A new peroxidase from garlic (*Allium sativum*) bulb: its use in H_2O_2 biosensing

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Peroxidase POX_1 isoenzyme was purified from garlic (*Allium sativum* L.) bulb by ammonium sulfate precipitation, gel filtration and anion-exchange chromatography. Native-PAGE profile showed two isoforms, designated POX_{1A} and POX_{1B}. POX_{1B} seems to be more attractive for biosensor design since its K_m (app) for H_2O_2 is lower than that of POX_{1A}. In addition to its storage and operational stability, POX_{1B} was found to be highly heat-stable, since almost 70% of its activity was conserved at 60°C, whereas full activity was retained at 50 and 40°C for 40 min. The optimal pH was approx. 5 and the optimal temperature was 30°C. Next, gelatin was used as a matrix for enzyme immobilization on a gold electrode surface and electrochemical measurements were performed by using cyclic voltammetry. POX_{1B}-based electrodes show great potential for application in H_2O_2 monitoring of biological samples.

Introduction

Peroxidases (EC 1.11.1.7) are widely distributed throughout plants. These haem-containing enzymes catalyse the oxidation of substrate by H_2O_2 [1]. They contain Fe(III) protoporphyrin IX (ferriprotoporphyrin IX) as a prosthetic cofactor. On the basis of sequence alignments and biological origin, three classes can be distinguished. The first one contains bacterial peroxidase (EC 1.11.1.6) and yeast cytochrome *c* peroxidase (EC 1.11.1.5), as well as plant ascorbate peroxidases (EC 1.11.1.11). The second and third classes (EC 1.11.1.7) include fungal and plant peroxidases respectively [2]. It has been demonstrated that peroxidases are involved in physiological processes (such as lignification, suberization, cell elongation and growth), in stress-related physiological processes (including browning [3], wound healing [4] and disease resistance [5–7]) as well as in plant–pathogen interactions [8–10]. Numerous cationic and anionic peroxidase isoforms have been described in the literature. However, research publications have been mainly devoted to the HRPC [cationic isoenzyme of HRP (horseradish peroxidase)]. Peroxidases have found many applications in biotechnology. They have been used as an important reagent for clinical diagnosis, various enzymatic assays, wastewater treatment to remove phenolics [11], synthesis of various aromatic compounds and removal of peroxides from foodstuffs, beverages and industrial wastes [12,13].

Novel applications in biosensor design have aroused much interest. Amperometric peroxidase-modified electrodes have been developed for the detection of H_2O_2, organic hydroperoxides, phenols and aromatic amines. The determination of H_2O_2 is especially of practical importance in chemical, biological and clinical as well as many other fields. These analytical devices combine speed, selectivity and sensitivity. Amperometric measurements are made by applying a potential between a reference and a working electrode, which allows one to measure a current when a electro-active analyte is oxidized or reduced, depending on the voltage at the working electrode [14]. The problem of electron transfer between the enzyme's active centre and the electrode makes the enzyme immobilization on to the electrode the key factor for the successful operation of electrochemical biosensors [15]. Peroxidases have been immobilized on various materials, such as chitosan [16,17], agar [18], alginate [19], carrageenan [20], nafion [21], TCAP (5,2′:5′,2″-terthiophene-3′-carboxylic acid polymer) [22], DDAB (didodecyldimethylammonium bromide) [23], Eastman AQ polymer [24] and epoxy Sepharose matrix [25]. There have been only a few reports on the use of gelatin as a matrix (e.g. [26]).

Many materials have been used for electrode fabrication [26–29]. Gold is very interesting in this regard, since it is chemically inert, easily modified (after 'piranha' cleaning) to be highly hydrophobic and favourable for direct electron transfer [30]. Many immobilization strategies are

Key words: amperometric measurement, biosensor, garlic (*Allium sativum*) peroxidase, gelatin matrix, gold electrode, hydrogen peroxide (H_2O_2).

Abbreviations used: CV, cyclic voltammogram; DAB, diaminobenzidine; HRP, horseradish peroxidase; R.S.D., relative standard deviation.

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known [31–35]. Peroxidases of different origin have been increasingly investigated as substitutes for HRP in order to enhance peroxidase-based electrode performances and to reduce its cost [36–38].

In the present paper, we describe the purification and some biochemical properties of a new peroxidase called POX_1 from garlic (*Allium sativum*) bulbs. POX_{1B} isoform with interesting properties such as great affinity for substrate, heat-stability, storage and immobilization in gelatin, was used for peroxidase-based gold electrode construction.

Experimental

Biological materials and reagents

Fresh garlic bulbs were obtained from a local market. They were picked and selected to discard any damaged ones.

Guaiacol, *o*-dianisidine, DAB (diaminobenzidine) and 3-amino-9-ethylcarbazol were obtained from Sigma. H_2O_2 (30%, v/v) was supplied by Prolabo. All chemicals were of the highest grade commercially available.

