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Polypyrrole/phytase amperometric biosensors for the determination of phytic acid in standard solutions

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A B S T R A C T
Amperometric biosensors based on the physical immobilization of phytase (PhyA) into polypyrrole (PPy) films were prepared in aqueous medium. The PPy/PhyA films were characterized by cyclic voltammetry, and surface and structural characterization techniques, SEM and FTIR. Both voltammetric and amperometric transduction methods were used in order to detect phytic acid in acetate buffer at pH 5.5 at room temperature. The biosensors exhibited a detection limit of 0.15 mmol L−1 and a linear range of phytic acid content from 0.5 to 2.0 mmol L−1, which are adequate values for typical analyses of phytic acids in most seeds, grains, and vegetables.

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1. Introduction

Phytase (myo-inositol hexaphosphate phosphohydrolase) (PhyA) catalyzes the hydrolysis of phytic acid (myo-inositol hexaphosphate or phytate) into phosphorous compounds, which is an essential nutrient for animals [1] according to the reaction shown below.

\[
\text{phytic acid} + \text{H}_2\text{O} \xrightarrow{\text{phytase}} \text{inositol} + \text{PO}_4^{3-}
\]

Phytic acid is the main storage form of phosphate in grain seeds, pollen, vegetables, and oilseeds [2,3], but it is also considered an antinutrient due to its chelation ability, binding dietary minerals such as magnesium, zinc, and calcium [2]. Monogastric animals, such as pig and poultry, and also humans, are not able to digest phytic acid because they lack sufficient amounts of the digestive enzyme, PhyA [4,5]. In this case, unabsorbed phosphorus compounds pass into the feces and can be degraded by aquatic microorganisms, being one of the causes of eutrophication of water bodies downstream the rural areas [6]. In order to alleviate adverse effects of high levels of phytic acid, PhyA has been used as supplementary feeding in swine and poultry rations [7–9].

The presence of phytic acid in food and feedstuffs can be determined by many methods; a well-known procedure involves the precipitation of iron (III)–PhyA complex, and further analysis of the endpoint of titration [10]. In addition, techniques such as nuclear magnetic resonance spectroscopy, high-performance liquid chromatography, and SDS–polyacrylamide gel electrophoresis [11,12] have been also used for the detection of phytic acid in different analytes. The use of biosensors based on the immobilization of PhyA into polymeric matrices appoints as an alternative for the detection of phytic acid. Previous studies have reported the preparation of poly(carbamoylsulphonate) hydrogel co-immobilized with PhyA and pyruvate oxidase onto Pt electrodes [13]; the authors obtained for these amperometric biosensors a detection limit of 2.0 × 10−3 mmol L−1 and a linear response ranging from 0.2 to 2.0 mmol L−1 of phytic acid at a fixed potential of 0.6 V vs Ag/AgCl [13]. Recently, amperometric biosensors based on the immobilization of PhyA into poly(allylamine) hydrochloride/Prussian Blue/ITO electrodes by the Layer-by-Layer technique were fabricated by our group [14]. These biosensors exhibited a detection limit of 0.19 mmol L−1 and a linear response ranging from 0.5 to 2.0 mmol L−1 of phytic acid at 0.0 V vs SCE [14].

The present study provides continuity for this research field mainly with the aim of improving properties of the PhyA biosensors. PhyA was immobilized into polypyrrole (PPy) films by a simple and fast protocol based on physical interactions that take place between the enzyme and the polymer matrix. PPy is regarded as a suitable matrix for most enzyme immobilization once pyrrole oxidizes at relatively low oxidation potentials in both aqueous and non-aqueous media. Besides, PPy is usually biocompatible [15]. The electronic properties of the PPy films also allow a fast, direct
electron charge transfer between the polymer film matrix and the immobilized enzyme, thus yielding sensitive and selective signals [16,17].

Previously, we have already shown the viability of biosensors based on the immobilization of PhyA onto LbL films [14]. However, the use of a polymeric matrix appoints as other alternative for obtaining simpler and cheaper biosensors, in particular when they are miniaturized, and require minimum sample amounts. Here, we describe the build of PPy/PhyA films and their characterization by electrochemical measurements, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR).

2. Experimental

Phytase (PhyA) from Aspergillus ficuum (EC 3.1.3.8) (1.1 units g−1, IP=4.5) (Sigma–Aldrich) and phytic acid (myo-inositol hexaphosphate) (Synth) were used as received. Pyrrole (Sigma–Aldrich) was freshly distilled before use. PhyA and phytic acid solutions were prepared by using 0.1 mol L−1 acetate buffer at pH 5.5.

