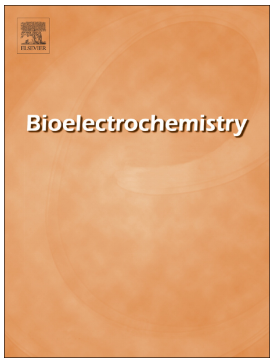

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A new peroxidase from leaves of guinea grass (*Panicum maximum*): A potential biocatalyst to build amperometric biosensors

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Abstract

A new plant peroxidase was isolated from the leaves of guinea grass (*Panicum maximum*) and partially purified using a biphasic polymer system (poly(ethylene glycol) - ammonium sulfate) followed by size-exclusion chromatography and ultracentrifugation until obtaining a homogeneous extract containing a high peroxidase activity. The novel peroxidase was characterized as having a specific activity of 408 U/mg and a molecular weight of 30 kDa. The pH for its optimum activity was 8.0 and exhibited a high thermostability at 66°C with a k_{inact} of $8.0x10^{-3}$ min^{-1}. The best substrates for peroxidase from guinea grass are *o*-dianisidine and 2,2´-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid). POD from guinea grass was directly immobilized on the surface of a graphene screen printed electrode and cyclic voltammograms in the presence of potassium ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) as a redox species demonstrated an increase in the electron transfer process. The graphene- modified electrode exhibits excellent electrocatalytic activity to the reduction of H_2O_2, with a linear response in the 100 μM to 3.5 mM concentration range and a detection limit of 150 μM. The new peroxidase from guinea grass allowed the modification of a graphene electrode providing a potential sensor detection system for determination of H_2O_2 in real samples with some biomedical or environmental importance.

Key words: Guinea grass; peroxidase; graphene; biosensor; hydrogen peroxide.

1. Introduction

Peroxidases (PODs) are included in the group of oxidoreductases (EC 1.11.1.7) due to their ability to oxidize amines and phenols by using hydrogen peroxide [1]. PODs are involved in the lignification process, cell elongation and in plant defence mechanisms [2]. They contain Fe (III) protoporphyrin IX as a prosthetic cofactor. Numerous peroxidases have been described in the literature [2], however, most of the publications have been mainly devoted to the Horseradish peroxidase (HRP). HRP is a redox enzyme with a pI 8.8 that belongs to class III of the plant peroxidase superfamily. Traditionally it has been the most commonly used oxidoreductase for the development of enzyme-based amperometric biosensors, clinical diagnosis and waste-water treatment among many others applications [3]. HRP has a very broad specificity towards amines and phenolic compounds and good stability at room temperature, however, the main disadvantage is its low stability towards high concentrations of H_2O_2 and other hydroperoxides [2]. These drawbacks have been a motivation for the search of new sources of plant peroxidases with higher stability and different specificity.

In recent years there has been a considerable interest in exploring new tropical plant peroxidases containing unique and special biochemical properties for different biotechnological applications. For example, Sakharov et al., 2001 [2] isolated an extreme highly thermostable POD from the leaves of Royal palm tree peroxidase (RPTP). Spring cabbage serves as an enzymatic source to isolate and purify partially a novel anionic POD and it was employed as a biocatalyst for the construction an enzymatic electrode [4]. A new POD from garlic (*Allium sativum* L.) bulb was purified and biochemically characterized. This POD was found to be highly heat-stable, since almost 70% of its activity was

conserved at 60°C [5]. In a similar work Sakharov et al., 2002 [6] purified a POD extracted from the peels of sweet potato tubers (*Ipomea batatas*), the POD purified exhibited a high specific activity of 4900 U/mg of protein. Csöregi et al. [3] studied the comparison of plant peroxidases for the construction of electrochemical biosensors and it was found that soybean peroxidase immobilized on graphite electrodes was the most efficient peroxidase for H_2O_2 detection.

To the best of our knowledge, not previous studies has investigated about purification of POD from leaves of Guinea grass (PGG) and its potential application in the construction of amperometric biosensors. We first present the partial purification of POD by means of different steps including protein precipitation combined with pigments elimination, exclusion size chromatography, and ultracentrifugation. Second, the obtained POD was immobilized on the surface of screen printed graphene electrodes (SPGE) by physical adsorption. The modified electrodes were characterized by cyclic voltammetry and, chronoamperometric methods. Finally, we utilized the SPGE modified electrodes to detect H_2O_2 and to determine analytical parameters (limit detection, linear range and interference studies). The integration of PGG into the surface of SPGE will provide a new biosensor for sensing H_2O_2 in real samples.