Crude extract preparation

Garlic bulbs were ground with an electric blinder. The smooth paste was crushed in the presence of liquid nitrogen until a fine powder was obtained, which was then homogenized in 25 mM Tris/HCl buffer (pH 7.5) containing 0.1 mM PMSF and 2 mM EDTA at 4°C overnight. After filtration, the extract was centrifuged (12000 ***g*** at 4°C for 20 min) in a high-speed brushless centrifuge (MPW-350R; MED Instruments), and the supernatant, considered as crude extract, was conserved for further enzyme purification.

Determination of proteins

Protein content was determined by the Bradford method [39] using BSA as the standard.

Enzyme assay

Peroxidase activity was measured by a continuous spectrophotometric quantification of oxidation products of *o*-dianisidine at 460 nm in the presence of H_2O_2, at optimal temperature (Beckman DU 640 B spectrophotometer, equipped with a temperature regulator). The final reaction mixture contained 0.033% (v/v) or 10 mM H_2O_2 and 0.25% (v/v) or 0.79 mM *o*-dianisidine in 100 mM sodium acetate buffer (pH 5) and suitable amounts of enzyme. All measurements were carried out within 5 min at 10 s intervals. One unit of activity is defined as the amount of enzyme that increases the absorbance by 1 unit per min [40].

Purification procedure

All steps were performed at 4°C. The crude extract proteins were precipitated with ammonium sulfate at 80% saturation. The pellet was collected by centrifugation at 12000 ***g*** for 60 min and dissolved in 25 mM Tris/HCl (pH 7.5). Gel-filtration chromatography was performed on a Sephacryl S200-HR column (90 cm long × 4 cm diameter; Sigma) equilibrated with 25 mM Tris/HCl buffer (pH 7.5). Elution was performed at a flow rate of 15 ml/h; fractions (2 ml each) were collected. Fractions that contain peroxidase activity were pooled. Anion-exchange chromatography was performed on a DEAE-Sepharose CL 6B column (11 cm × 3 cm; Sigma), which was equilibrated with 25 mM sodium acetate buffer (pH 7.5). Elution was carried out at a flow rate of 10 ml/h with 200 ml of NaCl gradient (0–1 M) in the same buffer. Fractions (2 ml) were collected. Active fractions tested by the *o*-dianisidine method were analysed by anodic native PAGE.

Gel electrophoresis

Protein extracts were mixed with 50% glycerol and 0.02% Bromophenol Blue in 25–192 mM Tris-glycine buffer (pH 8.3). Electrophoresis was performed at 4°C under non-constant current in 25–192 mM Tris/glycine buffer (pH 8.3). Peroxidase activity was revealed on the gel as described by Medjeldi Marzouki et al. [25], with some modifications. The gel was incubated in 0.1 M sodium acetate buffer (pH 5). Afterwards, it was transposed for 10–15 min in the same buffer containing freshly guaiacol solution (0.3%, v/v). The gel was re-incubated in a buffer solution containing 0.3% (v/v) guaiacol, 0.1% (v/v) H_2O_2, 0.02–0.05% (w/v) DAB or 3-amino-9-ethylcarbazol. Enzyme activity bands were allowed to develop within 5–10 min and then the gel was washed with distilled water and scanned.

Enzyme characterization

Enzyme characterization was carried out by submitting it to the following conditions and measuring the remaining activity against the substrate *o*-dianisidine as previously described [40]. Optimal temperature was determined by measuring peroxidase activity over a broad range from 4 to 75°C at pH 7 (standard condition). Then, optimal pH was measured by testing peroxidase activity in the following buffers: 25 mM sodium acetate buffer (pH 3.5–5), sodium phosphate buffer (pH 5–7) and Tris/HCl buffer (pH 8–9) at optimal temperature. Activity was also examined at various salt concentrations from 0 to 250 mM NaCl in 25 mM sodium acetate (pH 5) buffer. Thermal stability was analysed by measuring the remaining activity after heating the enzyme solution in 25 mM sodium acetate buffer (pH 5) at three temperatures (40, 50 and 60°C) during different times. The enzyme activity was then measured as indicated in the 'Enzyme assay' subsection above. Storage stability at three temperatures (–20, 4 and 25°C) was examined by measuring the remaining activity, every week during 2 months.