The electropolymerization of pyrrole was performed by cyclic voltammetry using a potentiostat/galvanostat PAR Versastat II with a three-electrode cell, a saturated calomel electrode (SCE) as the reference electrode, a Pt disk as the working electrode, and a Pt plate as the counter electrode. The electrochemical deposition of polypyrrole (PPy) films was carried out by cyclic voltammetry from 0.0 to 0.8 V vs SCE at 50 mVs−1 in an aqueous solution containing 0.07 mol L−1 pyrrole and 0.1 mol L−1 NaCl up to reach 10 complete cycles. For the PPy films obtained up to reach 10 cycles, we obtained a thickness of about 0.8 µm which was calculated by an equation reported in the literature [18]. Smaller numbers of cycles yielded thinner films, but not necessarily homogeneous; much thicker films, on the other hand, would hinder ionic movements into the film and, therefore, offer higher diffusion resistance of the analyte related to the polymeric matrix.

Freshly prepared PPy films were washed with water and a monomer-free electrolyte solution. After that, they were dipped into a PPy solution at 0.2 mg mL−1 at 40 °C, where were kept for 2 h; this procedure allowed us to physically immobilize PhyA into the PPy film. Previously, we verified that the immobilization of PhyA by the Layer-by-Layer technique occurred efficiently at about 40 °C [14]. This temperature has no direct effect on the enzymatic activity, since the optimum temperature of PhyA activity lies between 37 °C and 60 °C (at pH 5.5). The geometrical surface area of the Pt electrodes modified with PPy and PPy/PhyA films was 0.6 cm².

Chronoamperometry was used for the detection of phytic acid at a fixed potential of 0.0 V vs SCE. The current was monitored as a function of the time after successively adding aliquots of 50 µL of 0.1 mol L−1 phytic acid at 10 mL acetate buffer at pH 5.5.

The morphology of the PPy and PPy/PhyA films on Au coatings was studied by scanning electron microscopy (SEM) (Digital Scanning Microscope, DSM 960, Zeiss, West Germany). The infrared spectra of these films on Pt substrates were obtained using a Thermo Nicolet–Nexus 470/FT-IR spectrophotometer from 400 cm−1 to 2000 cm−1 after 64 scans.

3. Results and discussion

Polypyrrole (PPy) films were electrodeposited on Pt electrodes in an aqueous medium containing pyrrole and NaCl up to reach ten successive voltammetric cycles. During the electropolymerization process, the current density increases linearly with the number of cycles indicating the growth of an electroactive film (PPy) on the electrode surface. The PPy/PhyA film was then prepared by keeping the PPy film into a solution of 0.2 mg mL−1 PhyA in acetate buffer at 40 °C for 2 h.

The voltammetric responses of the PPy and PPy/PhyA films were obtained in a pyrrole-free solution, 0.1 mol L−1 acetate buffer at pH 5.5. The PPy film exhibited a fairly well-defined voltammetric response (Fig. 1); after modification with PhyA, higher values of current densities can be noticed in the voltammogram, but still with the typical electrochemical response of a native PPy matrix. The differences in current seen for the PPy and PPy/PhyA films indicate the occurrence of electrostatic interactions between PhyA and its matrix, PPy; this is the expectable behavior for films with immobilizing enzymes entrapped into a polymeric matrix [19]. The voltammogram recorded in the literature for a different matrix (a metallophthalocyanine–metalloporphyrin complex) without and with immobilized glucose oxidase [20,21] is very close in shape to the curves seen in Fig. 1, indicating that the presence of enzyme has a role in the voltammetric response of the electrode material. The enhanced capacitive background current seen for the PPy/PhyA film can be related to a typically high electron transfer capacity of this film, and consequently, its larger specific surface area. However, generally speaking, the voltammetric responses of the PPy ad PPy/PhyA films in the blank solution did not show remarkable differences.

