of analytical-reagent grade were obtained from Sigma (St. Louis, MO, USA). A stock standard solution of TMB (0.01 mol l^{-1}) was prepared in distilled water and kept in a dark bottle. *p*-Aminophenyl phosphate was synthesised in the laboratory using a published procedure.[45] Polystyrene microplates were obtained from Iwaki Glass (Iwaki City, Japan).

Apparatus

For electrochemical detection we used a thin layer cell for LCEC (liquid chromatography–electrochemistry) from Bio-Analytical Systems, West Lafayette IN, USA). This cell included a working electrode (glassy carbon disk, 3 mm diameter), a reference electrode (Ag/AgCl) and an auxiliary electrode (stainless steel). The current output was measured with a Metrohm (Herisau, Switzerland) 641 VA detector and recorded with a Model 868 recorder (Amel, Milan, Italy). For FIA, a Model 7125 HPLC valve (Rheodyne, Cotati, CA, USA) with a closed loop of 5 or 20 μl (Supelco, Bellefonte, PA, USA) was used. The working buffer was pumped with a Minipuls 3 peristaltic pump Gilson, (Villiers le Bel, France).

A Model 550 microplate reader (Bio-Rad, Hercules, CA, USA) was used for ELISA with spectrophotometric detection.

Cyclic voltammetric studies were carried out with an Amel Model 433 polarographic analyser. The voltammetric cell consisted of a glassy carbon working electrode (3 mm diameter)

from Metrohm, a reference electrode (SCE) and a Pt counter electrode.

Procedures

FIA

The working buffer was passed through the electrochemical cell by a peristaltic pump until a constant baseline current was reached. A transient current variation was recorded after the injection of the analyte solution into the flow stream by means of the Rheodyne valve loop.

ELISA

A 1 : 10 000 dilution of rabbit IgG (solution A) was prepared from a stock standard solution (10 mg ml^{-1}) in 0.1 mol l^{-1} phosphate-buffered saline (PBS) (pH 7.0). Anti-rabbit IgG–HRP conjugate solution (solution B) was prepared by 1 : 20 000 dilution of the Sigma stock standard solution (titre 1 : 40 000) in PBS. The substrate solution (solution C) consisted of 2×10^{-4} mol l^{-1} TMB and 10^{-3} mol l^{-1} H_2O_2 in 0.1 mol l^{-1} citrate–phosphate buffer (pH 5.0). H_2O_2 was added to the TMB solution just before the measurement.

In the competitive enzyme immunoassay format, solution A (250 μl) was added to immuno-plate wells for 1 h at room temperature and the coated plates were blocked with 5% bovine serum albumine (BSA) in PBS at 4 °C overnight. A 125 μl volume of solution B plus 125 μl of rabbit IgG standard solutions were then incubated in the wells at room temperature for 1 h. Between all the mentioned steps a three-cycle washing procedure with PBS was adopted. A 250 μl volume of solution C was added to the wells and the enzymatic reaction was allowed to proceed for 15 min. A 50 μl volume of 2.4×10^{-2} mol l^{-1} NaN_3 were used to stop the reaction and 20 μl were injected into the FIA system.

Results and discussion

Voltammetric studies

HRP catalyses the following redox general reaction:

$$SubH_2\ (red) + H_2O_2 \rightarrow Sub\ (ox) + 2\ H_2O$$

The enzymatic activity can be measured by amperometric detection of the reduction current generated by Sub (ox) at an appropriate working electrode. Carbon electrodes and potassium iodide, *o*-phenylenediamine, hydroquinone, ferrocene and ferrocene derivatives have been already used for this purpose and for enzyme immunoassays.[19,24,25,46]