Kinetics

The apparent Michaelis constants for H_2O_2 as the oxidant substrate and *o*-dianisidine as the reducing substrate were

determined by varying H_2O_2 concentration from 0.1 to 10 mM using an *o*-dianisidine concentration of 0.79 mM. Then, *o*-dianisidine concentration was varied from 0.079 to 0.79 mM in the presence of H_2O_2 at a saturation concentration of 10 mM in order to determine the K_m (app) for *o*-dianisidine. The K_m (app) for guaiacol as the reducing substrate and H_2O_2 as the oxidant were obtained by varying guaiacol concentration from 4.5 to 27 mM in a 1 ml reaction mixture containing 965 μl of sodium acetate buffer (0.1 M; pH 5), 3 μl of guaiacol and 2 μl of H_2O_2 (30%, v/v) [41]. These series of experiments were carried under optimal conditions (pH, ionic strength and temperature) (determined previously). K_m and V_{max} for each substrate was calculated from Lineweaver–Burk plots of $1/v_0$ versus $1/[S_0]$. Molar absorption coefficients (ϵ) of 11.3×10^3 and 3.8×10^3 $M^{-1} \cdot cm^{-1}$ were used respectively for *o*-dianisidine and guaiacol. For the free and the immobilized POX_{1B} enzyme, V_{max} values for H_2O_2 were expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$. All assays were performed in duplicate.

Effect of gelatin percentage on immobilized POX_{1B} activity

A gelatin solution at different concentrations, 8, 12 and 16%, was first prepared in 100 mM sodium acetate buffer (pH 5). An equal volume of the enzyme was taken from the pooled fractions after the DEAE-Sepharose CL 6B chromatography process and was mixed with the solubilized gelatin to obtain suitable percentages (4, 6 and 8%). The obtained mixture was left to solidify for 1–2 min at –20 °C.

Gold electrode preparation

Gold electrodes were used for electrochemical analysis. The gold surface was cleaned in an ultrasonic bath for 5 min in acetone and then for 5 min in pure ethanol and finally immersed for 1 min in a 'piranha solution' [3:1 (v/v) 98% H_2SO_4/30% H_2O_2] (**the solution can be explosive and needs to be handled with great care**). It was then rinsed two or three times with ultrapure water and dried under a nitrogen flow [42].

Enzyme immobilization

A gelatin solution was first prepared in 100 mM sodium acetate buffer (pH 5). A volume of 10 μl was taken from a mixture containing 0.5 ml of enzyme solution (45 units/mg), 0.5 ml of solubilized gelatin at adequate concentration and 20 μl of formaldehyde that we drop on the gold electrode surface. The same experiment was performed using the HRP enzyme with the same specific activity (45 units/mg). The electrode was stored at 4 °C for at least 24 h [43].

Electrochemical setup

Amperometric measurements with gold electrodes were performed in a three-electrochemical-cell set-up. The cell contained a calomel reference electrode, a platinum auxiliary electrode and a gold electrode as the working electrode. Electrochemical measurements were carried out with a Voltalab40 (Radiometer Analytical, Lyon, France) three-electrode potentiostat equipped with Voltmaster4 software. In the voltammograms obtained, one cycle corresponds to almost 1 min under the applied potential (–1 and 1 V). The current obtained after applying the same potential to an electrode coated with just gelatin was considered as the negative control. All experiments were performed at 25 °C [44] and in a Faradic box.

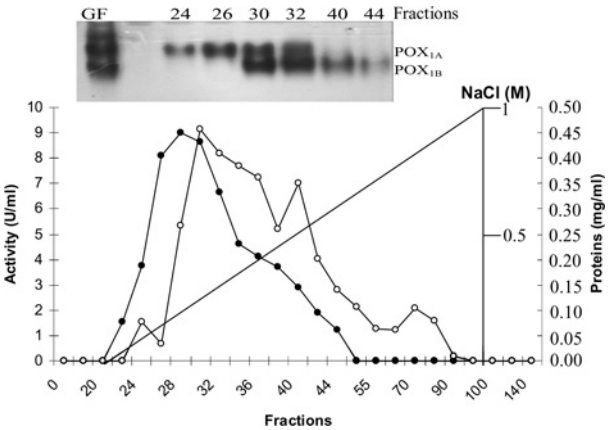

Figure 1 Elution profile of garlic peroxidase POX_1 obtained after DEAE-Sepharose CL6B chromatography

The column was equilibrated with 25 mM Tris/HCl buffer (pH 7.5). The Bradford method was used for protein content determination and peroxidase activity was measured as described in the Experimental section: ●, activity [units(U)/ml]; ○, proteins (mg/ml). The inset shows the native anodic PAGE (10% polyacrylamide gel) profile of garlic peroxidase isoenzymes; GF, pool of POX_1 after gel filtration; 24–44, anion-exchange-chromatography fractions; 24–26, POX_{1A}; 30–32, POX_{1A} and POX_{1B}; 40–44, POX_{1B}.

Results and discussion

Purification of POX_1

Two major peaks of peroxidase activity from garlic (designated POX_1 and POX_2) were reported previously [25]. We applied the same protocol to purify POX_1. The pooled active fractions (26 units/mg) of the first peak obtained from the gel-filtration column were loaded on to an anion-exchange column (DEAE-Sepharose CL 6B). Peroxidase activity was eluted with an NaCl gradient (0–1 M) into a single peak at approx. 250 mM NaCl.