Fig. 2 shows the FTIR spectra of the PPy and PPy/PhyA films from 650 cm−1 to 1750 cm−1 since in this spectral range they can be better comparable. The spectrum of the PPy film obtained by us is
in accordance with the literature [22–25]. The immobilization of PhyA into the PPy matrix was confirmed by the presence of sharp peaks shifted in the spectra of the PPy/PhyA film. Among them, the peak assigned to the C–H out-of-plane deformation of the pyrrole units [23] appeared at 915 cm\(^{-1}\) for the PPy film and shifted to 861 cm\(^{-1}\) in the spectra of the PPy/PhyA film. The two peaks at 1480 cm\(^{-1}\) and 1540 cm\(^{-1}\), which are typical of the PPy films, indicate the extent of the conjugation of this polymer [25]. They have been attributed to the symmetric and anti-symmetric ring stretching modes of PPy, and were shifted to 1442 cm\(^{-1}\) and 1566 cm\(^{-1}\) for the PPy/PhyA film and 1540 cm\(^{-1}\) for the PPy film. The peak at 1700 cm\(^{-1}\), which has been usually attributed to the presence of carbonyl groups in the PPy structure [22,24], is seen in the spectra of the PPy/PhyA film with a low intensity, indicating a slightly overoxidized polymer structure. The changes in the spectra of the PPy and PPy/PhyA film strongly evidence the occurrence of electrostatic interactions between negatively charged PhyA and its matrix, a positively charged PPy.

The PPy and PPy/PhyA films were also studied by scanning electron microscopy (SEM). Fig. 3 reveals the typical morphology of the PPy matrix, a spheroid structure with dispersed spheres with sizes of ca. 0.4 \(\mu\)m. For the PPy/PhyA film, the nodular structure from the polymeric matrix was maintained, but a narrower distribution of smaller sphere sizes of ca. 0.15 \(\mu\)m is also evident. Besides, the spheres are distributed more uniformly over the surface of the PPy/PhyA film as an indicative of the presence of immobilized PhyA on smooth PPy/PhyA films. Previously, it was shown that the morphology of the polymeric matrix (PPy) modifies, usually in a small extent, after introducing an insulating, bulky protein (glucose oxidase) into the electropolymerization medium [26]. Polymer films with entrapped enzymes generally exhibit smoother morphologies with low defect sites and absent enzyme clusters [27], these differences have been related to the influence of the enzyme on the whole growth process of the polymer film.

Fig. 4 shows the electrochemical responses of the PPy/PhyA film in 0.1 mol L\(^{-1}\) sodium acetate buffer at pH 5.5 containing different phytic acid concentrations. Between −0.2 V and +0.6 V vs SCE, it is clearly evident that the current density increases with the phytic acid concentration (see the inset of Fig. 4 for values of current densities obtained at a fixed potential, 0.0 V vs SCE). A linear increase of the current density is clearly seen from 0.5 mmol L\(^{-1}\) to 2.0 mmol L\(^{-1}\) of phytic acid; after that, it is evident a saturation regime. These results indicate that the PPy/PhyA film can be used to detection of phytic acid up to reach 2.0 mmol L\(^{-1}\) phytic acid at 0.0 V vs SCE where effects of interferents are expected to be negligible. The electrochemical responses of the PPy/PhyA biosensors are based on the monitoring of inorganic phosphorous compounds, which are produced by the enzymatic reaction (see Section 1). Without the presence of the immobilized enzyme, no significant changes in the voltammetric responses were verified for the PPy film after adding phytic acid ranged from 0.0 to 4.0 mmol L\(^{-1}\) (about 9.5% for the current densities obtained a fixed potential). For the PPy/PhyA film, the differences in current densities are higher than 54% as a strong evidence of the catalytic effect of the enzyme immobilized into the PPy matrix. After successive injections of phytic acid into the buffer solution, the PPy/PhyA film responded rapidly, and both anodic and cathodic currents varied. The substrate specificity of PhyA was also verified by using phenyl phosphate disodium salt, buffer, inositol, and dipalmitol phosphatidyl choline (DPPC), however, no significant changes in the current densities were also obtained by using these solution, which corroborates the conclusion that the PPy/PhyA film is only capable of detecting phytic acid and, consequently, free phosphorous ions.

Fig. 5 shows the chronoamperometric curves obtained for the PPy/PhyA film at a fixed potential of 0.0 V vs SCE in 0.1 mol L\(^{-1}\) acetate buffer at pH 5.5. After adding successive aliquots of 0.1 mol L\(^{-1}\) phytic acid (50 \(\mu\)L each) in 10 mL of acetate buffer, the current density increased with the concentration of phytic acid up the fourth addition at 2.0 mmol L\(^{-1}\). After that, the current density decays abruptly due to a saturation effect of the active sites of PhyA (denaturation) in the presence of phytic acid. After a long-term experiment, one expects modifications in temperature and pH of the film surface, with PhyA modifying its three-dimensional

**Fig. 3.** SEM images of PPy and PPy/PhyA with a 10.000× resolution.

**Fig. 4.** Cyclic voltamograms of PPy/PhyA in acetate buffer at pH 5.5 and different concentrations of phytic acid ranging from 0.0 to \(4.76 \times 10^{-3}\) mol L\(^{-1}\). Inset: current density as a function of the concentration of phytic acid obtained at a fixed value of potential, 0.0 V.
Table 1: Characteristics of phytic acid biosensors based on entrapped phytase (PhyA).