Cyclic voltammetric investigation of these substrates and others used for spectrophotometric detection, such as *o*-dianisidine and tetramethylbenzidine, were carried out at a glassy carbon electrode. The most interesting results in terms of the generated electrocatalytic current (difference between the cathodic waves in buffer and in the presence of HRP) were observed with hydroquinone and TMB. The structure of TMB is shown in Fig. 1. Fig. 2 shows the voltammograms of TMB and hydroquinone in the potential range −500 to +800 mV; voltammograms 1A and 1B were obtained in the presence of the couples TMB and H_2O_2 and hydroquinone and H_2O_2 respectively. The buffers used were 0.1 mol l^{-1} citrate–phosphate[40] plus 0.1 mol l^{-1} KCl for TMB and 0.1 mol l^{-1} phosphate[24] plus 0.1 mol l^{-1} KCl for hydroquinone. Two-electron redox behaviour was observed for TMB with oxidation peaks at +250 and +400 mV *versus* SCE. The formation of radical intermediates during the electrochemical oxidation of molecules such as benzidine and dianisidine has been evidenced in earlier polarographic studies.[47] This supported the hypothesis of two subsequent one-electrode step processes for the oxidation of benzidines by HRP in the presence of H_2O_2. The formation of a radical cation and the two-step mechanism were demonstrated for TMB using spectrophotometric and ESR data.[48,49] In solution the radical cation is a semiquinone–imine in equilibrium with a charge-transfer complex (blue) of the diamine (electron donor) and the diimine (electron acceptor).[49] Our electrochemical data for TMB confirmed the previous polarographic data for the oxidation of benzidines.

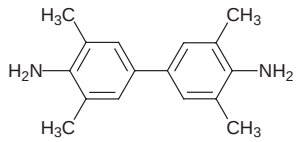

Fig. 1 TMB structure.

The electrochemistry of hydroquinone was as reported previously.[24] The addition of HRP (0.1 U ml^{-1}) to the two substrate solutions resulted in the consumption of TMB and hydroquinone and the formation of reaction products. Consequently, a decrease in the oxidation and an increase in the reduction currents (Fig. 2, voltammograms 2A and 2B) were observed.

Amperometric FIA hydrodynamic voltammetry for TMB was performed in the range +350 to 0 mV to assess the best working potential. As shown in Fig. 3, substrate oxidation was observed for potentials ranging between +350 and +150 mV (curve a); curve b represents the background currents in citrate–phosphate buffer.

A working potential of +100 mV *versus* Ag/AgCl was selected for the measurement of HRP enzymatic activity. In fact, at this potential, the current background was near to zero and no substrate oxidation occurred. These conditions are the optimum for enzymatic activity determination in which a small amount of product [TMB (ox) in this case] needs to be measured in the presence of high concentrations of substrate. The applied

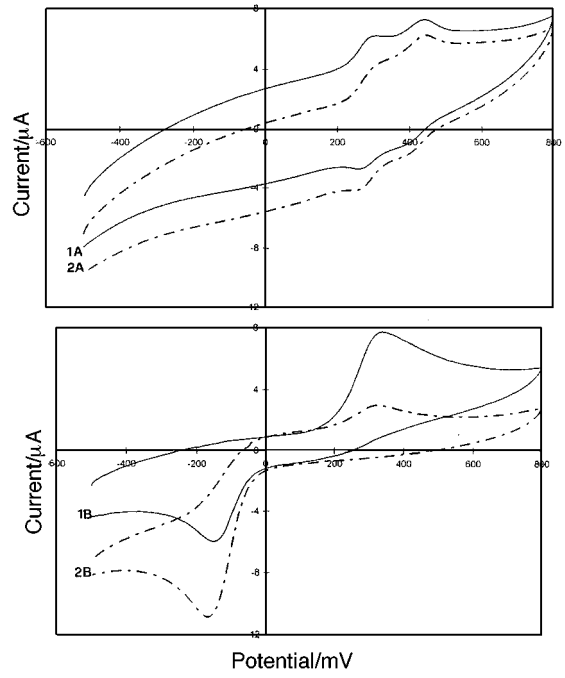

Fig. 2 Cyclic voltammetry of 2×10^{-4} mol l^{-1} TMB + 2×10^{-4} mol l^{-1} H_2O_2 in 0.1 mol l^{-1} citrate–phosphate buffer (pH 5) + 0.1 mol l^{-1} KCl (1A) and 3×10^{-4} mol l^{-1} hydroquinone + 3×10^{-4} mol l^{-1} H_2O_2 in 0.1 mol l^{-1} phosphate buffer (pH 7) + 0.1 mol l^{-1} KCl (1B). Voltammograms 2A and 2B were recorded 1 min after the addition of 0.1 U ml^{-1} HRP in solution. Scan rate, 50 mV s^{-1}.

potential selected for measurement with hydroquinone was −250 mV *versus* Ag/AgCl.

