ARTÍCULO DE REVISIÓN

From Rainforest to Lab: Electrochemical Biosensing with Colombian Plant Peroxidases

Del Campo al Laboratorio: Biosensores Electroquímicos de Peroxidasas de Plantas Colombianas

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ABSTRACT

The peroxidases are a class of enzymes found in various species of Colombian tropical plants. These enzymes belong to the larger group of peroxidases, which are heme-containing proteins involved in catalysing a wide range of reactions in living organisms. Peroxidases have emerged as promising biocatalysts with versatile biotechnological applications. This paper aims to provide a detailed analysis of peroxidases in Colombian tropical plants and their potential in electrochemical sensing. The review begins by elucidating the structural and functional characteristics of peroxidases in plants, exploring their classification, and highlighting their catalytic mechanisms. It then delves into the various substrate specificity and affinity of plant peroxidases and its comparison with other peroxidases. Furthermore, the diverse electrochemical techniques relevant to biosensing and their applications in biosensor development are thoroughly examined. The challenges and prospects of utilizing Colombian plant peroxidases in biosensing applications are critically evaluated.

In summary, this study highlights the significance of peroxidases in plants as valuable bioanalytical tool. Their multifaceted applications in environmental, agricultural, food, and pharmaceutical bioanalysis sectors make them indispensable in addressing contemporary challenges. The insights provided herein serve as a foundation for future research endeavours aimed at harnessing the full potential of Colombian tropical plant peroxidases for the construction of electrochemical biosensors.

Key words: peroxidases, biosensing, electrochemical biosensors, biocatalyst.

RESUMEN

Las peroxidasas son una clase de enzimas presentes en diversas especies de plantas tropicales colombianas. Estas enzimas pertenecen al grupo más grande de peroxidasas, que son proteínas que contienen el grupo hemo y catalizan una amplia gama de reacciones en organismos vivos. Las peroxidasas han surgido como biocatalizadores prometedores con aplicaciones biotecnológicas versátiles. Este artículo tiene como objetivo proporcionar un análisis detallado de las peroxidasas en plantas tropicales colombianas y su potencial en la detección electroquímica. El estudio comienza elucidando las características estructurales y funcionales de las peroxidasas en plantas, explorando su clasificación y destacando sus mecanismos catalíticos. Luego profundiza en la especificidad y afinidad de los diferentes sustratos de las peroxidasas de plantas y las compara con otras peroxidasas. Además, se examinan exhaustivamente las diversas técnicas electroquímicas relevantes para la detección y sus aplicaciones en el desarrollo de biosensores. Se evalúan críticamente los desafíos y las perspectivas de utilizar peroxidasas de plantas colombianas en aplicaciones de detección.

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En resumen, este estudio destaca la importancia de las peroxidasas en plantas como herramienta bioanalítica valiosa. Sus aplicaciones multifacéticas en los sectores de análisis ambiental, agrícola, alimentario y farmacéutico las convierten en elementos indispensables para abordar desafíos contemporáneos. La información proporcionada aquí sirve como base para futuros esfuerzos de investigación dirigidos a aprovechar todo el potencial de las peroxidasas de plantas tropicales colombianas para la construcción de biosensores electroquímicos.

Palabras claves: peroxidasas, biodetección, biosensores electroquímicos, biocatalizador.

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INTRODUCTION

Colombian flora boasts an unparalleled richness, with its diverse array of plant species harboring immense biotechnological potential (Alomía *et al*., 2022; Garcia-Vallejo & Alzate, 2024; Jaramillo *et al*., 2024). In recent times, the integration of cutting-edge biotechnological advancements with the wealth of natural resources provided by this flora has opened new vistas of research and innovation (Garcia-Vallejo & Alzate, 2024) Among the myriad of biologically active compounds found within tropical plants, peroxidases (PODs) have emerged as a group of enzymes that hold immense promise for biotechnological applications (Brusova *et al*., 2005).

PODs, as versatile biocatalysts, play a fundamental role in various physiological processes within plants (Freitas *et al*., 2024), ranging from growth and development to stress response (Vishwakarma *et al*., 2024) and defence mechanisms (Y. Liu *et al*., 2021). Their ability to catalyse the oxidation of a wide range of substrates using hydrogen peroxide (H_2O_2) makes them integral components of numerous biochemical pathways. Over the years, scientists have begun to unravel the diverse functions and potential applications of these remarkable enzymes (Mathé *et al*., 2010).

While PODs are found throughout various plants, horseradish roots currently stand as the primary commercial source of these enzymes. Horseradish Peroxidase (HRP) is a highly versatile and widely used enzyme derived from the roots of the horseradish plant (*Armoracia rusticana*) (Bilal *et al*., 2020). It plays a vital role in various biotechnological applications, such as immunoassays, molecular detection, and bioanalytical techniques, due to its ability to catalyse colour-producing reactions in the presence of specific substrates and H_2O_2 (Bilal *et al*., 2023; X. Zhang *et al*., 2023). Its broad substrate specificity and stable nature make it a valuable tool in research, diagnostics, and industry (Yang *et al*., 2023). Nevertheless, a significant drawback is its reduced stability when exposed to high concentrations of H_2O_2 and hydroperoxides. As a result, researchers have been motivated to explore alternative sources of plant peroxidases that offer greater stability and different substrate specificities (Kotchey *et al*., 2013). This pursuit aims to find enzymes that can better withstand oxidative conditions and cater to specific biotechnological needs.

In the context of bioanalytical tools, PODs play a pivotal role due to their ability to facilitate precise and sensitive detection of specific substrates (Bhapkar *et al*., 2023a; Han *et al*., 2022; Rafaqat *et al*., 2024). As is shown in Figure 1, the focus of this exploration lies in PODs derived from Colombian tropical plants and their unique contributions to the burgeoning field of electrochemical biosensors. Guinea grass, African oil palm, Royal palm, and

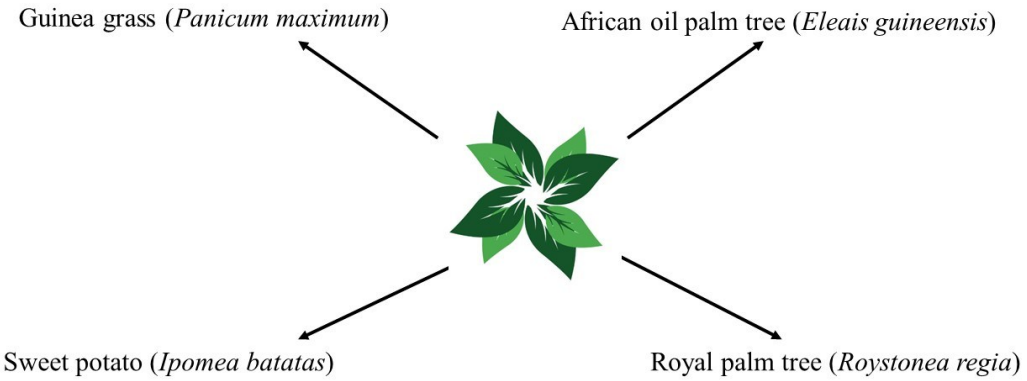


Figure 1. Peroxidases isolated and purified from some Colombian tropical plants.

sweet potato are plants grown in Colombia, and their peroxidases (PODs) exhibit unique catalytic characteristics, stability, and versatility, presenting an exciting avenue for researchers seeking innovative solutions in bioanalytical applications (Guo *et al*., 2024; Škulj *et al*., 2024).