2. Experimental

2.1. Partial purification of Guinea grass peroxidase

Guinea grass (*Panicum maximum)* leaves were harvested and collected in the territory state of Santander, Colombia. Leaves were triturated, gently washed and incubated with constant stirring in 30 mM phosphate buffer, pH 8.0, for 1h at ambient temperature. Then the homogenate obtained was filtered and centrifuged (7000 rpm, 10 min) and the pellet was discarded. For the extraction of pigments, a biphasic system containing 14% (w/v) poly(ethylene glycol) (PEG) and 10% (w/v) $(NH_4)_2SO_4$ was used. After 3h of incubation two phases were formed (the top PEG phase containing coloured compounds and the bottom aqueous phase containing peroxidase). The aqueous phase containing the enzyme was applied directly to a Sephadex G-50 column (2.5 x 41cm) equilibrated with TRIS buffer 3 mM, pH 8.0. Elution of the fractions containing PPG was eluted with the same buffer. The fractions having the PPG were collected and concentrated by ultracentrifugation (5000 rpm for 30 min at 4°C) using Amicons membranes (10000 kDa). Finally, the fractions containing POD activity were stored at 5°C awaiting the electrochemical measurements.

2.2. Enzyme assay

POD activity was measured by spectrochemical techniques: 10 μL of the enzyme was added to 2.5 mL of PBS 30 mM, pH 8.0 containing 10 mM guaiacol and 4.4 mM H_2O_2 as substrates and the absorbance change was monitored at 25°C (ε_{470}=5200 M cm^{-1}). One unit of activity (U) was defined as the amount of enzyme that caused the oxidation of 1 μmole

of substrate per min. The specific activity was expressed as units of activity per mg of protein. Protein contents were determined by Bradford assay [7].

2.3. Electrophoretical procedures

The molecular weight value of PGG was determined by SDS-PAGE in 8% polyacrylamide gel under denaturing conditions (1% 2-mercaptoethanol and 5 mM EDTA) [8].

2.4. pH and thermostability

pH-stability of PGG was studied in 10 mM universal buffer (CH_3COOH, H_3PO_4, H_3BO_3–NaOH). By the other hand thermal stability of PGG was studied as described [9]. After heating of 990 μL of 10 mM Tris–HCl (pH 8.0) to 66°C, a 10 μL of PGG was added and incubated at the same temperature. At different times, aliquots (10 μL) of the enzyme solution were removed, mixed quickly with 90 μL of the same buffer, incubated for 30 min at 25°C to restore the activity lost due to reversible inactivation, and finally, the activity was measured.

2.5. Km values

Km values were measured at 25°C for: ABTS (0.016-0.150 mM, pH 4.0 and 0.6 mM H_2O_2); *o*-dianisidine (0.08-0.5 mM, pH 8.0 and 9 mM H_2O_2) and guaiacol (0.1-10 mM, pH 8.0, 10 mM H_2O_2) and calculated from Lineweaver-Burke plots.

2.6. Electrochemical experiments

Cyclic voltammetry and chronoamperometric experiments were carried out with an Autolab PGSTAT101 (Echo Chemie, Utrecht, The Netherlands) controlled by NOVA 1.10.1.9 software (Metrohm, Filderstand, Germany) in a screen printed three electrode configuration. Screen printed graphene electrodes (SPGE, 110GPH) were provided by

DropSens (Oviedo, Spain). Each electrode consisted of a graphene working electrode (4 mm of diameter), a silver pseudoreference electrode, and a carbon counter electrode. SPGE was modified by physical adsorption of 5 μL of PPG. After 24h of incubation at 4°C the electrode was gently rinsed with phosphate buffer to remove unbounded PPG. The SPGE modified electrode was fitted into a methacrylate electrochemical cell (Dropsens, Spain). All the experiments were carried out at room temperature. Before each CV experiment, the work solution was degasified with pure nitrogen and magnetically stirred for 30 s. For chronoamperometric measurements, the working potential was set at -650 mV (vs Ag) and the solution was stirred gently with a magnetic stirrer before and during the addition of hydrogen peroxide solutions. ~~All the applied potentials used in this study are referred to the internal Ag pseudoreference electrode of SPGE.~~

Detection of potential interferences such as glucose, citric acid, and ethanol were evaluated in concentrations of 0.5 mM under optimized experimental conditions. The changes in the current signal of 1 mM was compared in the absence and in the presence of selected interferences.

