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Zein as biodegradable material for effective delivery of alkaline phosphatase and substrates in biokits and biosensors

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ABSTRACT

A biodegradable material, zein, is proposed as a reagent delivery platform for biokits and biosensors based on alkaline phosphatase (ALP) activity/inhibition in the presence of phosphatase substrates. The immobilization and release of both the substrate and/or the active ALP, in a biodegradable and low-cost material such as zein, a prolamin from maize, and in combination with glycerol as plasticizer have been investigated. Three zein-based devices are proposed for several applications: (1) inorganic phosphorus estimation in water of different sources (river, lake, coastal water and tap water) with a detection limit of 0.2 mg/L – compared to at least 1 mg/L required by legislation, (2) estimation of ALP in saliva and (3) chlorpyrifos control in commercial preparations. The single-use kits developed are low cost, easy and fast to manufacture and are stable for at least 20 days at −20 °C, so the zein film can preserve and deliver both the enzyme and substrates.

1. Introduction

Plastics have replaced glass and metals in many areas of use due to their properties such as elasticity, biocompatibility, stability, low cost and easy manufacture. However, the majority of them are derived from petrochemicals. In addition, huge amounts of wastes are generated and have to be treated, but it has been shown that recycling has failed to provide a safe and environmentally-friendly solution.

Biodegradable and biocompatible materials extracted from renewable resources have received much interest, since they offer less petroleum-dependent and contaminant-causing alternatives than plastics. According to ASTM ISO "Environmentally Degradable Plastics", biodegradable materials are defined as a material in which degradation results from the action of naturally-occurring microorganisms such as bacteria, fungi and algae (Briassoulis and Dejean, 2010). In this context, zein which is a storage protein isolated from the maize endosperm, has shown biodegradation under different environmental conditions (pH, T° and moisture) (Imam and Gordon, 2002) and also, *in vivo* and *in vitro* studies demonstrated its use in tissue engineering (Lin et al., 2011) and drug delivery (Paliwal and Palakurthi, 2014). Zein consists of a mixture of polypeptides, dominated by α-zein (~20–25 kDa, 70–85%), which is rich in leucine, proline, alanine, serine and glutamine, and γ-zein (~15–30 kDa, 10–20%), which also has a high cysteine content. This creates an amphipathic structure which combines an antiparallel α-helical structure from α-zein, with the N-terminal driven polyproline II (Argos et al., 1982) type structure from γ-zein, held together by disulfide bridges and inter- and intra-chain hydrogen bonding. Despite the polar glutamine and serine, this results in a rather hydrophobic insoluble material. Rhys and Dougan, for example have shown that hydrogen bonding between the side chain and backbone, associated with the polyglutamine sequence causes an insoluble structure due to side-chain/backbone internal hydrogen bond formation taking preference over hydrogen bonding with water (Rhys et al., 2013). Nevertheless, ~50% of the amino acid content of zein is polar and side chain interactions provide a potentially well-stabilized environment for incorporation and stabilization of other proteins.

Nowadays, there is an increasing demand to develop *in situ* devices. In order to find green alternatives for on site sensing, not only biodegradable materials are required, but also, biosensors have been explored, based on enzyme immobilization or using combinations of enzymes like alkaline phosphatase (where phosphate and organophosphorus compounds are inhibitors) or pyruvate oxidase (where phosphate is cosubstrate) (Villalba et al., 2009). The main mode of detection in this biosensor research has been electrochemical, linked with multi-enzyme systems that produce a better electroactive product or current amplification.

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Nevertheless, the same reagents can be used in an optical assay, by changing the enzyme's substrate, so that a fluorophore/colorimetric product is generated. For example, alkaline phosphatase is a non-specific phosphomonoesterase that is competitively inhibited by several analytes. Previous studies have used alkaline phosphatase for phosphate estimation developing the assay in solution (Coburn et al., 1998; Upadhyay and Verma, 2015) and also for heavy metals and pesticide analysis (Upadhyay and Verma, 2013; Upadhyay and Verma, 2014; Berezhetskyy et al., 2008; García Sánchez et al., 2003; Mazzei et al., 2004; Prieto-Simón et al., 2006), However, these papers have not included assays in the field with real environmental samples/biological samples or made the step to an integrated solid-state (and ultimately biodegradable) sensor.

This work takes the first steps to investigate whether devices, composed entirely of biodegradable materials, could be developed for biokits and biosensors. The systems investigated here are based on alkaline phosphatase (ALP) activity/inhibition in the presence of phosphatase substrates, such as 3-O-methylfluorescein phosphate (OMFP) or p-nitrophenyl phosphate (p-NPP). The enzyme and substrate were packaged in a solid film of zein and delivered to the test solution, so that both ALP and OMFP or p-NPP diffused from the zein disk into the solution. The potential applications presented here are: (1) inorganic phosphate (P_i) estimation, necessary to control nutrients and eutrophication (phosphate levels in water are regulated by the EU through the Urban Waste Treatment that underlines the maximum annual mean total phosphorus concentration of 1–2 mg/L and the Water Framework Directive 2000/60/EC), (2) the estimation of ALP in saliva, which allows distinction to be made between adult and child saliva and (3) the organophosphorus pesticide (OPs) estimation in control analysis of commercial formulations. In this instance, a comparative study was performed using the conventional ammonium molybdate method to validate the results obtained in water. The active ALP immobilization, substrate stabilization and delivery system were studied and it was shown that zein can be used for encapsulating/packaging and delivering enzymes and substrates to the sample during the assay. These simple, environmentally friendly and low cost devices can also avoid the need of time-consuming preparation of fresh substrate/enzyme solutions for carrying out the assays.