Plant PODs have gained attention for their inherent catalytic efficiency, stability, and versatility, making them promising candidates for advancing bioanalytical tools, particularly in the context of electrochemical sensing (Rafaqat *et al*., 2024). These enzymes exhibit remarkable resilience to various environmental conditions, making them well-suited for integration into electrochemical biosensing platforms (Kulkarni *et al*., 2022).

The focus on electrochemical applications is grounded in the growing significance of biosensors as powerful tools for real-time, sensitive, and selective detection of analytes (Lai *et al*., 2009).

METHODOLOGY

This bibliometric study aimed to systematically analyse the existing literature on Colombian plant PODs and their applications in electrochemical biosensing. The study employed various databases such as Web of Science, Scopus, and PubMed, identifying key research trends, citation networks, and patterns of collaboration. A total of 86 publications, from 1990 to 2023, were identified within a specified time range, providing a comprehensive overview of biosensing with PODs from Colombian plants. The analysis revealed a steady increase in publications, in the last three decades, indicating a growing interest in the use of plant PODs for biosensing applications. Specifically, key topics of focus included physiological role, biochemical properties, isolation of PODs, electrochemical detection techniques and immobilization methods. Collaborative networks showed a global span, with significant contributions from Colombian, Russian and Spanish institutions, underscoring the role of local expertise in advancing this field. The detailed bibliometric study not only allowed us highlighted the evolution of research but also pointed toward emerging trends and gaps that warrant further exploration.

PHYSIOLOGICAL FUNCTIONS OF PLANT PEROXIDASES

Figure 2 shows an overview of the diverse physiological functions performed by plant peroxidases, highlighting their roles in processes such as stress response, growth regulation, and defense mechanisms. In the plant kingdom, PODs belong to a family of enzymes and play vital roles throughout the plant's life cycle (Abdulwahhab Mohammed & M-Ridha, 2024). They are involved in numerous processes, such as cell wall metabolism, lignification (strengthening of cell walls), suberization (formation of protective layers), metabolism of reactive oxygen species (ROS), regulation of auxin (a plant hormone) levels, fruit growth and ripening, and defense against pathogens (S. Zhang *et al*., 2014). Overall, PODs serve as multifaceted

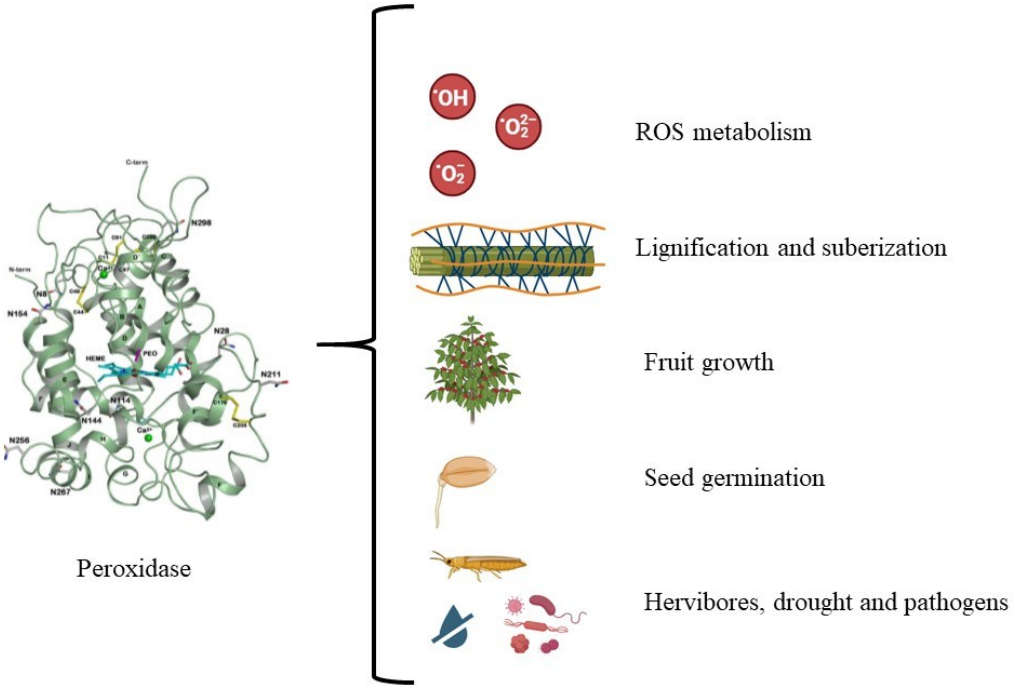


Figure 2. Diverse physiological functions played by plant peroxidases.

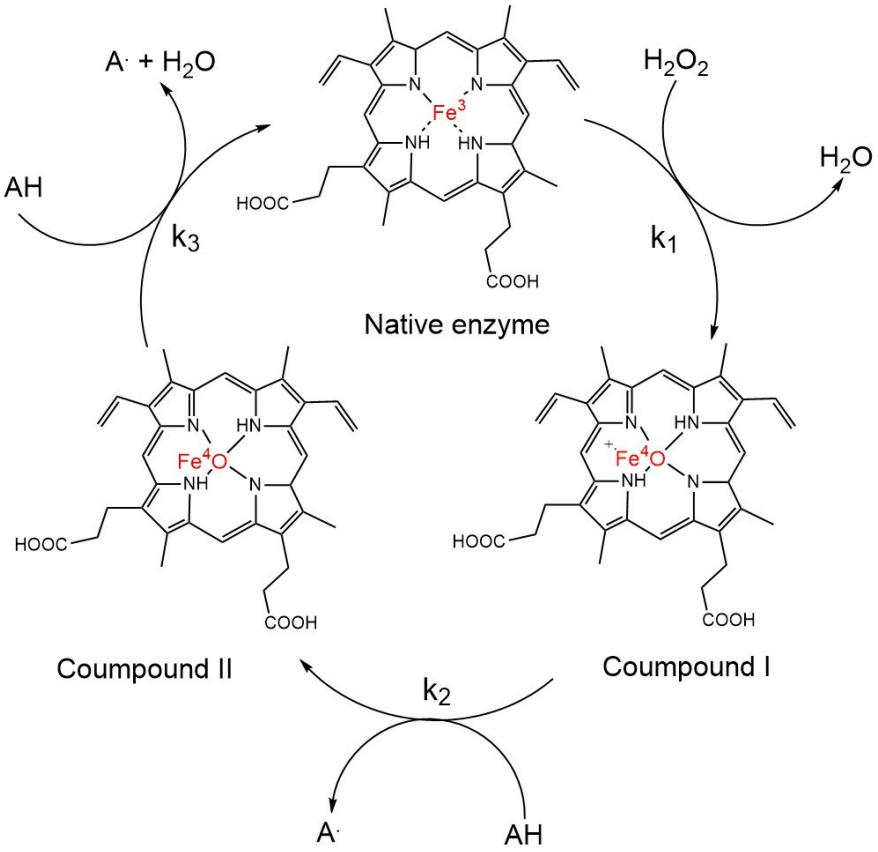


Figure 3. Mechanism of the catalytic cycle of plant PODs.

players in plant physiology, safeguarding against oxidative stress while actively participating in fundamental cellular processes essential for plant survival and growth.