FIA measurement of reaction products and HRP activity

The response of the two HRP reaction products [TMB (ox) and benzoquinone] in the FIA system at the selected potentials was tested. As the oxidised form of TMB was not commercially available, it was produced enzymatically using H_2O_2 five times more concentrated than TMB. The production of the oxidised TMB was monitored by the measurement of the absorbance at 450 nm until the value remained constant. The concentration of TMB (ox) was calculated using a constant absorbance value and the molar absorptivity.[49] The concentration obtained was in agreement with that expected.

Using a 5 μl loop and a flow rate of 200 μl min^{-1}, an RSD of 10% was observed for consecutive injections of TMB (ox), and 2–3% when a 1 min wash at a high flow rate was carried out between the injections. This was confirmed by the flow rate study shown in Fig. 4; the sensitivity and RSD decreased in the range 200–800 μl min^{-1}. A current signal about four times higher was obtained on replacing the 5 with a 20 μl sample loop. This loop and a flow rate of 800 μl min^{-1} were selected for the measurement of TMB (ox). Benzoquinone, the HRP reaction product of the hydroquinone, was detected using a 20 μl loop and a 200 μl min^{-1} flow rate. As shown in Fig. 5, the linearity range was 5×10^{-8}–1×10^{-3} mol l^{-1} with a sensitivity of 36 nA μmol l^{-1} and 5×10^{-8}–5×10^{-5} mol l^{-1} with a sensitivity of 16 nA μmol l^{-1} for benzoquinone and TMB, respectively (higher concentrations of benzoquinone were not soluble in the working buffer). The response (oxidation) of *p*-aminophenol (product of the reaction of PAPP in the presence of AP) at the applied potential of +100 mV *versus* Ag/AgCl is reported in Fig. 5(B) for comparison. The sensitivity was similar to that obtained with TMB (ox) and the linearity range was 10^{-7}–10^{-4} mol l^{-1} in 0.1 mol l^{-1} diethanolamine (DEA) buffer (pH 8.0). The optimum concentration of substrates for the measurement of HRP activity was then studied. The current change was recorded after 1 min of incubation with 10^{-3} U ml^{-1} of HRP and plotted for different substrate to H_2O_2 ratios. The results for TMB, reported in Fig. 6, indicated that the highest current output was achieved using 2×10^{-4} mol l^{-1} TMB and 10^{-3} mol l^{-1} H_2O_2. The apparent K_m for H_2O_2 under these experimental conditions was 5.4×10^{-4} mol l^{-1} (V_{max} = 355 nA min^{-1}). These parameters were not calculated for TMB because of the limited solubility in the working buffer (3×10^{-4} mol l^{-1}). It should be noted that when working at the selected concentration of H_2O_2 (10^{-3} mol l^{-1}) a twofold increase in current was obtained on switching from 1×10^{-4} to 2×10^{-4} mol l^{-1} TMB (Fig. 6). This indicates that 2×10^{-4} mol l^{-1} TMB is not the ideal substrate concentration. The sensitivity of the system could be improved by increasing the solubility of TMB provided that no electroactive compounds are

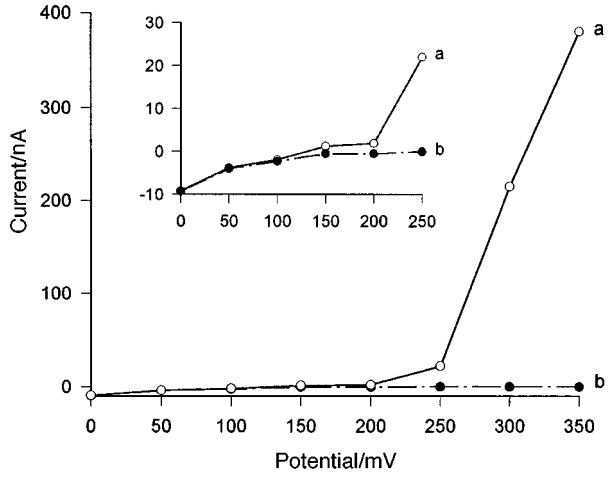

Fig. 3 (a) FIA hydrodynamic voltammetry of 2×10^{-4} mol l^{-1} TMB in 0.1 mol l^{-1} citrate–phosphate buffer and (b) background current value. Injection loop, 5 μl; flow rate, 200 μl min^{-1}. Inset, expanded scale.