<table>
<thead>
<tr>
<th>Transducer</th>
<th>LOD (mM)</th>
<th>Linear range (mM)</th>
<th>Interferents tested</th>
<th>Matrix</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amperometry</td>
<td>0.15</td>
<td>0.5–2.0</td>
<td>Phosphate ions, inositol, and phenyl phosphate</td>
<td>PPy(PhyA)</td>
<td>This work</td>
</tr>
<tr>
<td>Amperometry</td>
<td>0.002</td>
<td>0.2–2.0</td>
<td>–</td>
<td>Poly(carbamoylsulphonato) hydrogel (PhyA and pyruvate oxidase)</td>
<td>[13]</td>
</tr>
<tr>
<td>Amperometry</td>
<td>0.19</td>
<td>0.2–2.0</td>
<td>Phosphate ions, inositol, DPPC, and phenyl phosphate</td>
<td>Poly(allylamine) hydrochloride/Prussian Blue (PhyA)</td>
<td>[14]</td>
</tr>
<tr>
<td>Voltammetry</td>
<td>–</td>
<td>6.0–50</td>
<td>–</td>
<td>Phospholipid LB films (PhyA)</td>
<td>[28]</td>
</tr>
<tr>
<td>Impedance</td>
<td>0.004</td>
<td>–</td>
<td>–</td>
<td>Poly(vinylsulfonate) (PhyA)</td>
<td>[29]</td>
</tr>
</tbody>
</table>

Fig. 5. Amperometric curves for PPy/PhyA at 0.0 V vs SCE in acetate buffer at pH 5.5. The arrows indicate the addition of aliquots of 50 μL of a 0.1 mol L−1 phytic acid in 10 mL of acetate buffer solution. Inset: maximum values of the current density as a function of the concentration of phytic acid as indicated by the arrows.

structure and, consequently, its properties. Since this process is irreversible, the current density decay is, in fact, very fast.

The PPy and PPy/PhyA films were tested when in the presence of phytic acid, inositol, and phosphate ions (Fig. 6). For inositol, no current signal was noticed in the amperometric curves for both films, PPy and PPy/PhyA; for phosphate ions, both films gave current signals, and for phytic acid, only the PPy/PhyA film detected its presence in solution. These results indicate that the amperometric response of the biosensor can be in fact related to the adsorption of phytic acid into the polymer matrix, as it was verified before for Prussian Blue/PAH/PhyA biosensors [14].

We verified that both voltammetric and amperometric detection methods show good performances in detecting phytic acid by using the PPy/PhyA films. The detection limit obtained for these films was 0.15 mmol L−1 (three times the noise), with a linear range between 0.5 and 2.0 mmol L−1 of phytic acid at a low operating potential. The sensitivity of the PhyA/PPy film towards phytic acid was calculated by slope of the linear correlation curve, and gave a value of 0.2 μA mmol−1 cm−2. Apparent Michaelis– Menten constant (K_M) was found to be 2.44 mmol L−1 for the PhyA immobilized into the PPy film, with value similar to that obtained by Mak et al. [13], 2.25 mmol L−1. These results are consistent with our previous results from the use of PhyA immobilized in Layer-by- Layer film onto ITO-coated glass and modified with Prussian Blue [14] and also, bi-enzyme sensors based on the co-immobilization of PhyA and pyruvate oxidase [13]. In Table 1, we comparatively show recent results on biosensors fabricated from PhyA entrapped into different matrices. Although the detection limit obtained by us is typically lower than that obtained previously [14], the use of PPy/PhyA films provide a suitable means of obtaining low-cost biosensors. Furthermore, the Pt electrode used for the electrodeposition of the PPy/PhyA films is reusable and requires only the presence of PhyA and pyrrole in the electrolyte solution, decreasing costs and fabrication steps.

4. Conclusions

PPy/PhyA biosensors were successfully fabricated by the electrochemical synthesis of the PPy matrix with physically immobilized PhyA. Techniques such as cyclic voltammetry, SEM, and FTIR allowed us to determine differences in the responses of the PPy and PPy/PhyA films. We obtained for the PPy/PhyA film a detection limit of 0.15 mmol L−1 and a linear range of concentrations between 0.5 and 2.0 mmol L−1 of phytic acid. These results indicate that the PPy/PhyA biosensors are an alternative for the detection of phytic acid in solution with relatively low costs and at low operating potentials.

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