3. Results and discussion

3.1. Partial purification and stability of guinea grass peroxidase

In a previous work [10] it was found that crude extract from the guinea grass exhibit high peroxidase activity. Prior to partial purification the conditions for PGG extraction were optimized.

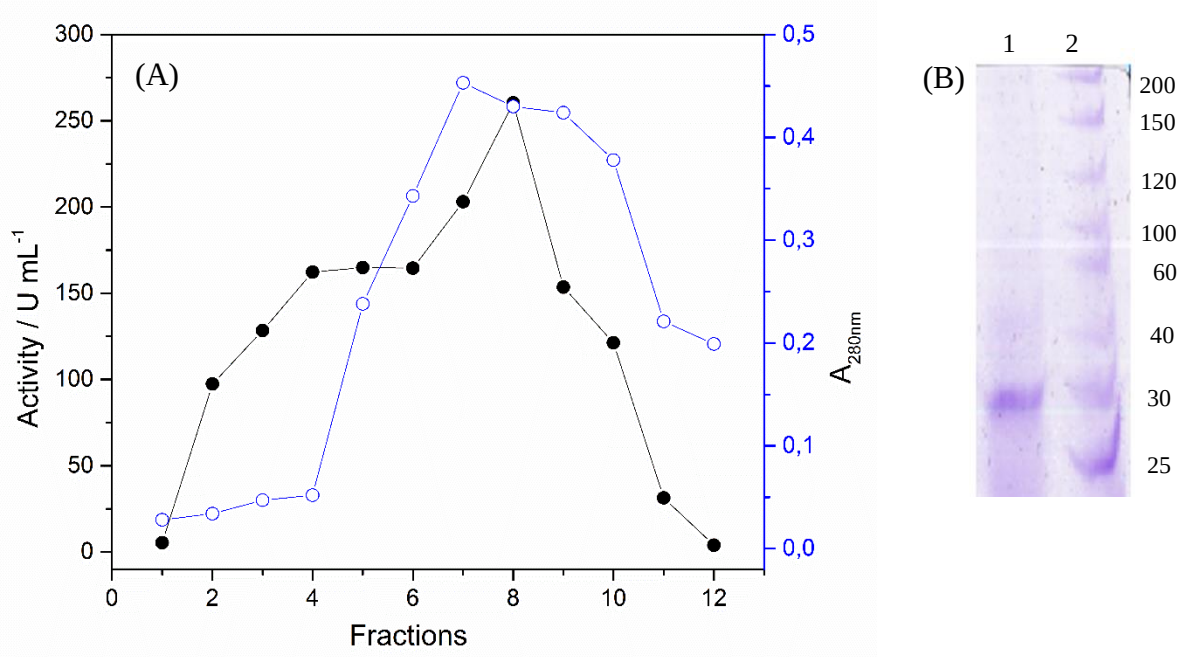

Fig. 1. (A) Size-exclusion chromatography of PGG on a Sephadex G-50 column. (B) SDS-PAGE of purified PGG (8% polyacrylamide gel under denaturing conditions).

Extraction in 30 mM phosphate buffer, pH 8.0 was the most efficient and an analysis of the specific activity of PGG extraction showed that 6 h of extraction was needed to achieve maximum extraction. Longer extractions time resulted in the decrease of the peroxidase activity, probably due to the presence of phenols and their oxidation products that could

inactivate the enzyme. As is reported by Sakharov *et al.* [2] many extract of plant tissues present a high concentration of pigments. In order to obtain a clear extract, the pigments were removed by using an aqueous two-phase system, namely PEG- ammonium sulfate. The PGG specific activity of the extract after elimination of pigments was 263 U/mg. The further purification was carried out using size exclusion chromatography on a Sephadex G-50 column equilibrated with Tris buffer 3 mM, pH 8.0. PGG was eluted using the same buffer and the chromatographic step increased the specific activity to 335 U/mg (Fig. 1A). Finally after the last step of purification of PGG a maximum specific activity of 408 U/mg was obtained. Figure 1B shows the SDS-PAGE of PGG with an approximately molecular weight of 30kDa, this result is within the range reported in previous reports for plant peroxidases [6].