The native anodic PAGE profile of POX_1 step DEAE-Sepharose CL 6B is shown in Figure 1. Two isoforms were revealed, designated POX_{1A} and POX_{1B}. We resolved POX_1 into two isoforms that migrated differently when native 10% acrylamide gel was used for staining peroxidase activity (Figure 1). POX_{1A} fractions (22–26; 105 units/mg)

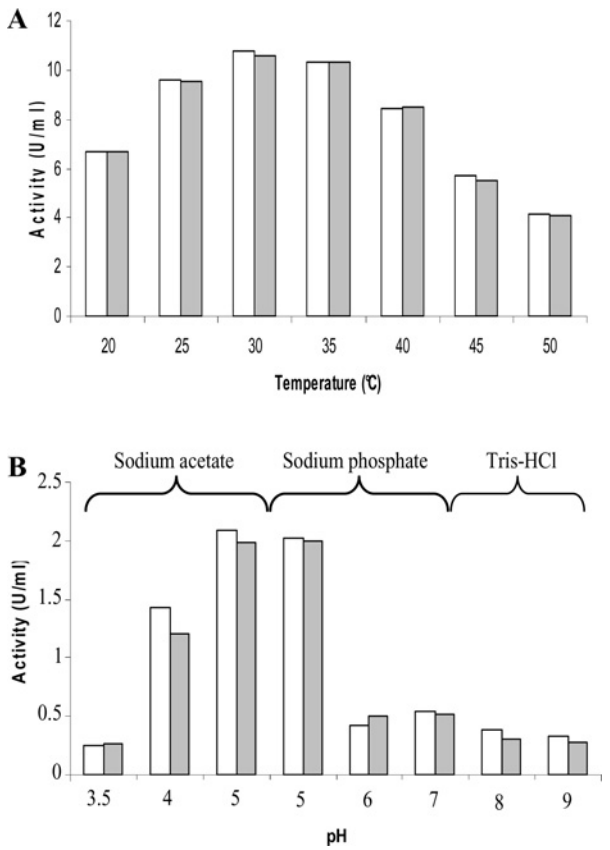

Figure 2 Effect of temperature and pH on POX_{1B} activity

(**A**) Effect of temperature on POX_{1B} activity; optimal temperature was determined by measuring peroxidase activity by using *o*-dianisidine as the substrate over a broad range from 4 to 75 °C under standard conditions (pH 7) and then at optimal pH. The Figure shows the measured peroxidase activities when temperature was varied between 20 and 50 °C. For the other temperatures, the obtained activities were lower; bar, POX_{1B}; POX_{1A}. (**B**) Effect of pH on POX_{1B} and POX_{1A} activity; optimal pH was measured by testing peroxidase activity using *o*-dianisidine as the substrate in the following buffers: 20 mM sodium acetate (pH 3.5–5.5), sodium phosphate (pH 6–7), Tris/HCl (pH 8–9) at optimal temperature; open bar, POX_{1B}; closed bar, POX_{1A}.

and POX_{1B} fractions (36–44; 18.4 units/mg) were pooled separately and used for further experiments.

Biochemical characterization of POX_{1B}

The optimal temperature of POX_{1B} activity is approx. 30 °C (Figure 2A). This value is also optimal for POX_{1A}. There is no considerable variation of POX_{1B} activity for temperatures between 25 and 35 °C. This value is less than that reported for POX_2 [25], which was between 35 and 40 °C. It is also far less than optimal temperatures reported for isoperoxidases from turnip [C1 (40 °C), C2 (45 °C) and C3 (55 °C)] [44] and for HRP (45 °C) [48]. The optimal pH for POX_{1B} enzyme was determined to be approx. 5 against the substrate *o*-dianisidine. The same value was obtained for POX_{1A} (Figure 2B). This value is similar to that reported for POX_2 enzyme [25] as well as for cationic and anionic isoperoxidases from turnip [44] and the six isoperoxidases purified from Korean radish (*Raphanus sativus*) (A1, A2, A3n, A3, C1 and C3), which have an optimal pH of approx. 4.5 and 5.0 [45]. It is not far from that of palm-tree (*Phoenix dactylifera*) peroxidases (5.4) [46] but it is less than the optimum (pH 6.6) reported for *Opuntia ficus-indica* (Indian fig cactus) [47]. The optimal pH for HRP activity is between 7.5 and 8.6 [48]. The commercially available enzyme (for example, HRP commercialized by Toyobo Enzymes: PEO-131, PEO-301 and PEO-302) presents an optimal pH varying between 6 and 7, but it is stable for pH between 5 and 10 (20 h at 25 °C).

POX_{1B} activity was slightly affected by NaCl concentrations from 0 to 250 mM in contrast with POX_2, which was inhibited when NaCl concentration exceed 50 mM [25]. In this way, the presence of salts in the sample does not affect the reliability of the dosage protocol. This has also been demonstrated in some previous studies [47,49].