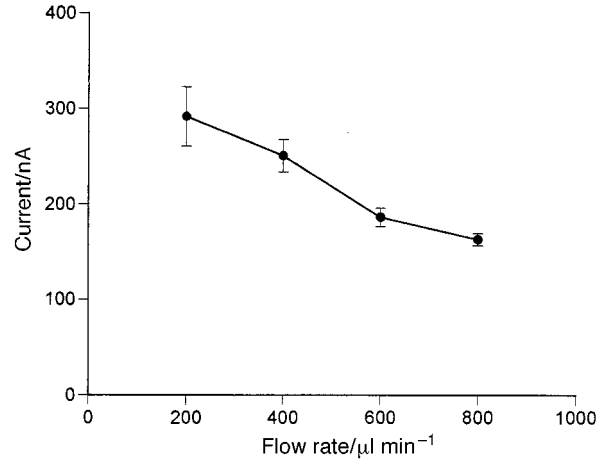

Fig. 4 Response of 5×10^{-5} mol l^{-1} TMB (ox) at different flow rates. Injection loop, 5 μl; applied potential, +100 mV *versus* Ag/AgCl. Each point is the mean of nine determinations.

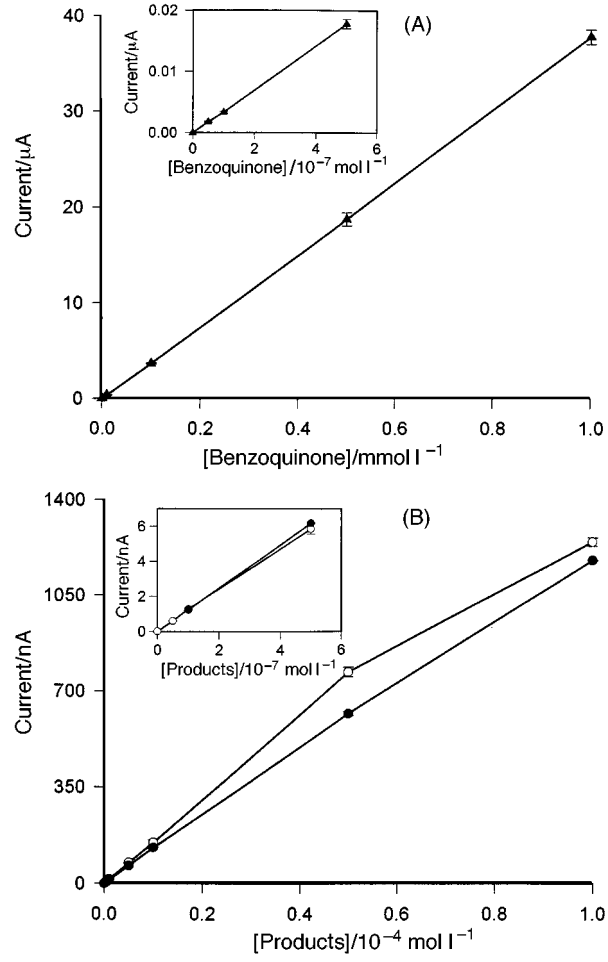

Fig. 5 (A) Calibration curve for benzoquinone. Applied potential, −250 mV *versus* Ag/AgCl; injection loop, 20 μl; flow rate, 200 μl min^{-1}. (B) Calibration curves for (○) TMB (ox) and (●) PAP. Flow rate, (○) 800 μl min^{-1} and (●) 200 μl min^{-1}; injection loop, 20 μl.

used. The optimum concentration for hydroquinone was found to be 10^{-3} mol l^{-1} with a 1 : 1 molar ratio of H_2O_2 owing to the spontaneous reaction of the substrates.

Calibration curves for HRP were constructed for a 15 min incubation of the enzyme with the substrate mixture at room temperature. Under the optimum conditions it was possible to measure as low as 1.7×10^{-12} mol l^{-1} HRP (10^{-4} U ml^{-1}) with hydroquinone [Fig. 7(A)] and 8.5×10^{-14} mol l^{-1} HRP (5×10^{-6} U ml^{-1}) with TMB [Fig. 7(B)]. Using TMB as the enzymatic substrate, concentrations of HRP higher than 8.5×10^{-12} mol l^{-1} gave currents outside the linearity range obtained for TMB (ox). Although the electrochemical detection of benzoquinone was found to be more sensitive than that of TMB (ox) (Fig. 5), TMB appears to be more suitable for use as a substrate for HRP determination because of its limited spontaneous reaction with H_2O_2 (blanks after 15 min: TMB and H_2O_2 = 4 nA, hydroquinone and H_2O_2 = 300 nA; Fig. 7). Spontaneous oxidation of HRP substrates by H_2O_2 has already been observed in other studies.[19,44] Using 10^{-3} mol l^{-1} PAPP at +100 mV *versus* Ag/AgCl in 0.1 mol l^{-1} DEA buffer (pH 9.0), we were able to detect 4.5×10^{-15} mol l^{-1} of AP (2×10^{-6} U ml^{-1}) after 15 min of incubation (data not shown).