3.2. Stability of peroxidase from guinea grass

Stability of enzymes is highly affected by pH because it can alter the activity affecting ionizations states of the side chains. It is well documented that enzymatic activity of peroxidases is pH-dependent. Therefore, the stability of PGG at different pHs was tested at room temperature. Interestingly PGG is a highly stable enzyme over a broad range of pH from pH 4 to 12 with maximum stability at pH 8.0 (Fig 2A). A similar type of pH-stability has been reported of HRP and peroxidase from Royal palm tree (RPP) at 25°C and 70°C, respectively. However HRP shows low stability at pH below 4.5 and given that under these conditions the synthesis of some electronic polymers such as PANI is of utmost importance [11], PGG thus becomes a potential alternative for synthesis of PANI due to stability shown at pHs between 3.0 and 4.0.

On the other hand, PGG preserves 60 % of the enzymatic activity after 1 h of incubation up to 66°C at pH 8.0. At the same conditions, PODs from different sources were rapidly inactivated [4]. For example, POD from tobacco was inactivated at 65°C after 1 h of incubation [12]. In a similar report pepper fruit, acidic POD preserves only 25% of the total activity after 1 h of incubation at 65°C [13].

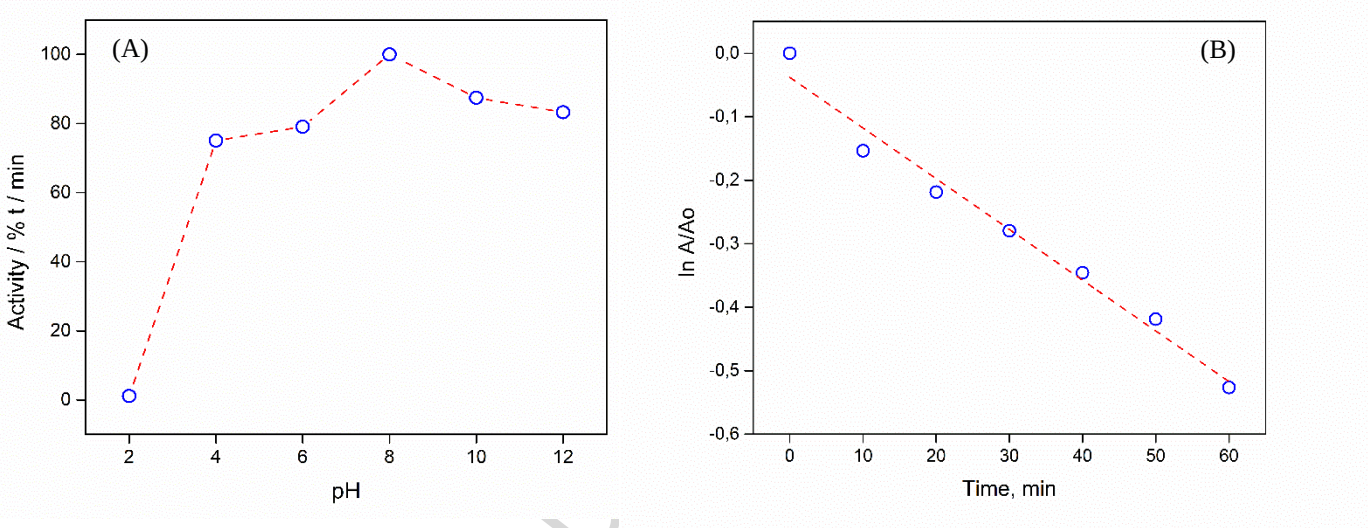

Fig. 2. Effect of pH (A) and temperature (B) on activity of peroxidase from guinea grass.

As is shown in Fig. 2B the thermal inactivation of PGG follows a first order equation, with k_{inact} equal to $8x10^{-3}$ min^{-1}. HRP has a significantly lower thermostability, already around 64°C this enzyme inactivated about two times faster with k_{inact} of $1.6x10^{-2}$ min^{-1} [12]. The reason for the high stability of PGG could be attributed to the high thermal resistance due to the carbohydrate domain present in most of the plant peroxidases [14]. The stability of PGG along with the abundance of the raw material for purification of the enzyme opens up new possibilities for future analytical applications.

