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Alkaline Phosphatase−Polyresorcinol Complex: Characterization and Application to Seed Coating

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An alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli* ATCC27257 was immobilized by copolymerization with resorcinol. The phosphatase−polyresorcinol complex synthesized retained about 74% of the original enzymatic activity. The pH and temperature profile of the immobilized and free enzyme revealed a similar behavior. Kinetic parameters were determined: K_m and K_i values were 2.44 and 0.423 mM, respectively, for the phosphatase−polyresorcinol complex and 1.07 and 0.069 mM, respectively, for free phosphatase. The thermal and storage stabilities of the immobilized phosphatase were higher than those of the native one. On addition to soil, free enzyme was completely inactivated in 4 days, whereas the phosphatase−polyresorcinol complex was comparatively stable. Barley seed coated with the immobilized enzyme exhibited higher rhizosphere phosphatase activity. Under pot culture conditions, an increase in the soil inorganic phosphorus was detected when the seed was encapsulated with the phosphatase−polyresorcinol complex, and a positive influence on biomass and inorganic phosphorus concentration of shoot was observed.

KEYWORDS: Alkaline phosphatise; enzyme immobilization; enzyme−resorcinol complex; *Escherichia coli*; hollow-bead; seed coating

INTRODUCTION

Deficiency of soil phosphorus (P) is one of the most important chemical factors restricting plant growth in soils. Therefore, a large quantity of soluble forms of P fertilizers is applied to achieve maximum plant productivity. However, a great part of the soluble P introduced into soil as fertilizer reacts with soil components forming insoluble P-bearing products (*1*), which are not efficiently taken up by the plants (*2*). This unmanaged excess of P application is known to cause environmental and economic problems. In fact, the overfertilization of P leads to pollution due to soil erosion and runoff water containing amounts of soluble P. The runoff from P-loaded soil is accepted as the main factor in the eutrophication of natural water reservoirs (*3*).

Soil contains a wide range of organic substrates, which can be a source of P for the growth of plants (*4*). To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Mineralization of most organic phosphorus compounds is carried out by means of extracellular phosphatases (*5*). Phosphatases, synthesized by plant root and microorganisms, are adaptive enzymes produced in response to a need for P by plants (*6*).

Many attempts have been made to modify the biological processes taking place in soil in order to generate a more suitable medium for plant growth (*7*). One such approach may be to apply stabilized enzymes (e.g., cellulase, Pronase, phosphatase), capable of expressing their activity at a high level and for an extended period of time, to increase soil biochemical activities (*8*). A soil enriched in phosphatase activity would be expected to support an effective and economic use by plants of organic P present in soil organic matter, a rapid mineralization of exogenous organic P added in organic wastes (compost manure, crop residues, etc.) adapted to metabolic needs of plants. Another biotechnological application to agriculture is the acceleration of any process conducive to more efficacious plant nutrition in the rhizosphere. Thus, the use of a stable phosphatase seed coating as a means of bringing about a localized solubilization of soil organic phosphate is a way (*9*) particularly important in the early stages of seedling establishment.

Many extracellular enzymes in soil are stabilized by their association with clays or polyaromatic supports (e.g., humates moieties). Several authors have prepared synthetic humic-enzymes based on the fact that phenolic compounds respond to enzymes, such as peroxidases and laccases, to form radical and quinones, which subsequently polymerize through covalent bond formation or nucleophilic addition (*10*−*12*). The formation of polyphenol−enzyme complexes is characterized by chemical and structural properties comparable to the naturally occurring enzyme complexes of soil (*10*). Garzillo et al. (*12*) synthesized an acid phosphatase−polyresorcinol complex more stable than the free enzyme toward alkaline pH, high temperature, and

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proteasas (trypsin and chymotrypsin); conversely, when added to soil, free and immobilized phosphatase showed comparable stability.

The objective of this work was to synthesize a stable alkaline phosphatase−phenolic complex using resorcinol through a copolymezation process catalyzed by peroxidase. The phosphatase−polyresorcinol complex obtained was characterized. The effect of encapsulation of barley seeds in the hollow bead with the immobilized enzyme on plant growth and phosphate uptake were also studied. To the best of our knowledge, these complexes have not been previously applied in the seed coating. Specifically, this study may help developing sustainable agricultural systems that will require new techniques to minimize fertilizer application rates while maintaining adequate crop yields.