The enzyme–substrate incubation time in ELISA ranges from a few minutes to 1 h or more depending on the analysis time and sensitivity required. Hence the stability of the reagents under the working conditions is one of the major issues to be addressed when developing new ELISA methods. It has been already reported that *p*-aminophenol is not stable in alkaline media.[31] We observed a decrease of 35% in the electrochemical signal after 30 min in 0.1 mol l^{-1} DEA buffer (pH 9.5) and of 23% in DEA (pH 9.0), whereas the signal was stable in the pH range 8–8.5. The highest sensitivity for the measurement of AP activity was attained by performing the enzymatic reaction (with 10^{-3} mol l^{-1} PAPP) at pH 9.0 and then measuring at pH 8.0 (by addition of HPO_4^{2-}). TMB was stable in the working buffer [0.1 mol l^{-1} citrate–phosphate (pH 5.0)] for at least 2 h; TMB (ox) was stable for 30 min; a 15% decrease in the signal was observed after 45 min.

Electrochemical ELISA

The reaction of TMB with H_2O_2 in the presence of HRP gives rise to a coloured product that is measured at 655 or 450 nm after H_2SO_4 blockage.[40] A comparison of chromogenic substrates for HRP as the label in enzyme immunoassays demonstrated that TMB gave a high sensitivity, comparable to that of a fluorimetric method using 3-(*p*-hydroxyphenyl)propionic acid.[13] A model system using rabbit IgG as analyte in an ELISA competition format has been developed; assay conditions are reported in the Experimental section. Calibration curves for rabbit IgG in the 5×10^{-3}–10 μg ml^{-1} range were obtained with both amperometric and spectrophotometric detection (Fig. 8). The calibration curves exhibited similar sensitivity (expressed as the amount of IgG needed to displace 50% of the antirabbit IgG conjugate): 0.51 μg ml^{-1} for the spectrophotometric and 0.59 μg ml^{-1} for the amperometric method. However, a 30 min incubation time was used in the former and 15 min in the latter method. Hence, using TMB as the HRP substrate, the amperometric procedure is more efficient than the spectrophotometric procedure.

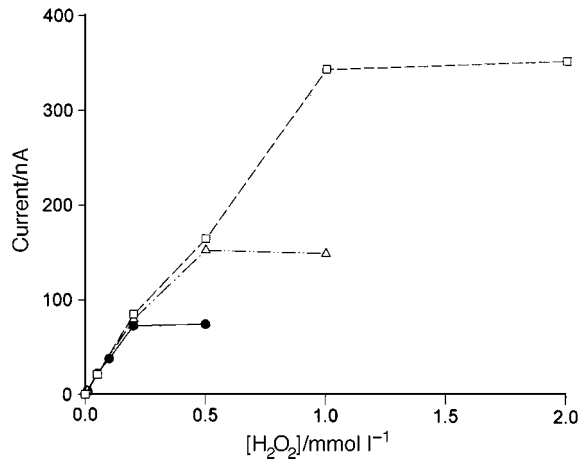

Fig. 6 Current response after addition of HRP (10^{-3} U ml^{-1}; incubation time, 1 min) *versus* H_2O_2 concentration. Concentration of TMB; (●) 5×10^{-5}; (△) 1×10^{-4}; and (□) 2×10^{-4} mol l^{-1}.

Conclusions

The use of TMB as an HRP substrate for ELISA with electrochemical detection has been investigated. This substrate exhibited better performance in terms of sensitivity than other substrates already used for the amperometric detection of HRP activity. The detection limit for HRP activity was of the same order of magnitude as that with the PAPP–AP electrochemical system at the same applied potential. This is very important for the future development of immunosensors for measurements in the field; in fact, using a carbon working electrode at an applied potential of +100 mV *versus* Ag/AgCl, both AP- and HRP-based ELISAs can be carried out.