3.3. Substrate specificity and Km values

The substrate specificity of PGG has been examined using three well-known peroxidases substrates: ABTS, guaiacol, and *o*-dianisidine. Table 1 shows experimental optimal conditions for the determination of PGG substrate specificity.

Table 1. Experimental conditions for the determination of guinea grass peroxidase substrate specificity

Substrate [AH]	λ (nm)	ε (M^{-1} cm^{-1})	[AH] (mM)	[H_2O_2] (mM)	pH
ABTS	414	31000	0.15	0.6	4.0
Guaiacol	470	5200	9.99	8.0	5.0
o-dianisidine	420	2640	0.5	9.0	8.0

Apparent K_m values for PGG were: 0.18 mM (for ABTS), 0.29 mM (for *o*-dianisidine) and 9.70 mM (for guaiacol). According to our results the highest affinity ($1/K_m$) was for ABTS followed by *o*-dianisidine and guaiacol. As is shown in table 2 the highest turnover, k_{cat}, of PGG was found for the substrate *o*-dianisidine (1734 min^{-1}) followed by ABTS (1054 min^{-1}) and guaiacol (186 min^{-1}). These results are in good agreement with previous studies carried out with palm tree peroxidases that showed high reactivity towards ABTS followed by aromatic amines and, finally, by phenolic compounds [15]. A similar behavior was exhibited by spring cabbages peroxidases [4].

Table 2. Kinetic parameters of guinea grass peroxidase with different substrates

Substrate [AH]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/ K_m($mM^{-1}s^{-1}$)
ABTS	0.18	1054	5860
o-dianisidine	0.29	1734	5980
Guaiacol	9.70	186	19

3.4. Electrochemical studies

To study the electrocatalytic activity of PGG towards the reduction of hydrogen peroxide, cyclic voltammograms (CVs) were used. The PGG was adsorbed on the surface of SPGE and CVs of ferri/ferrocyanide couple using modified and unmodified SPGE are shown in Fig. 3A. The electrochemical response of a biosensor to $K_3Fe(CN)_6$/ $K_4Fe(CN)_6$ by CV is established to evaluate the barrier created after each modification step.