BIOCHEMICAL OVERVIEW OF PLANT PEROXIDASES

PODs, specifically classified as EC.1.11.1.x enzymes, are essential enzymes that break down H_2O_2 while oxidizing a wide range of substrates, both phenolic and non-phenolic compounds (AH). The catalytic mechanism can be observed in Figure 3. These enzymes are found ubiquitously in nature, present in bacteria, fungi, algae, plants, and animals (Freitas *et al*., 2024).

Figure 3 illustrates the catalytic cycle mechanism of plant peroxidases (PODs), highlighting the key steps and intermediates involved in the enzymatic process. The POD binds to its substrate, typically a peroxide compound like H_2O_2. The enzyme reacts with the peroxide substrate, leading to the formation of an intermediate compound known as Compound I. This step involves the oxidation of the heme iron at the active site. Compound I, now an active oxidizing agent, reacts, causing the oxidation of the substrate. This step is crucial for the peroxidase's function in breaking down or transforming specific substrates. After substrate oxidation (AH), Compound I is reduced to Compound II. This reduction involves the transfer of electrons within the enzyme. The enzyme is then restored to its original state through the introduction of an external reducing agent. This process often involves reducing Compound II back to the enzyme's native state, thereby concluding the catalytic cycle.

PODs from Colombian tropical plants belongs to the family of secretory Class III PODs characterized by the presence of a single polypeptide chain, two calcium atoms, an a heme prostetic group located in the crevice by two anti-parallel α-helices. Of particular interest in this Class III family, plants such as sleepy plant (*Mimosa pudica*), castor oil plant (*Ricinus communis*), lemongrass (*Cymbopagon citratus*) and pod marigold (*Calendula officinalis*), among others, exhibited a high amount of POD (Sakharov *et al*., 2001). Watanabe et al., (Watanabe *et al*., 2007) report for first time the X-ray structure of native POD, extracted from the royal palm tree (RPTP) (*Roystonea regia*), which has been refined to a resolution of 1.85 Å. RPTP exhibited a folding pattern consistent with that of the plant peroxidase superfamily, featuring a single heme group and two calcium-binding sites located similarly.

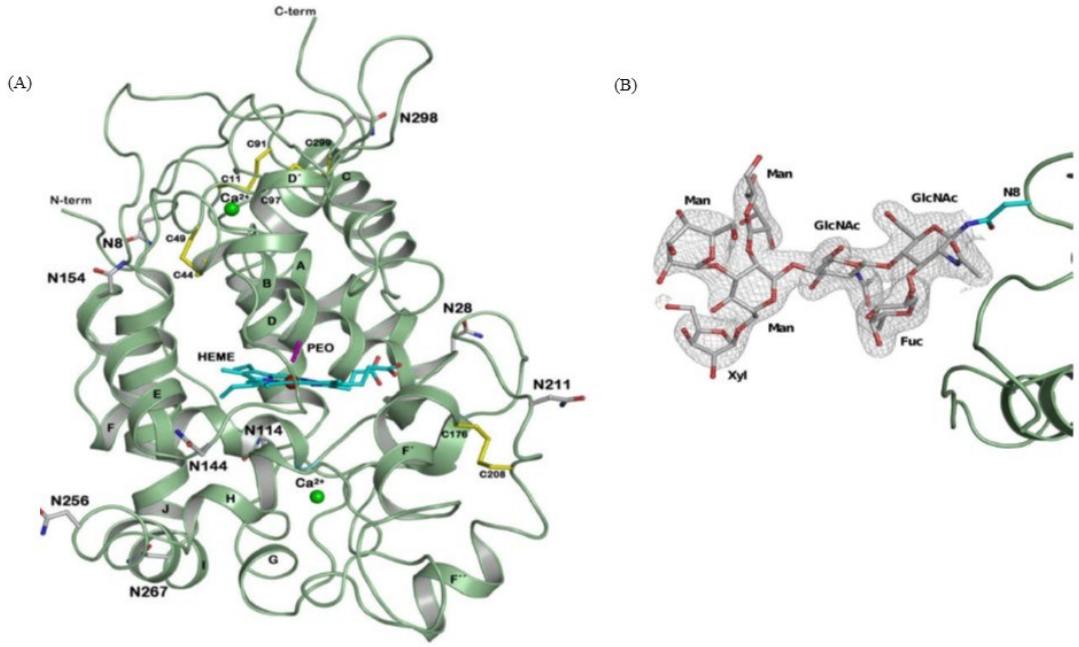


Figure 4. (A) Three-dimensional of RPTP elucidated by X-ray crystallography; (B) Schematic representation of seven N-linked glycan chains found in RPTP (adapted from Watanabe *et al.*, 2007).

Figure 4 shows the three-dimensional structure of RPTP determined by X-ray crystallography. The three-dimensional analysis of RPTP, conducted in the context of a hydroperoxide complex state, uncovered the presence of a bound 2-(N-morpholino) ethanesulfonic acid molecule (MES) situated at a potential secondary substrate-binding site (Watanabe *et al.*, 2007). Additionally, the electron-density maps of RPTP clearly delineate nine N-glycosylation sites, providing novel insights into the conformational arrangement of the glycan chains of this extensively glycosylated enzyme.

Thermodynamic characterization provides valuable insights into the fundamental properties and behavior of enzymes, enabling their rational design and optimization for various practical applications in biotechnology, medicine, and beyond. From this perspective, Zamorano *et al.*, (Zamorano *et al.*, 2008) characterized the structural stability of RPTP using techniques such as high-sensitivity differential scanning calorimetry, circular dichroism, steady-state tryptophan fluorescence, and analytical ultracentrifugation across various solvent conditions. The results unveiled a reversible thermal and chemical folding/unfolding process of RPTP at pH 7, demonstrating a highly cooperative transition between folded dimers and unfolded monomers. This transition exhibited a notable free stabilization energy of approximately 23 kcal/mol of monomer at 25°C. Furthermore, the structural stability of RPTP was found to be pH-dependent. Specifically, at pH 3, where ion pairs are disrupted due to protonation, the thermally induced denaturation of RPTP was irreversible and strongly influenced by the scan rate, indicating kinetic control over the process. Additionally, thermal transitions at this pH were observed to vary with protein concentration, suggesting a dimeric behavior of RPTP in solution, which undergoes thermal denaturation concomitant with dissociation.

Researchers have successfully isolated and purified PODs to understand their physicochemical properties better. Table 1 provides a comparison of several PODs derived from different plant sources, showing a range of properties including molecular weight, optimal pH, temperature optimum, substrate affinity, and thermostability.

According to Table 1, most peroxidases have a molecular weight between 30-50 kDa and maintain their catalytic activity for more than one hour at temperatures above 70°C, exceeding the values shown by HRP (Lay, *et al.*, 2009). On the other hand, a broad substrate specificity towards organic molecules such as amines and phenols, with optimal activity typically at pH values between 5.0 and 7.0.

ISOLATION AND CHARACTERIZATION OF PLANT PEROXIDASES

Numerous studies of tropical plants have showed that certain tropical Colombian plants leaves demonstrate elevated peroxidase activity (Rodríguez *et al.*, 2002).