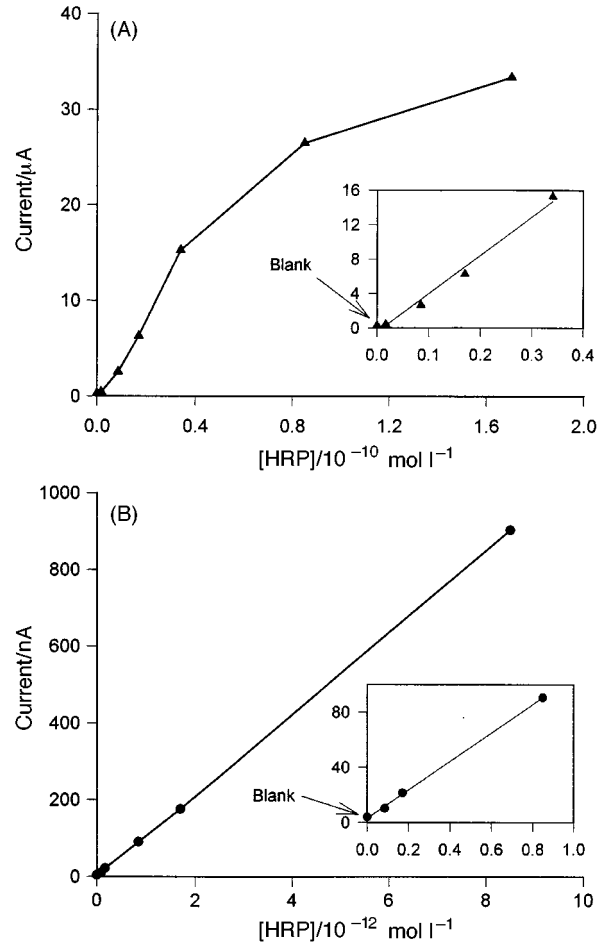

Fig. 7 Calibration curves for HRP obtained after 15 min of incubation of the enzyme (A) with 10^{-3} mol l^{-1} hydroquinone + 10^{-3} mol l^{-1} H_2O_2 and (B) with 2×10^{-4} mol l^{-1} TMB + 10^{-3} mol l^{-1} H_2O_2. Insets, expanded scales.

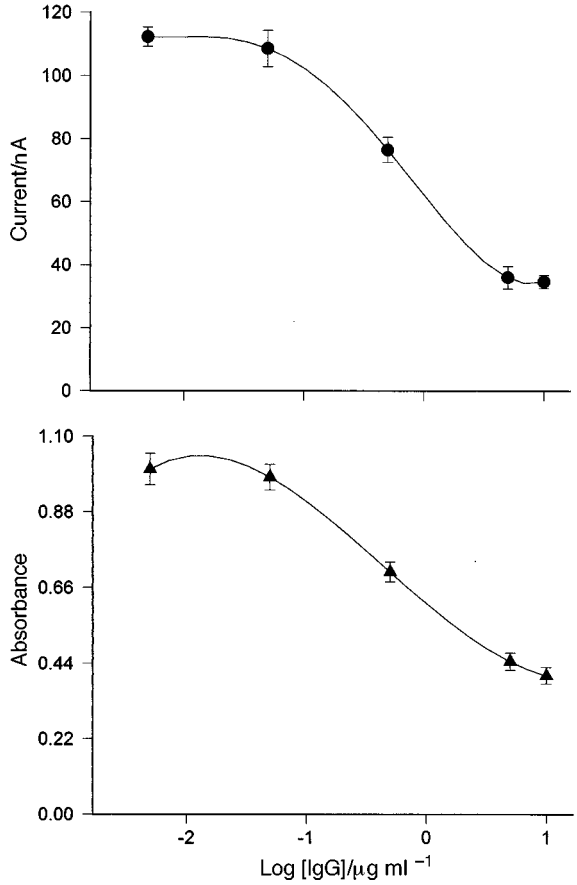

Fig. 8 Calibration curves of rabbit IgG in the range 5×10^{-3}–10 μg ml^{-1} obtained with (●) amperometric and (▲) spectrophotometric detection. Each point is the mean of four determinations.

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