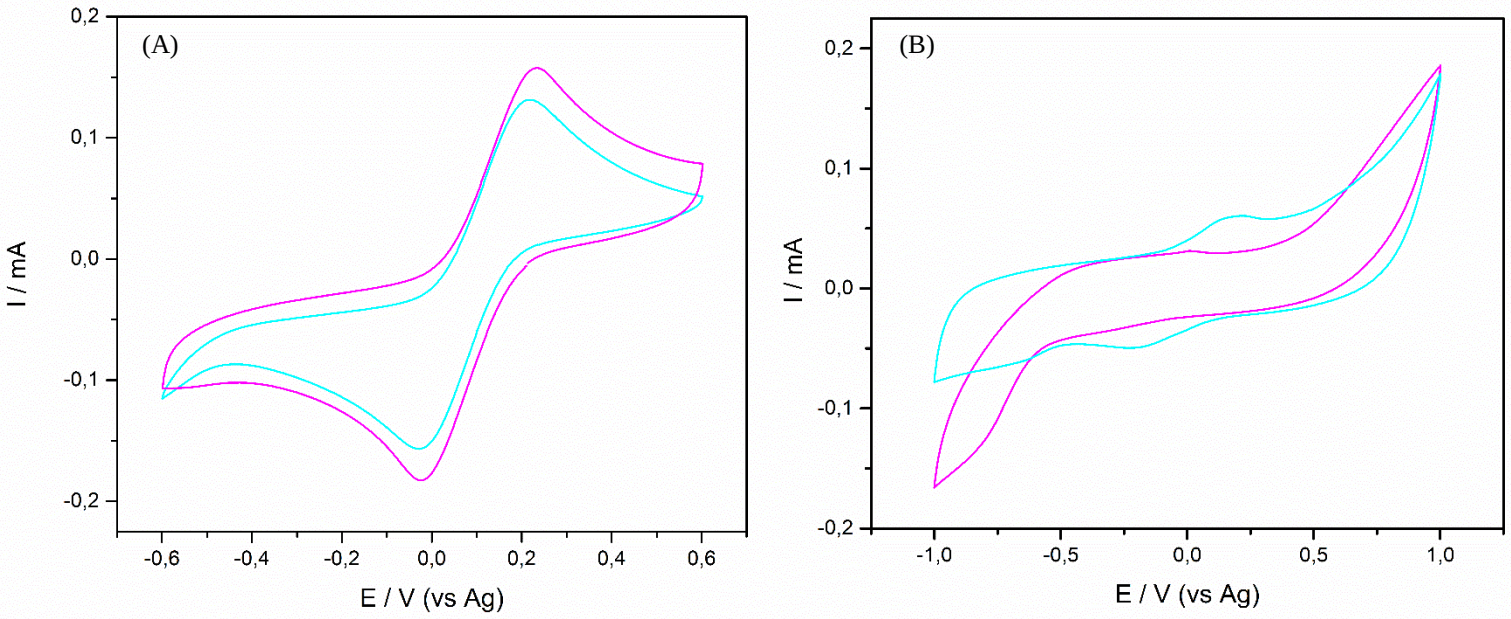

Fig 3. (A) Cyclic voltammograms of bare SPGE (cyan) and, PGG-SPGE (magenta) in the presence of 10 mM $K_3Fe(CN)_6$ containing 0.1 M KCl, scan rate of 100 mV s^{-1}. (B) Cyclic

voltammograms of PGG graphene modified electrode in the absence (cyan) and in the presence (magenta) of 4 mM of H_2O_2, scan rate of 100 mV s^{-1}.

Fig. 3A shows CV for bare graphene electrode (cyan color) which exhibit a well pair of redox peaks at 218 mV and at -31 mV for anodic and cathodic peak, respectively. On the other hand, it can be observed an increase in both cathodic and anodic current when PGG was deposited on the surface of SPGE (magenta color). Thus the presence of PGG plays an important role in the electron transfer between redox species in solution and the graphene electrode surface probably due to the increasing of the electroactive area of the transducer. A surface-controlled quasi-reversible process was exhibited by the PGG-graphene electrode when the increased of different scan rate produces the diffusion of the electroactive species to the transducer surface (see supporting information SI 1).

Graphene, an allotrope of carbon in the form of a two-dimensional honeycomb, displays special electrochemical properties showing to be potentially useful in the construction of electrochemical biosensors [16]. The combination of graphene with PGG shows an increase in the surface area of the electrode, which is reflected in the current values obtained for the modified electrode. The presence of the peroxidase combined with the porosity and the presence of different functional groups in the graphene structure improves the electron transfer between PGG and the electrode surface compared with unmodified graphene electrode.

In order to evaluate the bioelectrocatalytic reduction of hydrogen peroxide by the electrode modified with PGG a CV in the presence of 4 mM of H_2O_2 was measured. As it is shown in Fig. 3B there was no increase in the cathodic response of the CV for unmodified graphene

electrode in the absence of H_2O_2. It is interesting to note that when 4 mM H_2O_2 is added to the buffer solution, the CV shows a high reduction current (cathodic peak at -650 mV), which could be attributed to the electrocatalytic reduction of H_2O_2 by the presence of PGG acting as an efficient biocatalyst. In contrast to the majority of biosensor based on plant peroxidases which report high potential values [4, 16–18], our modified electrode exhibited an applied potential of -650 mV (vs Ag) that probably would minimize the detection of interferences prone to oxidation in real samples.

In order to demonstrate the success of H_2O_2 detection at the PGG graphene modified electrode, chronoamperometric technique was selected to monitor the response of successive additions of H_2O_2. A potential of -650 mV (vs Ag) selected from the CV of figure 3B was used as a working potential for the chronoamperometric measurements.