2. Materials and methods

2.1. Materials

Zein, p-nitrophenylphosphate (p-NPP), 3-O-methylfluorescein phosphate cyclohexammonium salt (OMFP) and alkaline phosphatase (ALP) from bovine intestinal mucosa (lyophilized power $\geq$10 units/mg solid), were purchased from Sigma Aldrich (Saint Louis, USA). While absolute ethanol was obtained from Romil (Cambridge, UK) and sodium monobasic phosphate from Merck (Darmstadt, Germany).

2.2. Apparatus

All the emission measurements were made in a spectrofluorometer Jasco FP-750 (Tokyo, Japan) and the absorbance measurements in a spectrophotometer Agilent 8453 (Palo Alto, USA).

2.3. Preparation of the biokits

Protein film casting was carried out by dissolving zein (10% w/v) in aqueous ethanol (90% v/v) along with glycerol (0%, 30%, 50%, 70% and 90% on a zein weight basis) as a plasticizer and then the enzyme, alkaline phosphatase (Commercial ALP, 10 units/mg) was added. Fresh alkaline phosphatase (ALP) aqueous solution (80 μL of 10 mg/mL) was added and the mixture (1.6 mL) was stirred for 20–30 min. Finally, it was placed into a well-plate mold with 8 positions, 200 μL was used for each biodevice, containing 1 unit of ALP (disk S1). After 6 h at room temperature, the biosensor reagent packages were obtained and were frozen (−20 °C) until further usage. The disks S2, S3, S4 and S5 were synthetized following the same process with 70% of glycerol and using in each case different volumes of ALP aqueous solution of 10 mg/mL: 70, 60, 40 and 8 μL for S2, S3, S4 and S5, respectively. The disks S2, S3, S4 and S5 contained 0.875, 0.75, 0.5 and 0.1 units of ALP.

The substrate embedded into the film was also prepared. The substrate 3-O-methylfluorescein phosphate cyclohexammonium salt (2.4 μM) was dissolved in ethanol and was added to the zein mixture, which is zein (10% w/v) in aqueous ethanol (90% v/v) along with glycerol 70% on a zein weight basis. The same experimental process was used for p-nitrophenyl phosphate (2.02 μM) immobilization in zein. Therefore, three types of disks were prepared: one disk was made of ALP immobilized in a zein film, other disk was made of OMFP immobilized in zein film and the last disk was made of p-NPP immobilized in zein film. All these reagent disks were stored at −20 °C for 20 days.

Kit A for P_i estimation in waters contains 2 separate disks (one of OMFP immobilized in zein and the other of ALP immobilized in zein) and the buffer (Tris-HCl 100 mM, pH=9.0), The kit B for ALP in saliva contains only one disk (p-NPP immobilized in zein) and buffer (Tris HCl 100 mM, pH=8) and the kit C for organophosphorus in commercial preparations contained 2 separate disks (p-NPP immobilized in zein and the other of ALP immobilized in zein) and buffer (Tris HCl 100 mM, pH=8).

2.4. Methods

2.4.1. Fluorescence measurements

For free alkaline phosphatase in solution, fluorescence measurements were carried out in a vial containing Tris HCl buffer (2 mL, 100 mM, pH=9.0), alkaline phosphatase and substrate 3-O-methylfluorescein phosphate (OMFP) (2.4 μM). The OMFP was hydrolyzed by alkaline phosphatase to yield 3-O-methylfluorescein (OMF) that was detected at 485 nm excitation/513 nm emission in a quartz cuvette. The fluorescent signal was captured every 30 s for up to 5 min and the fluorescent intensity was plotted versus time (see Figs. 1 and 2), the slope obtained of each line represented the initial rate (V) for each ALP concentration. Therefore, initial rate as the variation of the fluorescence intensity (RFI) over time (t), gives $V=\Delta(\mathrm{RFI})/\Delta t$. The initial rate ($V$) increased with increase the enzyme concentration. All the experiments were carried out in triplicate (n=3). For the calibration of free ALP activity in aqueous solution, a calibration curve ($V=2.047\cdot[\mathrm{ALP}]+2.2321$, $R^2=0.998$) was calculated by initial rate vs ALP concentration from 0.25 to 50 mg/L (Commercial ALP used is 10 units/mg). A calibration curve of inhibition by P_i was calculated by initial rate vs inorganic P_i concentration from 0.5 to 5 mg/L in presence of 10 mg/L of ALP. Finally, the inhibition efficiency was obtained by the logarithm of P_i concentration vs the percentage inhibition. The percentage inhibition (% INH) was calculated as follows:

$$\%INH = \frac{V - V_p}{V} \times 100 \quad (1)$$

Where V is the initial rate without phosphate and, V_p is the initial rate with phosphate.

For the ALP disk, measurements were carried out in a vial containing the enzyme-zein film disk and Tris HCl buffer (2 mL,