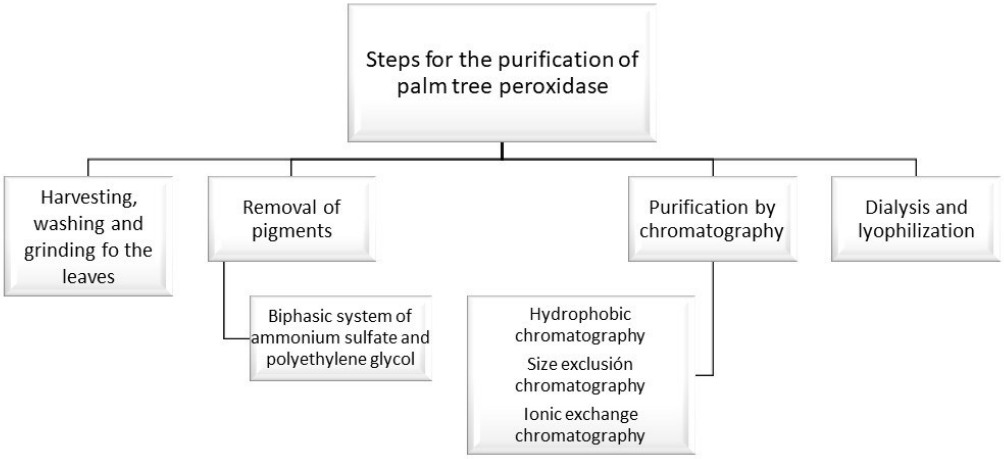
Table 1. Physicochemical properties of purified tropical plant peroxidases.

Source	Molecular weight (kDa)	pH optimum	Temperature optimum (°C)	Substrate affinity	Thermostability	Ref.
Roystonea regia	51	7.0-8.0	55	ABTS and ferulic acid	Preserved its catalytic activity after 1 h up to 70 °C	(Sakharov *et al*., 2001)
Eleais guineensis	57	5.0	55	ABTS and ferulic acid	Exhibited very high resistance at temperature as high as 80 °C.	(Rodriguez *et al*., 2002)
Panicum maximum	30	8.0	66	ABTS, *o*-dianisidine	Remained active up to 80 °C with maximum activity at 64 °C	(Centeno *et al*., 2017)
Ipomea batatas	37	4.5-5.5	-	ABTS, *o*-phenylenediamine	-	(Leon *et al*., 2002)
Trachycarpus fortunei	50	7.5	50	Ferulic acid and *o*-phenylenediamine	Retained its catalytic activity after 1 h up to 80 °C	(Baker *et al*., 2014)
Phoenix dactylifera	55	5.0-8.0	55	*o*-dianisidine and guaiacol	Remained active up to 80 °C with maximum activity at 55 °C	(Al-Senaidy & Ismael, 2011)
Chamerops excelsa	50	3.0-8.0	50	ABTS and guaiacol	Retained its catalytic activity after 1 h up to 70.5 °C	(Cuadrado *et al*., 2012)

The most substantial activity was observed in leaves from RPTP (*Roystonea regia*) (Alpeeva & Sakharov, 2007), guinea grass (GGP) (*Panicum maximum*) (Uribe *et al*., 2019), African oil palm (AOPTP) (*Elaeis guineensis*) (Rodríguez *et al*., 2002), and sweet potato (SPP) (*Ipomea batatas*) (Leon *et al*., 2002). Furthermore, the peroxidase activity exhibited no dependence on the age of plants and remained consistently constant throughout the entire year (de Oliveira *et al*., 2021). Consequently, plant POD can be classified as a constitutive enzyme. The isolation and purification of PODs from any plant species typically involve several standard laboratory techniques (Al-Madhagi *et al*., 2023).

For example, Sakharov *et al*., isolated and purified a POD from the leaves of RPTP (Sakharov *et al*., 2001), a kind of palm planted as an ornamental tree in parks and collected from a botanical garden located in Bucaramanga (Colombia), the enzyme purification steps included homogenization, $(NH_4)_2SO_4$ precipitation, extraction of palm leaf-colored compounds and consecutive chromatography on Phenyl-Sepharose, Sephacryl S100 and DEAE -Toyopearl. In a similar study, a high POD activity was found in the leaves of *Chamaerops excelsa* palm tree (Cuadrado *et al*., 2012). The following scheme summarizes the basic steps for the purification of plant PODs.

POD derived from the leaves of African oil palm, from crops in Sabana de Torres (department of Santander), presents a versatile enzymatic tool for various applications, particularly in environmental and industrial sectors (Yuan *et al*., 2021). This palm species, extensively cultivated in Colombia, offers a promising reservoir for the large-scale production of POD for industrial applications. For first time, this POD was purified from the leaves of this palm, the initial extraction process involved homogenization and pigment extraction using an aqueous two-phase polymer system. Initially, a conventional system comprising polyethylene glycol/K_2HPO_4 was employed. Substituting K_2HPO_4 with $(NH_4)_2SO_4$ facilitated the direct utilization of the salt phase containing accumulated peroxidase, which was subsequently



Scheme 1. Basic steps for the isolation and purification of plant peroxidases.

purified using a Phenyl-Sepharose column. Final purification steps were executed through liquid chromatography on Sephacryl S200 and DEAE-Toyopearl columns. The purified POD exhibited a specific activity of 4300 units per mg of protein toward guaiacol. Palm POD displayed a molecular weight of 57,000 and an isoelectric point of 3.8 (Rodríguez *et al*., 2002).

During a tropical plant screening (Alpeeva *et al*., 2005; Leon *et al*., 2002), sweet potato (*Ipomoea batatas*) tubers acquired from a local market, exhibited high POD activity with the major pool of PODs localized in the peel of sweet potatoes. The purification process involved the homogenization of the peel, extraction of colored compounds and finally, consecutive chromatographies on Phenyl-Sepharose and DEAE-Toyorpearl. The purified POD showed a specific activity of 4900 U/mg protein and a molecular mass of 37 kDa in a good concordance with similar plant peroxidases.

Finally, a newly discovered peroxidase from guinea grass (*Panicum maximum*) leaves, a type of grass used as animal fodder in Santander, and in some areas, it grows as a weed, was isolated and partially purified using a biphasic polymer system (polyethylene glycol-ammonium sulfate), followed by size-exclusion chromatography and ultracentrifugation (Centeno *et al*., 2017; Uribe *et al*., 2019). The resulting homogeneous extract displayed substantial POD activity. This novel enzyme exhibited a specific activity of 408 U/mg and a molecular weight of 30 kDa. Its optimal pH for activity was measured at 8.0, and it demonstrated remarkable thermostability at 66 °C with a k_{inact} of 8.0×10^{-3} min^{-1}.

ELECTROCHEMICAL FUNDAMENTAL RELEVANT TO BIOSENSING

Understanding the basic principles of electrochemistry is paramount in the field of biosensing (Bai *et al*., 2020). Electrochemical biosensors rely on the conversion of biochemical information into measurable electrical signals. The fundamental concepts include redox reactions at electrode interfaces, where electron transfer occurs between the biomolecules of interest and the electrode surface. The potential applied to the electrode influences these reactions, and the resulting current or voltage is proportional to the concentration of the target analyte. Key components in electrochemical biosensors, such as working electrodes, reference electrodes, and counter electrodes, play pivotal roles in facilitating and measuring these electron transfer events. Grasping these foundational electrochemical principles enables the design and optimization of biosensing devices for accurate and sensitive detection of various biological molecules, contributing to advancements in diagnostics and biotechnology (Cerdeira Ferreira *et al*., 2024; Vatankhahan *et al*., 2024).