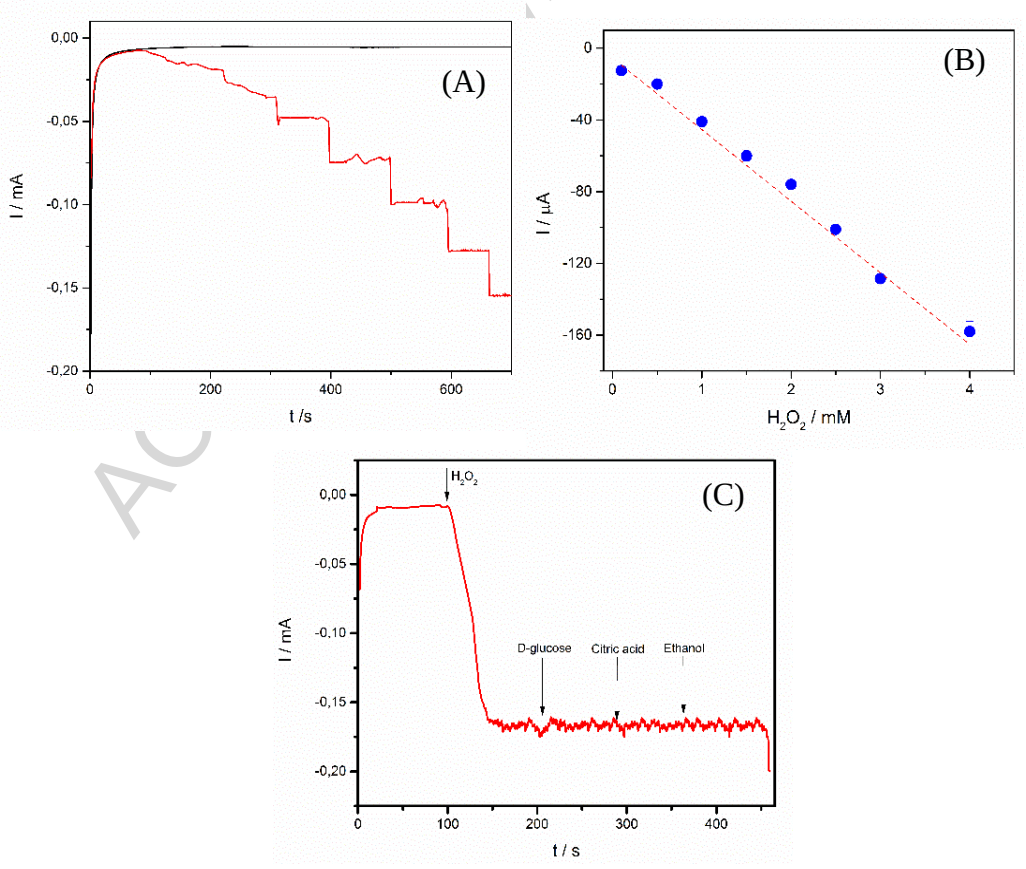

Fig. 4. (A) Chronoamperometric response of PGG at the graphene electrode (-) in the presence of different amounts of H_2O_2 into 7 mL of PBS 10 mM pH 7.0 and control experiments for bare SPGE (-) under successive additions of H_2O_2; (B) calibration curve of the cathodic current vs concentration of H_2O_2; (C) Chronoamperometric response of PGG at the graphene electrode at -650 mV (vs Ag) in the presence of different amounts of H_2O_2, glucose, citric acid and ethanol with constant stirring of 7 mL of PBS 10 mM pH 7.0.

Fig. 4A shows the amperometric response recorded after successive additions of H_2O_2 0.5 mM H_2O_2 to 7 mL pH 7.0 PBS solution at an applied potential of -650 mV (vs Ag). PGG modified electrode exhibited a reduction current with stable values upon addition of an aliquot of H_2O_2 to the stirring buffer solution which strongly evidences a fast electron transfer process between redox center of the PGG and the modified surface electrode.

Control experiments for the chronoamperometric response of the unmodified graphene electrode at -650 mV (vs Ag) does not show any current response after additions of H_2O_2 (Fig. 4A). A linear response range of the modified electrode in the presence of different H_2O_2 solutions was from 100μM to 4 mM, and the linear equation regression was I = -39.93 - 5.46 [H_2O_2], with a correlation coefficient of 0.99102. The limit of detection was 150 μM (S/N = 3), and the linear range was in a wide range of 100 μM to 4 mM with a sensitivity of 0.019 mA/μM (Fig. 4B). Table 3 compares the analytical performance obtained in previous reports and results obtained in this study.

Table 3. Comparison of HRP and PGG modified electrodes for sensing of H_2O_2.