Effect of soluble gelatin was measured first on free POX_{1B} activity. Peroxidase activity decreased, but diminution was negligible when gelatin was used until a certain percentage (4%). The stability of POX_{1B} enzyme is of interest for use in an immobilization protocol involving its inclusion in a 4% gelatin matrix.

Kinetics

The K_m (app) values for H_2O_2 obtained for POX_{1A} and POX_{1B} in the presence of *o*-dianisidine were respectively approx. 3 and 0.5 mM. This result suggests that POX_{1B} has better affinity for H_2O_2 than POX_{1A}. For POX_{1B}, the K_m (app) values for *o*-dianisidine and guaiacol were respectively 0.2 and 5.5 mM. The V_{max} values were respectively 0.56 and 31.8 mM · min^{-1}. The K_m (app) value for *o*-dianisidine was less than those reported for isoperoxidases extracted from the Korean radish (C_s: 1.18 mM; C_1: 0.81 mM; C_3: 1.2 mM). For H_2O_2, the K_m (app) value determined in the presence of *o*-dianisidine was less than that of C_s (1.27 mM) and C_3 (0.77 mM) but higher than that of C_1 (0.19 mM) [50]. The K_m (app) value for guaiacol is less than that reported for palm-tree peroxidases (12.5 mM) [46] and for POX_2 (9.5 mM) [25]. In a previous work, a comparison between peroxidase activities from *O. ficus indica* in the presence of three substrates (guaiacol, 4-chloro-1-naphtol and *o*-dianisidine) was performed [47]. Results showed that *o*-dianisidine is the most recognized substrate to test peroxidases, even if guaiacol is the most used substrate in plant peroxidases monitoring. This catalytic behaviour may be explained by several factors such as the highest concentrations of secondary metabolism products [8].

Thermal stability

Peroxidase stability assays at 40, 50 and 60 °C showed good thermal stability (Figure 3). In fact, POX_{1B} retained full activity

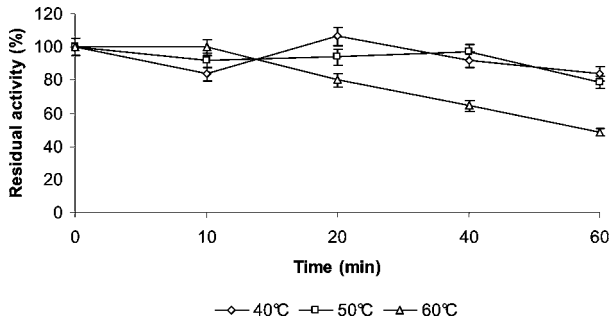

Figure 3 Heat inactivation of free POX_{1B} at 40, 50 and 60 °C over time

The enzyme was tested against *o*-dianisidine as the substrate as indicated in the Experimental section. The remaining activity was measured as indicated in the Experimental section. It was expressed as a percentage of the initial enzyme activity. The displayed points per dataset represent the average of the residual activity obtained after three replicates (represented by deviation bars) for each enzyme.

after 40 min incubation at 40 °C as well as at 50 °C. At 60 °C, 90% of activity was retained after 20 min, but 70% of the initial activity was lost after 40 min incubation. POX_{1B} exhibits the same stability against temperature as HRP [51–53]. However, POX_{1B} is more stable than POX_2, since this enzyme lost 50% of its activity after 15 min incubation at 60 °C [25]. POX_{1B} enzyme is also more stable than palm peroxidases, which can retain 58% of their activity after 30 min at 50 °C. Peroxidases from *O. ficus indica* can retain 40% of their activity after 10 min incubation at 70 °C, whereas for other members of Cactaceae, such as *Pachycereus pringlei* (Mexican giant cactus), there is total inactivation [54]. Thermal inactivation of peroxidases from hairy-root cultures of red beet (*Beta vulgaris*) extracted at acidic and neutral pH was negligible up to 50 °C, with almost 95% of the activity being retained, even after 40 min. Peroxidases with basic pH were very sensitive to temperature, with 50% loss at 50 °C in 40 min and with total loss of activity at 60 °C. At the latter temperature (60 °C), the acidic and neutral peroxidases retained more than 70% of the activity up to 40 min, whereas these enzymes were completely inactivated at 70 °C [39].

Storage stability

POX_{1A} and POX_{1B} were incubated at –20, 4 and 25 °C (Figures 4A and 4B). Results showed that POX_{1B} is more stable on storage than is POX_{1A}. In fact, POX_{1B} retained full activity after almost 2 months when stored at –20 and 4 °C. POX_{1B} activity slightly decreased when stored at 25 °C. However, POX_{1A} did not retain full activity except when it was frozen. Freezing seems to have a beneficial impact on the enzyme, since full activity was retained for both enzymes. However, after the same duration, 50 and 80% of the initial POX_{1A} activity was lost at 4 and 25 °C respectively.