In Colombia, a rich biodiversity offers a unique source of PODs from native plant species, which can be harnessed for novel biosensing applications. These plant-derived peroxidases hold potential for developing eco-friendly and cost-effective amperometric biosensors, contributing to advances in areas such as environmental monitoring, food safety, and medical diagnostics.

Amperometry

Amperometry is an electroanalytical technique that involves applying a constant reducing or oxidizing potential to an indicator electrode (working electrode) while measuring the resulting steady-state current flow (Guille-Collignon & Lemaître, 2021).

Amperometry serves as a technique for detecting ions in a solution based on electric current or changes in electric current. In amperometry, a constant potential is applied to the working electrode, and the current is measured over time. As the potential remains constant, amperometry does not yield a voltammogram. Figure 5 shows a

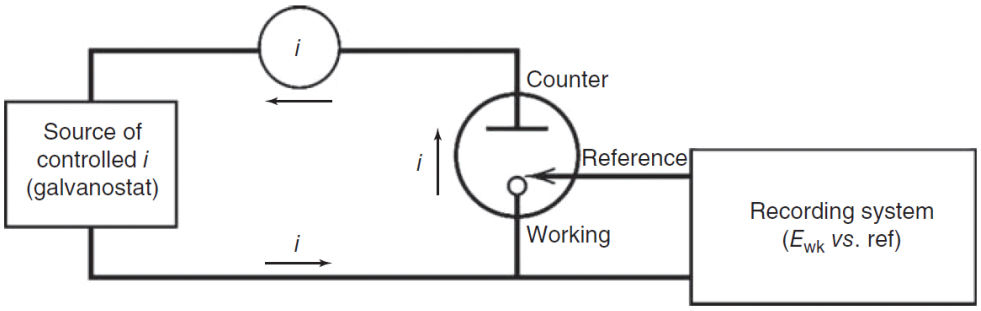


Figure 5. Block diagram of apparatus for amperometric measurements.

diagram of apparatus for amperometric measurements. The magnitude of the current is correlated with the amount of analyte present. In the context of biosensors, amperometry involves measuring the current generated by redox reactions at the electrode interface, providing a direct and sensitive means of quantifying the analyte concentrations. This technique is particularly valuable for real-time monitoring of biological events due to its high sensitivity and rapid response (Palsaniya *et al.*, 2023).

The working electrode in a biosensor serves as the site for these electrochemical reactions, with the current being directly proportional to the concentration of the target biomolecule. Amperometric biosensors have demonstrated remarkable utility in diverse applications, from clinical diagnostics to environmental monitoring, showcasing their potential for transformative impact in the field of biosensing (Smart *et al.*, 2023; Tapak *et al.*, 2022). The continuous refinement and integration of amperometric techniques contribute significantly to the advancement of biosensor platforms, enhancing their precision and broadening their scope in analytical sciences (Octobre *et al.*, 2024).

In the realm of amperometric biosensors, peroxidase-based systems offer remarkable sensitivity and selectivity for the detection of analytes ranging from phenolic compounds to environmental pollutants. Notably, a study by Izadar *et al.*,(Izadyar *et al.*, 2021) demonstrated the application of peroxidase from plants in fabricating a bienzymatic amperometric glucose biosensor combining POD from corn and glucose oxidase. Furthermore, the review by Bollella, (Bollella, 2022) described the importance of amperometric biosensor through six decades of progress highlighting the important role of PODs from plants.

Cyclic voltammetry

Cyclic voltammetry is a powerful electrochemical technique used to study the redox behavior of chemical species in solution. It involves applying a potential waveform to an electrode and measuring the resulting current (Wong *et al.*, 2022). The potential is typically swept linearly with time, creating a cyclic voltammogram that plots current against applied potential. This technique provides valuable information about the electrochemical properties of a system, including the number of electrons involved in a redox process, the kinetics of electron transfer reactions, and the stability of electroactive species (Di Noto *et al.*, 2022; Y. Zhang *et al.*, 2020).

Figure 6 shows a voltammogram where potential axis represents the applied potential (in volts) on the electrode. It exhibited the range over which the potential is swept during the experiment. The current axis represents the measured current (in amperes or its subunits) passing through the electrode as a response to the applied potential. This axis indicates the magnitude and direction of the electron flow during the redox processes.

By the other hand, the forward scan corresponds to the increasing potential sweep in one direction. It captures the oxidation or reduction reactions occurring at the electrode surface and the reverse scan is the portion of the

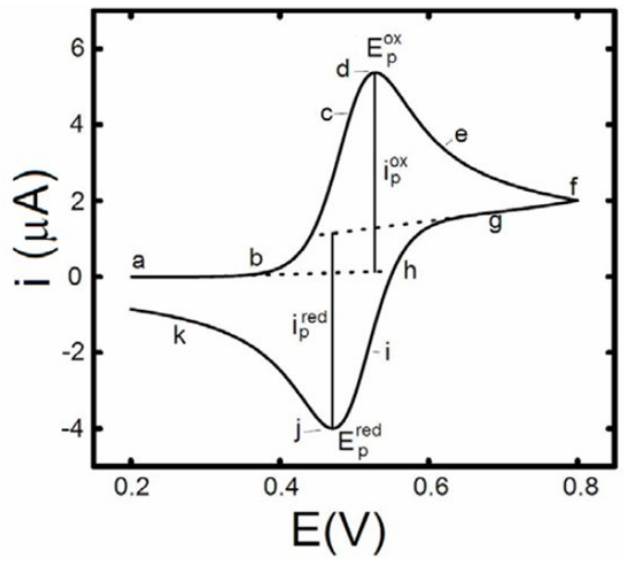


Figure 6. Cyclic voltammetry of ferrocene 0.5 mM in acetonitrile (scan rate 100 mVs^{-1}).

voltammogram where the potential is swept back in the opposite direction. It reflects the reverse redox processes that can occur as the potential is cycled. The anodic and cathodic peaks correspond to specific electrochemical events such as oxidation or reduction of analytes. Peaks provide information about the nature of the redox reactions and the concentration of electroactive species in the solution. Finally, the baseline is the line connecting the current values in the absence of any electrochemical activity. It represents the background current and is used as a reference point for determining the magnitude of current changes associated with redox processes.

CV plays a crucial role in biosensing (Olgaç *et al*., 2023; Shashaani *et al*., 2021; Xiao *et al*., 2021), offering a sensitive and versatile method for detecting biomolecules. In this electrochemical technique, the potential applied to a working electrode is varied linearly with time, causing the oxidation or reduction of species present in the solution. In biosensing applications, specific biomolecules, such as DNA, proteins, or enzymes (Z. Shi *et al*., 2024), can modify the electron transfer kinetics at the electrode surface, leading to characteristic voltametric signals. By monitoring these changes, researchers can quantify the concentration of target analytes in complex biological samples with high sensitivity and selectivity. Moreover, the reversible nature of CV allows for the investigation of dynamic interactions between biomolecules and electrodes, facilitating the design of robust biosensors for various diagnostic and analytical applications.