MATERIALS AND METHODS

Materials and Reagents. Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) was obtained, as described below, from *Escherichia coli* ATCC27257, a phosphatase hyperproductive strain obtained from the Spanish Official Culture Collection (Valencia, Spain).

Agar, horseradish peroxidase type I, resorcinol, phenolphthalein phosphate, and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO). Sodium alginate was purchased from Aldrich Chemical Co. (Sigma, St. Louis, MO). Bacto-pectone and Agar Noble were from Difco (Difco Laboratories Inc., Detroit, MI). All other chemicals were of reagent grade.

The designated soil was collected from the surface (0−10 cm) from a farm field at Ribera del Arlanza in Burgos (Spain). Prior to use, the soil was air-dried at room temperature (22 ± 1.0 °C) and then gently ground to pass through a 2 mm sieve. Relevant soil properties are pH 8.7, organic C 0.593% (w/w), C:N ratio 7.15, organic P 0.27 g kg^{-1}, and inorganic P 0.52 g kg^{-1}. Soil pH was measured in 1:5 (w/w) soil/H_2O extract. Soil organic carbon was determined following the wet digestion method of Walkley and Black (*13*). C:N ratio was determined using an elemental analyzer (LECO CHN A-932). Inorganic and organic P were estimated as described by Saunders and Willians (*14*).

Cell Growth and Enzyme Production. The bacterial strain was cultivated on agar slants at 37 °C for 24 h in a medium with 3% (w/v) special agar (Noble), 0.5% (w/v) Bacto-pectone and 0.05% (w/v) glucose as carbon source. The salt solution medium used was the one described by Echols et al. (*15*): 120 mM THAM (trishydroxymethylaminomethane), 80 mM NaCl, 20 mM KCl, 17 mM $(NH_4)_2SO_4$, 0.95 mM $MgCl_2$, 0.18 mM $CaCl_2$, 7.3 μM $ZnCl_2$, and 6.2 μM $FeCl_3$. The pH was set at 7.5 with 0.1 M HCl.

In order to produce alkaline phosphatase, *E. coli* was cultivated in batch cultures with the salt solution already described supplemented with 0.5% Bacto-pectone and 0.05% glucose. Several loops of a freshly grown agar slant culture were transferred to 250 mL Erlenmeyer flasks containing 100 mL of sterile medium and incubated at 37 °C for 24 h on a rotary shaker (150 rpm) (*16*).

To extract the alkaline phosphatase from *E. coli*, samples of the bacterial cultures were centrifuged at 7,800*g* for 15 min at 4 °C, and the supernatant was then discarded. The bacterial pellet was washed three times with 0.01 M Tris-HCl buffer (pH 7.7) and then subjected to osmotic shock (*17*). The pellet (1 g wet weight) was suspended in a solution (20 mL) of 0.5 M sucrose dissolved in 0.03 M Tris buffer at pH 8.0 containing 0.5 mM EDTA. The suspension was gently swirled for 10 min at 23 °C in a 2 L flask. The cells were then separated from the sucrose solution (10 min centrifugation at 13,000*g*), and then resuspended in the same volume of water at 3 °C. After 10 min of gentle swirling at 3 °C, the cells were separated from the supernatant (centrifugation for 10 min at 13,000*g*), which contained the alkaline phosphatase. The crude extract was diluted with distilled water (4%, v/v) and stored at 4 °C for further use.