Immobilization

The effect of gelatin was tested first on free POX_{1B} activity. Peroxidase activity decreased slightly, but diminution was negligible when gelatin is used, until a certain concentration, namely 4%. Thus three gelatin percentages (4, 6 and 8%) were chosen to immobilize POX_{1B} activity. Almost full activity was retained when a percentage of 6% was used. This percentage was applied in further immobilization experiments.

Immobilized enzyme showed a better affinity for H_2O_2 than the free one. In fact, immobilized POX_{1B} in gelatin has a K_m (app) value for H_2O_2 in the presence of *o*-dianisidine of almost 0.13 mM, which is far less than that of the free enzyme ($K_m = 0.5$ mM). For the immobilized POX_{1B}, the V_{max} value (3.51 $\Delta A \cdot min^{-1} \cdot ml^{-1}$) was higher than that of the free enzyme ($V_{max} = 2.06\ \Delta A \cdot min^{-1} \cdot ml^{-1}$). The enhanced affinity may be explained by a favourable change in the structural organization of the enzyme due to the immobilization procedure [55]. Consequently, the active sites of the enzymes could be more readily available for enzymatic interactions.

Storage experiments showed a remarkable stability of immobilized POX_{1B} in gelatin. The enzyme retains full activity after 1 month conservation at 4 °C (Figure 4C).

POX_{1B} enzyme exhibits interesting optimal operating conditions since it is highly active at 30 °C and at acidic pH. Kinetics showed that POX_{1B} enzyme had good affinity towards H_2O_2, *o*-dianisidine and guaiacol. In addition, good thermal and storage stability was observed at different temperatures. A simple immobilization method using gelatin showed that immobilized POX_{1B} exhibits an enhanced affinity towards H_2O_2 in the presence of *o*-dianisidine which permits a better sensitivity of measurement. The fixed enzyme preserved full activity when stored at 4 °C. All these properties make POX_{1B} an interesting candidate for a peroxidase-based biosensor designed to measure H_2O_2 in biological samples.

Electrochemical studies

The immobilization experiments were performed with native POX_{1B} using gelatin as a matrix. To study the electrocatalytic behaviour of the gelatin-immobilized POX_{1B} electrode towards H_2O_2, CVs (cyclic voltammograms) were used. With increasing the scan rate, the CV peak currents of the POX_{1B}-modified electrode were enhanced. The peak potentials are kept constanti over the scan-rate range of 50–100 mV/s (results not shown). A scan rate of 100 mV was used for all experiments. The CV of the POX_{1B}-electrode was recorded between –1 and + 1 V (Figure 5). In the presence of 10 mM H_2O_2 (saturating concentration), the anodic (oxidation) peak at + 300 mV increases significantly, and the cathodic (reduction) peak decreases slowly. This result indicates a typical H_2O_2 electrocatalytic redox process. No change in the anodic (respectively cathodic) peak current was observed when a saturating H_2O_2 concentration was added