In the case of POD from plants many studies have play a pivotal role in biosensor technology, particularly when coupled with CV techniques. For example, an electrochemical biosensor for H_2O_2 detection was developed by using soybean peroxidase-copper phosphate mediated organic inorganic hybrid (Bhapkar *et al*., 2023). CV analysis showed that the developed biosensor could detect H_2O_2 in the linear range of 20–100 µM with R^2 value of 0.963. The limit of detection (LOD) and sensitivity values calculated for H_2O_2 are 0.19 µM and 27.44 µA/(µM.cm^2) respectively. In a similar study, a biosensor based on the ionic liquid 1-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide (BMI·Tf_2N) and a novel source of POD (tissue from the pine nuts of *Araucaria angustifolia*) was constructed (dos Santos Maguerroski *et al*., 2009). The POD in the presence of hydrogen peroxide catalyzes the oxidation of rosmarinic acid to quinone and the electrochemical reduction of the product was obtained at a potential of +0.15V vs Ag/AgCl.

Impedance

Impedance is a measure of the opposition that a circuit presents to the flow of alternating current (AC) (Lazanas & Prodromidis, 2023; Robinson *et al*., 2023). It is a complex quantity that incorporates both resistance and reactance. Resistance represents the opposition to the flow of current in a circuit due to the material properties and geometry of the conductors, while reactance arises from the effects of capacitance, inductance, or both. Impedance is expressed in ohms (Ω) and can be represented using complex numbers, where the real part represents resistance, and the imaginary part represents reactance. In practical terms, impedance characterizes how a circuit responds to alternating current, affecting the amplitude and phase relationship between voltage and current. It plays a crucial role in the analysis and design of electrical circuits, especially in fields such as electronics, telecommunications, and biosensing (Sopoušek *et al*., 2020; Suthar *et al*., 2022).

Figure 7 shows an example of an impedance diagram of an electrochemical reaction, where there is a series resistance to the RC parallel circuit, representing the solution resistance, Rsol. This has the effect of shifting the semicircle to higher values on the real impedance axis (Z´) of the graph. C is the double layer capacitance (Cdl), which is in parallel with the impedance of the reaction. Therefore, R is the charge transfer resistance (Rct).

Impedance plays a crucial role in biosensing due to its sensitivity to changes in the electrical properties of biological systems (Robinson *et al*., 2023). Impedance biosensors enable label-free detection of biological analytes. Changes in impedance can occur due to interactions between the target analyte and the sensing surface, eliminating the need for fluorescent or radioactive labels, which can be costly and time-consuming. A high sensitivity can be reach in impedance measurements due to small changes in the dielectric properties or conductivity of the biological medium. This sensitivity allows for the detection of low concentrations of analytes, making impedance biosensors suitable for applications such as disease diagnostics and monitoring of biomolecular interactions. The rapid response time of impedance measurements allows for dynamic monitoring of cellular responses (Sopoušek *et al*., 2020), enzyme kinetics (L. Liu & Wang, 2024), and biomolecular binding events (Moghtaderi *et al*., 2024), providing valuable insights into biological phenomena.

Impedimetric techniques have contributed significantly to the development of new biosensors based on plant PODs. Several studies have underscored the utility of plant PODs in impedance biosensors for various applications, ranging from environmental monitoring to clinical diagnostics. For instance, research by Berti *et al*.(Forzato *et al*., 2020) demonstrated the efficacy of soybean peroxidase-based impedance biosensors for the detection of phenolic compounds in environmental samples, showcas-

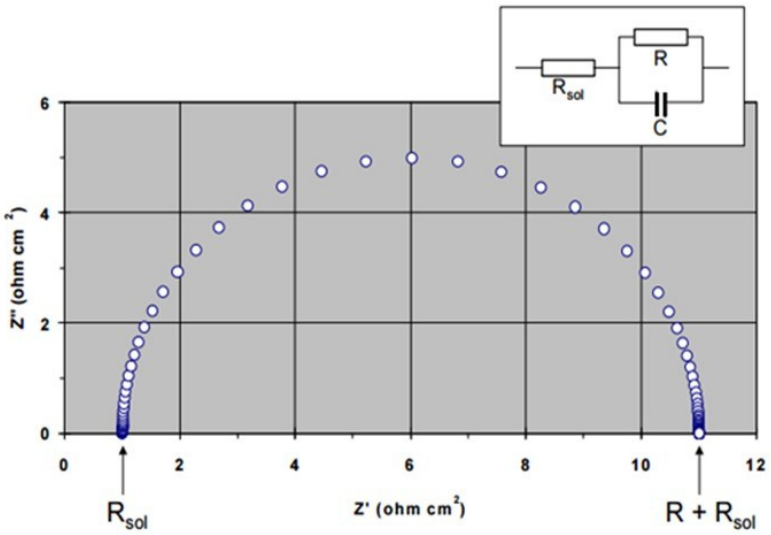


Figure 7. Real (Z´) and imaginary (Z´´) components of the total impedance (Z) for a parallel circuit resistance (R)-capacitance (C), considering the solution resistance (Rsol), at different frequencies (Rsol =1 ohm, R =1' ohm, C =0.0001 F cm-2, f ma)

ing their potential in environmental monitoring. Similarly, the work of Zhang *et al*. (Vallés *et al*., 2019) showcased the applicability of HRP in impedance biosensors for the rapid detection of biomarkers associated with diseases, highlighting the versatility and robustness of plant-derived PODs in biosensing technologies.

COLOMBIAN PLANT PEROXIDASES IN BIOSENSING

PODs sourced from Colombian plants play a pivotal role in enhancing the efficacy of electrochemical biosensors, marking a significant advancement in sensor technology. Incorporating peroxidase into electrochemical biosensors such as enhances their sensitivity but also enables the detection of analytes at lower concentrations, thus expanding the scope of applications in fields like environmental monitoring (Sridhar *et al*., 2022), healthcare (Tripathi & Bonilla-Cruz, 2023), and food safety (Su *et al*., 2022). Colombian plants offer a rich source of PODs with diverse properties, potentially offering a wide array of options for biosensor development. Harnessing these enzymes in electrochemical biosensors underscores the importance of biodiversity conservation and sustainable utilization of natural resources, while also paving the way for innovative solutions in biosensing technology (Gaspar *et al*., 2000).

Various immobilization methods have been explored to effectively attach PODs from plants onto electrode surfaces, enhancing their stability and activity in biosensing applications (Mohan *et al*., 2015; T.Sriwong & Matsuda, 2022). One common approach involves physical adsorption, in which PODs are simply adsorbed onto the electrode surface through non-covalent interactions (Di Risio & Yan, 2010). This method is relatively simple and cost-effective but may suffer from enzyme leaching over time. Covalent attachment (Torabi *et al*., 2007) is another widely used method, where PODs are covalently bound to the electrode surface via chemical linkers or functional groups. This approach offers enhanced stability and prevents enzyme leaching but may sometimes affect enzyme activity due to potential steric hindrance. Additionally, entrapment within polymer matrices or hydrogels (Caglar *et al*., 2021) provides a protective environment for PODs, shielding them from harsh conditions and prolonging their lifespan on the electrode surface. Each immobilization method presents unique advantages and challenges, and the selection depends on factors such as the intended application, desired stability, and compatibility with the electrode material.

For example, RPTP was immobilized on screen printed graphene electrodes modified with chitosan and glutaraldehyde (Villamizar *et al*., 2016). Cyclic voltammograms conducted in the presence of potassium ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) as a redox agent revealed a notable increase of 50mA, indicating the electron transfer process of a surface-modified screen-printed graphite electrode (SPGE) with chitosan-glutaraldehyde-reduced graphene oxide (CS -GA-RPTP).