Preparation of the Alkaline Phosphatase−Polyresorcinol Complex. The alkaline phosphatase−polyresorcinol complex was synthesized following the method reported by Sarkar and Burns (*10*) and modified by Ortega et al. (*18*). Resorcinol (102 mM) and peroxidase (288.8 U mL^{-1}) were dissolved separately in 0.1 M 2-amine, 2-methyl, and 1-propanol (2A2M1P) at pH 10.5. The resorcinol solution (20 mL) and 0.5% (w/w) H_2O_2 (20 mL) were added separately and slowly (1.7 mL h^{-1}) to 10 mL of the peroxidase solution at 4 °C, and the mixture was gently stirred. About 5 min after the start of the oxidative coupling reaction, when the quinones started to appear in the mixture, addition of the alkaline phosphatase (10 mL) was begun and continued dropwise for 10 h (1 mL h^{-1}). The reaction mixture was carried out at 4 °C. The mixture was flocculated by the addition of 15 mL of 0.5 M $CaCl_2$ and shaken gently (150 rpm) for 6 h at 20 °C. The suspension was centrifuged (15,400*g*) for 15 min at 4 °C, and the pellet was washed five times with 0.1 M calcium acetate buffer (pH 4.5). The copolymer was frozen and lyophilized. The insoluble enzyme−resorcinol copolymer was dissolved in 6 mL of 0.1 M 2A2M1P buffer (pH 10.5) and separately assayed for alkaline phosphatase activity.

To evaluate the effect of pH on the immobilization process, different buffer solutions in the pH range 4−12 were used: 0.1 M acetate (pH 4.0−5.4), 0.1 M tris-maleate (pH 5.4−8.6), 0.1 M glycine (pH 8.6−10.5), and 0.1 M 2A2M1P (pH 9.5−12.0).

Alkaline Phosphatase Assay. The activity of alkaline phosphatase was assayed by using *p*-nitrophenyl phosphate (*p*NPP) as an artificial substrate (*19*). 2A2M1P buffer was chosen to evaluate the activity because its phosphoryl acceptance abilities and aptitude to activate alkaline phosphatase are well known. Activity of both the immobilized and free enzyme was determined by incubating 1 mL of 25 mM *p*NPP, 3 mL of 0.1 M 2A2M1P (pH 10.5, containing 1 mM $MgSO_4 \cdot 7H_2O$ and 2 mM $ZnSO_4 \cdot 7H_2O$), and 1 mL of the enzyme solution at 37 °C for 60 min. For the soil phosphatase, samples of 1 g of fresh soil were added to 4 mL of 0.1 M 2A2M1P buffer at pH 10.5. Thereafter, 5 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ were added to the mixtures (to flocculate the organic matter and extract the *p*-nitrophenol), first shaking and then allowing to stand for 10−15 min at 20 °C. The contents were filtered (Whatman No. 6), and the *p*-nitrophenol (*p*NP) released was measured at 410 nm using a spectrophotometer (Hitachi U-2000). In parallel, a calibration curve using concentrations of *p*NP in the range 0−0.14 mM was run. Values shown in figures and tables represent the average of at least three triplicate assays. Duplicate controls, to which substrate was added after incubation, were assayed in all cases to deduct any nonenzymatic activity. Statistical controls (with variation coefficients <5% in all cases) show the high reproducibility of the experimental methods used.

Phosphatase activity was referred to 1 mL of enzyme solution or 1 g of dry weight soil and expressed in terms of enzymatic units. One unit (U) of alkaline phosphatase is defined as the amount of enzyme that releases 1 μmol of *p*NP per min under the assay conditions.

Determination of Kinetic Parameters. The kinetic parameters (K_m and V_{max}) of free and immobilized enzyme were determined by measuring initial rates of the reaction (under the conditions mentioned above) at substrate concentrations ranging from 1 to 40 mM. The Michaelis constant was calculated by analyzing the data according to the Enzyme Pack Program (Biosoft).

The inhibition of the enzyme by phosphate ions was determined by changing the substrate *p*NPP concentrations (1 − 40 mM) in the presence of trisodium phosphate (0.25, 1.25, and 2.50 mM) and in its absence. The mechanism was judged by the Lineweaver−Burk plots, and the inhibition constant (K_i), corresponding to each trisodium phosphate concentration, was calculated by replots of data taken from the reciprocal plot (K_{mapp} versus inhibitor concentration). In addition, the alkaline phosphatase activity was evaluated at different concentrations of the inhibitor trisodium phosphate (0−20 mM).

pH and Temperature/Enzyme Activity Curve. The effect of pH of both free and immobilized phosphatase was studied using three types of buffer solutions with pH values ranging from 5.2 to 12.4. The buffers were 0.1 M Tris-maleate (pH 5.2−8.6), 0.1 M borate (pH 8.4−10.2), and 0.1 M glycine (pH 10−12.4).