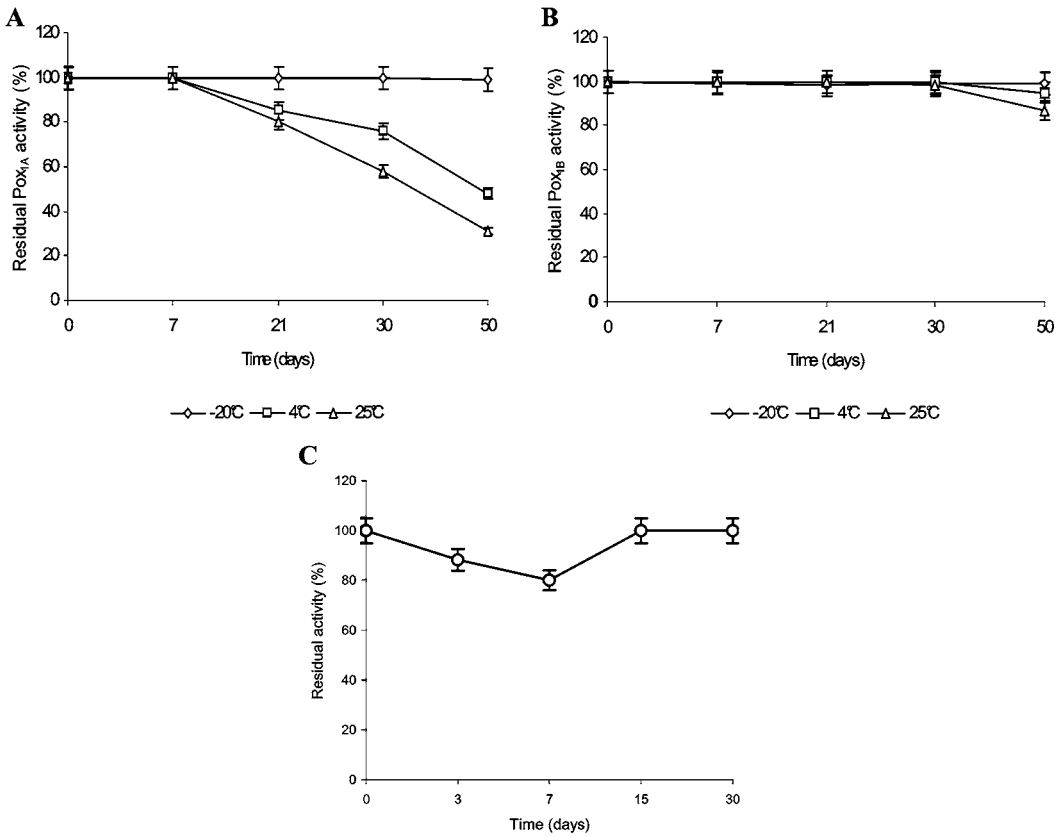

Figure 4 Effect of storage on POX_{1A} and POX_{1B} activities

(**A**) Free POX_{1A}; (**B**) free POX_{1B}. The remaining activity was tested by using *o*-dianisidine as the substrate, as indicated in the Experimental section and expressed as a percentage of the initial enzyme activity. (**C**) Effect of storage at 4 °C on 6% gelatin-immobilized POX_{1B}. Enzyme matrix corresponding to 6% gelatin and 18.4 units/ml was stored at 4 °C for different time periods. The remaining activity was measured by *o*-dianisidine as the substrate as indicated in the Experimental section. The displayed points per dataset represent the average of the residual activity obtained after three replicates (represented by deviation bars) for each enzyme.

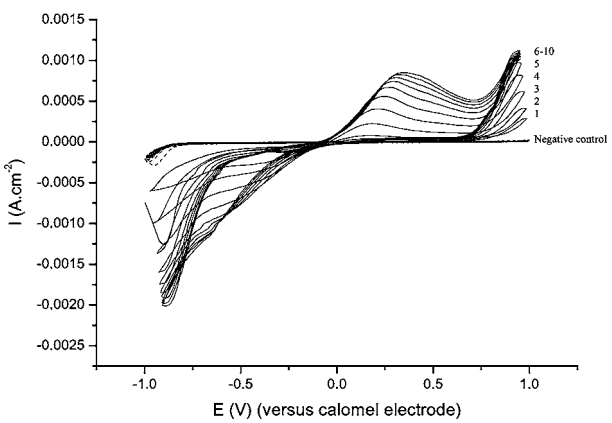

Figure 5 Voltammogram of immobilized POX_{1B} tested in the presence of H_2O_2 for different time periods

1–10, time (min); negative control, electrochemical response of the gold electrode coated with just gelatin to 10 mM H_2O_2.

to bare gold or gold modified with gelatin (negative control). Thus it can be concluded that the peak current increase is due to the kinetics of the oxidoreduction reaction on the material surface. Cyclic voltammetry has already been used to measure the response of the HRP-based biosensor to H_2O_2. A pair of well-defined and nearly reversible peaks was reported [56,57]. The POX_{1B}-based electrode showed high response at pH 5.0. Figure 5 shows that the measured current is proportional to the increase in reaction products and therefore to substrate concentration in the solution. The curve obtained is similar to those obtained with a UV–visible spectrometer (see the 'Enzyme assay' subsection).

The cyclic-voltammetric response of the biosensor in the absence and presence of H_2O_2 was used to assess the biosensor activity. After addition of H_2O_2 at concentrations varying between 1 and 8 mM, an increase in current was observed. The increase in the current depended on the added H_2O_2 concentration; interestingly, POX_{1B} has a linear activity even at a high concentration of approx. 8 mM of

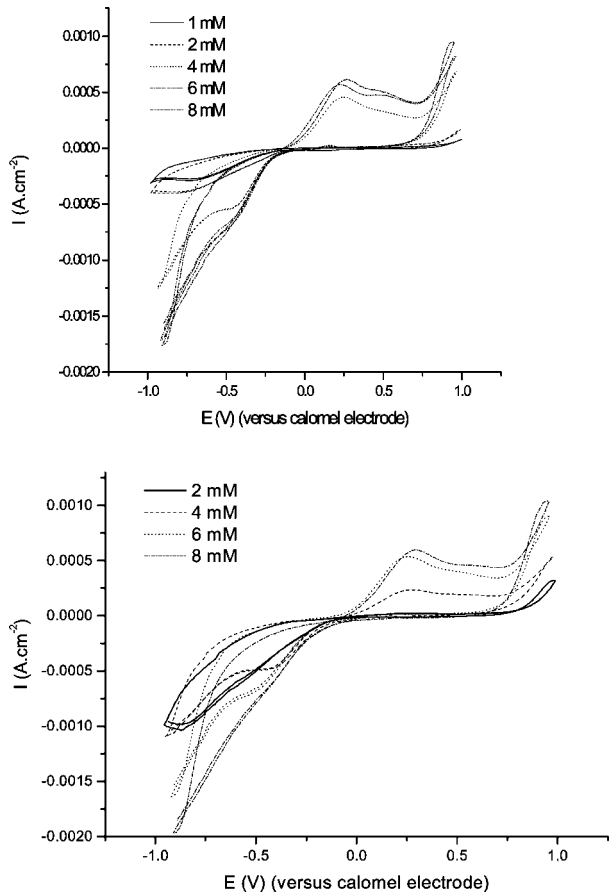

Figure 6 Enzyme cyclic-voltammetric response in the presence of H_2O_2 concentrations between 1 and 8 mM

Upper panel: POX_{1B}-based electrode; lower panel: HRP-based electrode.