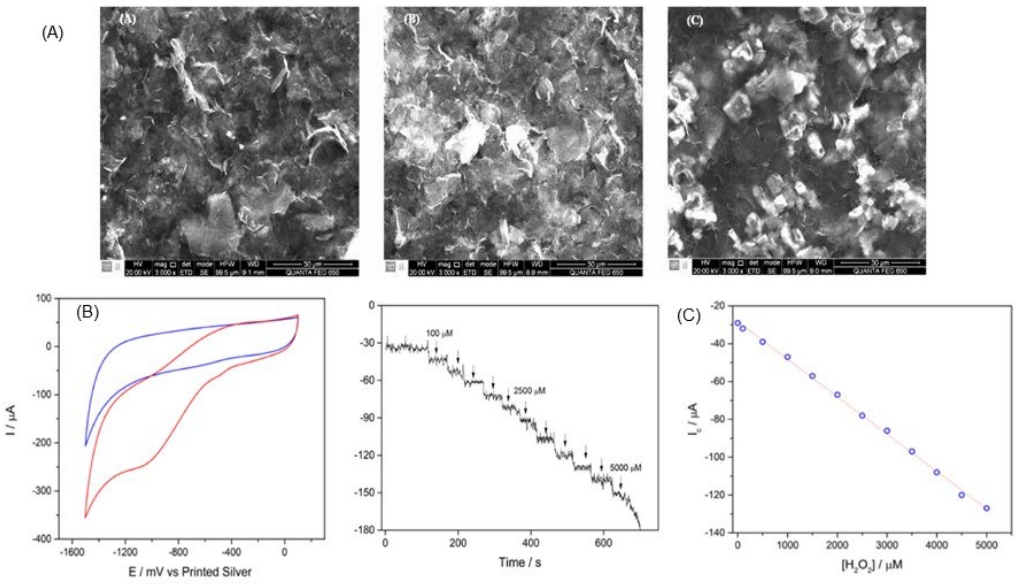


Figure 8. SEM images (A) of bare electrode and modified with RPTP, (B) CVs in the presence (0.5 mM) and the absence of H_2O_2 and (C) chronoamperometric measurements of different concentrations of H_2O_2 using the graphene modified electrodes with RPTP (adapted from Villamizar *et al.*, 2016)

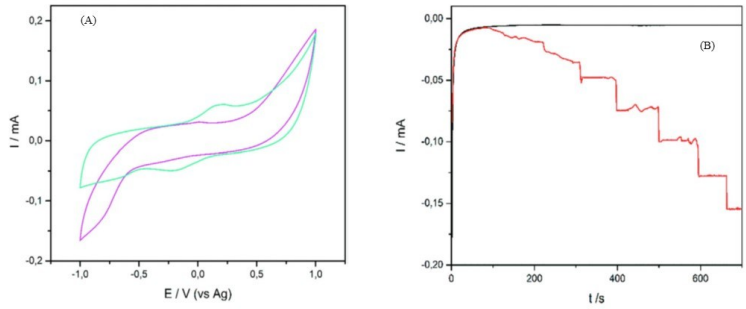


Figure 9. CVs of GGP graphene modified screen printed electrode without (cyan) and with H_2O_2 4mM, scan rate 100 mV s^{-1} (adapted from Centeno *et al.*, 2017).

The electrode, enhanced with graphene, demonstrated exceptional electrocatalytic performance in the reduction of H_2O_2, displaying a linear response within the concentration range of 100 mM to 5 mM and showing a detection limit of 87 mM as shown in Figure 8.

A new POD from the leaves of guinea grass was directly anchored onto the surface of a graphene screen-printed electrode (Uribe *et al.*, 2019). Cyclic voltammograms conducted in the presence of potassium ferrocyanide ($[Fe(CN)_6]^{3-/4-}$) as a redox species revealed an enhancement in the electron transfer process. This graphene-modified electrode exhibited exceptional electrocatalytic activity towards the reduction of H_2O_2, displaying a linear response within the concentration range of 100 µM to 3.5 mM and a detection limit of 150 µM as is shown in Figure 8. This novel POD from guinea grass facilitated the adaptation of a graphene electrode, offering a promising sensor detection platform for the determination of H_2O_2 in real samples of biomedical or environmental significance.

Precisely, this enzyme was effectively employed to create an analytical tool for rapidly determining TCS (triclosan). The authors devised a novel amperometric biosensor by modifying SPCNE (single-walled carbon nanotube electrode) with GGP (graphene-gold nanoparticles) using N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide (EDC) as a linking agent. The GGP-modified SPCNE showed an excellent electrocatalytic activity to TCS oxidation, at a redox potential of 370 mV, in the presence of H_2O_2, exhibiting a linear response between 20 mM to 80 mM and a detection limit of 3 µM (Orduz *et al*., 2019).

Electrochemical biosensors play a crucial role in detecting bacteria due to their rapid response, sensitivity, and cost-effectiveness (Yaghoobi *et al*., 2022). Gold electrodes are a common substrate in electrochemical biosensors because they can be easily functionalized with thiolated biomolecules (Hrdlička *et al*., 2018). Researchers have developed a rapid electrochemical biosensor to detect *Staphylococcus aureus* using screen-printed gold electrodes modified with cysteine and GGP(Guarín *et al*., 2020). Figure 10 shows a schematic representation of gold modified electrode with cys and GGP for the detection of 1mM H_2O_2 in the absence and the presence of *S. aureus*.

The SPGE, after modification, demonstrated excellent electrocatalytic activity toward the reduction of H_2O_2. By measuring the reduction in H_2O_2 current at -780 mV, which was consumed by the catalase enzyme from *S. aureus*, and it was electrochemically detected. The modified electrode exhibited sensitivity within a concentration range of $3x10^2$ to $3x10^8$ CFU/mL^{-1}, with a low detection limit of 102 CFU/mL^{-1}. The detection process took approximately 20 minutes, and the sensitivity was measured at 0.020 mA per CFU.

Electrochemical biosensors utilizing quantum dots offer remarkable sensitivity and selectivity in detecting biomolecules (Carter-Searjeant *et al*., 2023). Quantum dots, with their tunable properties, enhance signal amplification, making them ideal for precise measurements in biological samples (Adegoke *et al*., 2023). These sensors exploit the unique electrochemical behavior of quantum dots, enabling real-time monitoring of biomolecular interactions with high accuracy and efficiency. Additionally, their compatibility with miniaturized devices holds promise for point-of-care diagnostics and personalized healthcare applications (Tripathi & Bonilla-Cruz, 2023). Castillo *et al*., developed and electrochemical sensor for detection of H_2O_2 using GGP immobilized on screen-printed quantum dots electrodes (Castillo *et al*., 2022). GGP revealed a well-defined pair of redox signals at 17mV/-141mV corresponding to the redox process of the heme group (Fe^{2+}/Fe^{3+}) of PODs. The bioelectrocatalytic reduction of H_2O_2 showed a redox potential of -645 mV vs Ag/AgCl. Chronoamperometry studies allows the construction of calibration curves of reduction current vs H_2O_2 concentration for the determination of analytical parameters such as sensitivity, linear range and minimum detection level.