The optimum temperature for hydrolysis of *p*NPP was determined by measuring the alkaline phosphatase activity at eight different incubation temperatures over a range of 30−110 °C.

Thermal and Storage Stability. The thermal stability of both types of enzyme was studied by incubating the enzyme solution at three

temperatures (65, 70, and 80 °C) and withdrawing samples for the assay at fixed intervals over an incubation period of 7 h. The activity of these samples was determined under the standard conditions outlined previously. Thermal inactivation rate constants (k_d) were determined by linear regression analysis of a semilogarithmic plot of percentage activity remaining versus time. The half-life of enzymes was calculated from the values of thermal decay constants at different temperatures.

The enzyme preparations were stored in 2A2M1P buffer at pH 10.5 at 4 and −20 °C for a period of 3 months. Periodically, the remaining phosphatase activity was tested.

Stability in Soil. As test of stability, in the context of our attempt to mimic soil immobilized enzymes, native phosphatase (0.025 U g^{-1} soil) and the phosphatase−polyresorcinol complex (0.01 g g^{-1} soil) were incorporated in soil samples. The soil was held at 20 °C for 66 days and the residual phosphatase activity measured periodically. Phosphatase activity of soil control (without exogenous enzyme) was also determined.

Seed Coating. Barley seeds were coated with the phosphatase−polyresorcinol complex by encapsulation in calcium alginate hollow beads following the method described by Patel et al. (*20*). Three grams of barley seeds was mixed with 5 mL of 2% (w/v) carboxymethylcellulose solution containing 1% (w/v) calcium chloride and 0.3 g of the phosphatase−polyresorcinol complex. Then, the barley seeds were dropped into 500 mL of 1% (w/v) sodium alginate solution gently stirred in a 1 L beaker. At the surface of each seed, a calcium alginate layer formed from the inside to the outside, while the core remained liquid. After 10 min of gelation, about 300 mL of alginate solution were withdrawn, and the alginate solution containing the coated seeds was diluted with 500 mL of deionized water. The diluted solution was decanted, and the coated seeds were washed twice with water (250 mL), preventing the capsules from sticking together. The hollow beads were transferred into a stirred 1% (w/v) calcium chloride solution and left to harden for another 20 min. Finally, the calcium chloride solution was decanted, and the coated seeds were put on filter paper to dry.

Rhizosphere Phosphatase Activity. In order to determine the effect of the seed coating on the rhizosphere phosphatase activity, a qualitative visualization employing phenolphthalein phosphate in agar plates was used (*21*). The culture solution applied contained 2.5 mM $Ca(NO_3)_2$, 1 mM $NaH_2PO_4 \cdot H_2O$, 1 mM KCl, and 0.75 mM $MgSO_4 \cdot H_2O$ (pH adjusted with 1 M NaOH to 7). Four grams of agar was added to 1 L of the culture solution and autoclaved for 20 min at 121 °C. After cooling the solution to 30−40 °C, the medium was supplemented with phenolphthalein phosphate (1 g L^{-1}).

Barley seeds, pregerminated for 3 days, were placed in the Petri dishes near the edge, where a slot allowed the shoots to grow out of the dishes. The covers of the dishes, also supplied with slots at the edge, were held on with tape. Twenty-five milliliters of the medium (at 30−35 °C to prevent damage in the seeds and coating) were poured into the Petri dishes with an inner depth of 0.5 cm. After the agar solidified, the dishes were set on a slope so that the roots grew downward, mainly in the agar medium and less on its surface. The embedded plants were kept at room temperature for 15 h. Then paper disks wetted with 1 M NaOH were placed for about 1 min on the agar. After alkalinization of the medium, bright purple-colored zones occurred on the roots, indicating enzymatic hydrolysis of the reagent.