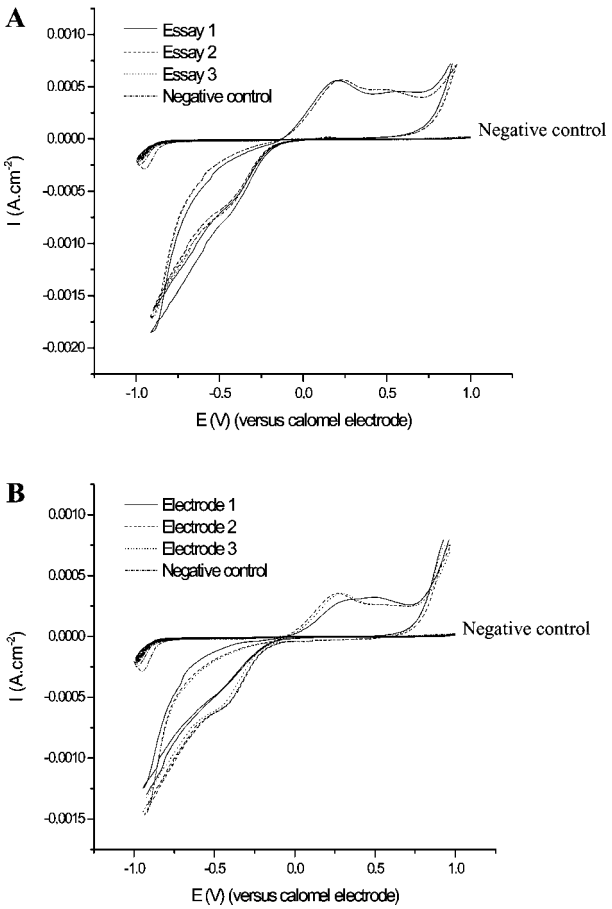

Figure 7 Cyclic voltammetry response of POX_{1B}-based biosensor in the presence of H_2O_2 as the substrate

(**A**) The same electrode is used three times; (**B**) three electrodes were used for measuring the H_2O_2 concentration.

H_2O_2 (Figure 6, upper panel). The same experiment was performed using the HRP enzyme with the same specific activity (45 units/mg). A similar electrochemical response was noticed when HRP activity decreased at elevated concentrations, 6 and 8 mM H_2O_2 (Figure 6, lower panel). When the H_2O_2 concentration exceeds 4 mM, HRP peroxidase denaturization may occur. An inactive oxidized form of the peroxidase may be obtained. However, elevated H_2O_2 concentrations must be avoided. Consequently, the advantage of using POX_{1B} enzyme as an alternative to HRP is the possibility of measuring H_2O_2 concentrations up to 8 mM in the biological sample solution. For concentrations under 1 mM, the redox couple Fe^{4+}/Fe^{3+} should be used for signal amplification.

The repeatability and reproducibility of the developed POX_{1B}-based electrode were studied. Figure 7 shows the electrochemical response obtained for two consecutive assays. The reproducibility of the current response of the proposed enzyme electrode to a solution containing 10 mM (saturating concentration) H_2O_2 was examined. The R.S.D. (relative standard deviation) was 0.5% for three successive assays. The repeatability was determined from the response of three different enzyme electrodes to a solution containing 10 mM H_2O_2. These yielded 1.2% R.S.D. for the slope of the analytical curve. The high reproducibility may be due to the high stability and efficiency of the immobilized enzyme. These results suggest that the POX_{1B}-electrode enzyme could be a reliable tool for serial tests.

Conclusion

Commercially available HRP is the most commonly used peroxidase in biosensor construction. However, new peroxidases are now offering a greater choice of substitutes with enhanced properties. In fact, the main drawback of HRP is its low stability with respect to temperature, H_2O_2 and acids. Therefore HRP biosensors are inconvenient for

the monitoring of H_2O_2 in solutions with high concentration of analyte or in samples having acidic pH, which is frequently demanded for the control of some industrial processes. In the present study, a novel garlic peroxidase (POX_1) was isolated and partially purified from *Allium sativum* L. Two isoforms were revealed (designated POX_{1A} and POX_{1B}). Special attention was devoted to POX_{1B}, which seems to be a good candidate for peroxidase-based enzyme electrode design for H_2O_2 monitoring. Immobilization using gelatin as a matrix was performed successfully.

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