Electrochemical biosensors leveraging PODs extracted from sweet potato (*Ipomoea batatas*) offer a sustainable and cost-effective solution for detecting various analytes (Leon *et al*., 2002). The use of SPP as biorecognition elements provides high specificity and sensitivity due to their inherent catalytic activity and stability under diverse environmental conditions. These biosensors can detect target molecules with remarkable precision, making them valuable tools in biomedical, environmental, and food safety applications (Smart *et al*., 2023). Moreover, harnessing natural enzymes from batata promotes eco-friendly prac-

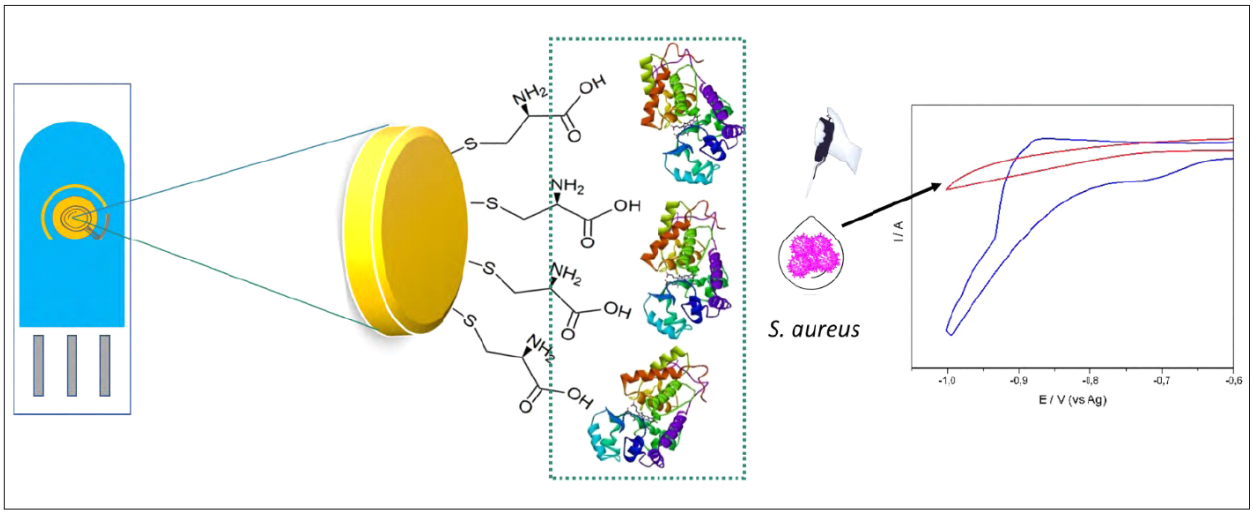


Figure 10. (A) Depiction of a gold screen-printed electrode modified with cysteine and GGP. (B) Cyclic voltammograms illustrating the detection of H_2O_2 at a concentration of 1 mM in the presence (indicated by the red line) and absence (shown by the blue line) of *S. aureus* bacteria (adapted from Guarín *et al.*, 2020).

tices in sensor development, aligning with the growing demand for sustainable technologies in the field of biosensing. Within this context, Csöregi *et al*., developed a bi -enzyme biosensor for glucose, ethanol and putrescine built on oxidase and sweet potato peroxidase (SPP) (Csöregi *et al*., 2003). The SPP-based electrodes displayed higher sensitivity and better detection limits for putrescine than those using HRP and were also shown to retain their activity in organic phase much better than the HPR based ones as is shown in calibration curves of Figure 10.

Figure 10. Calibration curves for glucose (a), putrescine (b), and ethanol (c) obtained with biosensors based on SPP and HRP (adapted from Csöregi *et al*., 2003).

Electrochemical sensing employing electrodes derived from various allotropes of carbon, such as graphene (Sethi et al., 2020), carbon nanotubes (W. Shi et al., 2021), and diamond-like carbon (Ficek et al., 2023), has revolutionized the field of analytical chemistry. These carbon-based electrodes offer exceptional properties including high electrical conductivity, large surface area, chemical stability, and biocompatibility (González-Hernández et al., 2022). Their unique structural characteristics enable the sensitive detection of analytes through electrochemical reactions, making them ideal platforms for biosensing applications. Carbon allotrope-based electrodes have been utilized in diverse fields ranging from environmental monitoring to medical diagnostics (Vatankhahan et al., 2024), showcasing their versatility and efficacy. Their integration into electrochemical sensing devices holds immense promise for rapid, accurate, and cost-effective detection of various substances, thereby driving advancements in sensor technology for improved quality of life and environmental sustainability (Tsounidi et al., 2023). GGP was deposited on the surface of SPEG and graphene oxide electrode (SPEGO) to study the bio-electrocatalytic reduction of H_2O_2 (Castillo et al., 2022). The sensors exhibited a couple of well-defined redox peaks at 120 mV/10.5 mV and 184 mV/59 mV for anodic and cathodic peaks, respectively. The GGP-modified electrodes exhibited a good electrocatalytic activity for the reduction of H_2O_2 reduction at a redox potential of -0.6 V and -0.5 V for SPEG and SPEGO, respectively.

CHALLENGES AND LIMITATIONS

PODs from Colombian tropical plants have emerged as promising candidates for use in electrochemical biosensors due to their remarkable catalytic properties and widespread availability. However, several challenges must be addressed to fully exploit their potential in this application. One significant challenge is the stability of plant peroxidases under the harsh conditions often encountered in electrochemical sensing, such as high temperatures and extreme pH levels. Ensuring the long-term stability of PODs immobilized on electrode surfaces is crucial for the consistent and reliable performance of biosensors. Another challenge is optimizing immobilization techniques to achieve efficient electron transfer between the POD and the electrode surface. Various methods, including physical adsorption, covalent binding, and entrapment within polymer matrices, have been explored to immobilize PODs while maintaining their catalytic activity and stability. Additionally, the interference from other electroactive compounds present in complex sample matrices poses a significant challenge for selective detection using plant PODs. Strategies such as selective membrane barriers and signal amplification techniques need to be implemented to overcome this issue and enhance the specificity of electrochemical biosensors. Furthermore, the development of portable and miniaturized biosensor platforms for on-site detection applications requires additional research to optimize the sensitivity, reproducibility, and cost-effectiveness of plant peroxidase-based biosensors. Despite these challenges, ongoing advancements in enzyme immobilization techniques, electrode materials, and signal amplification strategies hold promise for the widespread use of plant peroxidases in electrochemical biosensing applications, paving the way for sensitive, selective, and reliable detection of various analytes in environmental, clinical, and food safety context.

CONCLUSIONS

In conclusion, Colombian plant PODs offer immense potential for the development of electrochemical biosensors, bridging the rich biodiversity of the rainforest with cutting-edge laboratory technologies. Despite facing challenges such as stability under harsh conditions, efficient immobilization techniques, and selectivity in complex sample matrices, the versatility and catalytic efficiency of these enzymes make them valuable candidates for sensitive and selective detection applications. Through innovative approaches in enzyme immobilization, electrode modification, and signal amplification, significant strides have been made in harnessing the capabilities of Colombian plant PODs for electrochemical biosensing. As research continues to unravel the intricacies of these enzymes and their interactions with electrode surfaces, we anticipate further advancements in biosensor design, paving the way for real-world applications in environmental monitoring, clinical diagnostics, and food safety. The integration of Colombian plant PODs into electrochemical biosensing platforms not only showcases the potential of natural resources but also underscores the importance of interdisciplinary collaboration in addressing global challenges through bio-inspired solutions. This attempt represents a significant step forward in utilizing local biodiversity for technological innovations, offering sustainable solutions

that can adapt to various industrial needs while promoting conservation efforts in Colombia's rich ecosystems.

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