Modified electrode	Redox potential (V)	Linear range (μM-mM)	Detection limit (μM)	Ref.
Dendrimer/Au/CS/HRP/ITO electrode (vs NCE)	-0.28	165-1.5	200	[20]
HRP/PE/MWCNTs GC electrode (vs Ag/AgCl)	-0.4	0.5-0.82	0.16	[21]
HRP/PANI/Pt electrode (vs SCE)	-0.25	5-60	3.2	[22]
HRP/nafion-sonogel/carbon electrode (vs Ag/AgCl)	-0.25	4-100	1.6	[23]
PGG/graphene electrode (vs Ag)	-0.65	100-4	150	This work

Figure 4C shows the interference studies of the PGG modified electrode response after additions of 5mM of glucose, citric acid, and ethanol. The study showed no significant signal to the interferences molecules assayed thus indicating a high selectivity and specific response towards H_2O_2.

4. Conclusions

In conclusion, the presented study was designed to purify partially the peroxidase from Guinea grass (*Panicum maximum)* and to evaluate the bio-electrocatalytic properties in the

reduction of hydrogen peroxide. Peroxidase from the leaves of Guinea grass immobilized on the surface of screen printed graphene electrode has shown to be an alternative biocatalyst to the commercial available HRP and other plant peroxidases. The resulting graphene- modified electrode exhibited a fast direct electron transfer and a good performance towards H_2O_2 determination with a wider linearity range and a lower detection limits. The modified electrode exhibited a negligible amperometric response towards glucose, citric acid, and ethanol. Further research in this novel biosensor could lead to obtaining a new sensing alternative for the detection of H_2O_2 in real samples

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Supporting information

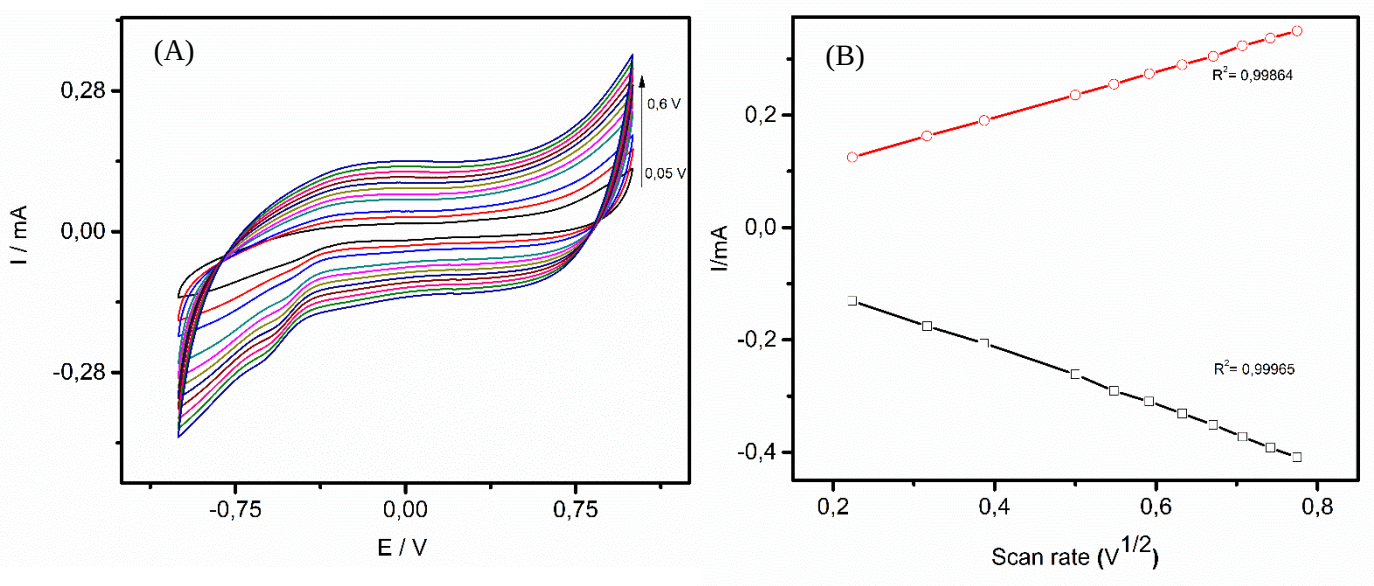

Fig. S1. (A) Cyclic voltammograms of PGG graphene modified electrode at different scan rates; (B) plots of square root of scan rate vs anodic and cathodic current.

Highlights

- A novel peroxidase was isolated from guinea grass plant.
- The electrode modified exhibits excellent electrocatalytic activity to the reduction of H_2O_2.
- The peroxidase will allow the fabrication of a biosensor for determination of H_2O_2 in real samples.