Plant Growth. Pot culture experiments were carried out to study the effects of coating barley seeds with the immobilized enzyme on the plant growth. Untreated and coated seeds were germinated on a wet filter paper at 26 °C in a growth chamber (Conviron E7) for 96 h. Five seedlings were transplanted to a plastic pot (containing 150 g of soil) at 10 mm below the soil surface, and then the pots were transferred to an artificial climatic chamber. The plants were developed under environmental conditions of 20/15 °C photo/dark period temperature and 16 h photoperiods per day. The pots were watered everyday with 15 mL of water. Each experiment was conducted five times.

One plant from each replicate was carefully freed from the soil at 7, 14, 21, 28, and 35 days after planting. After each harvest, plant growth was determined by measuring the length, dry weights, and concentration of inorganic phosphorus of the shoots. The shoots were oven-dried for dry weight determination (70 °C/48 h). Shoot samples were subsequently ashes (16 h at 550 °C) and the residue dissolved in 0.9 M H_2SO_4 (~1 mL for each 10 mg of oven-dry tissue wt) (*22*). The inorganic P content of the acid solutions was then measured according to the ascorbic acid−molybdate method of Murphy and Riley (*23*).

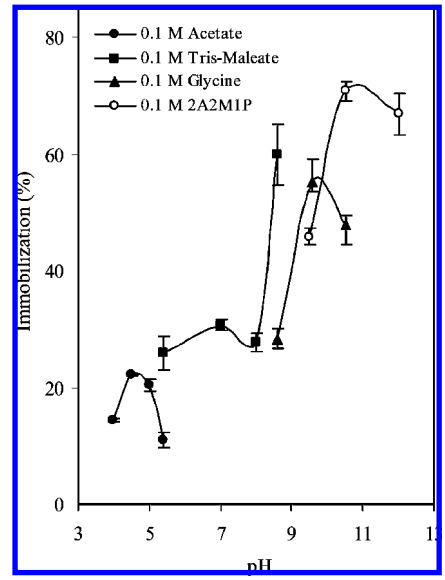

Figure 1. Effect of immobilization pH on the synthesis of the alkaline phosphatase−polyresorcinol complex. Immobilization conditions: 1.45 U mL^{-1} alkaline phosphatase added.

Table 1. Effect of Enzyme Concentration on Alkaline Phosphatase Immobilization[a]

enzyme concentration (U mL^{-1})[b]	phosphatase−polyresorcinol complex (U mL^{-1})[b]	activity retention (%)
0.029 ± 0.001	0.021 ± 0.001	72.41
0.050 ± 0.002	0.037 ± 0.001	73.87
0.132 ± 0.004	0.085 ± 0.002	64.39
0.240 ± 0.006	0.147 ± 0.008	61.25
1.095 ± 0.035	0.474 ± 0.017	43.28

[a] Immobilization pH 10.5. [b] Results are the mean values of triplicate determinations ± standard deviation.

Statistical Procedure. Multiple analysis of variance (MANOVA) was applied to all data with Statgraphics 4.0 plus for Windows. The results were expressed as level of significance (P) obtained for the effects of each variable studied ($P > 0.05$ was considerate not significant). The results given below for phosphatase activity are the average values of three replicates for each experimental condition, and for the pot cultivation experiment are the average value of five replicates (each plant for different pots).

RESULTS AND DISCUSSION

Synthesis of the Alkaline Phosphatase−Polyresorcinol Complex. The synthesis of the phosphatase−polyresorcinol complex was performed by reacting resorcinol with peroxidase in the presence of alkaline phosphatase. The optimal pH value for the immobilization of phosphatase was investigated in the pH range 4−12. As can be seen in **Figure 1**, the phosphatase−polyresorcinol complex synthesized in 2A2M1P buffer at pH 10.5 (optimum pH of performance of the enzyme) presented the highest level of enzyme activity, retaining about 70% of the added enzyme. There was a drastic reduction in relative activity as the pH values were less than 8.6. Moreover, Garzillo et al. (*12*) also reported that exposing the phosphatase to high peroxidase concentrations produced a complex with low activity and thermal stability, suggesting that the polymerization process must be carried out at pH and temperature values suboptimal for the peroxidase catalysis. In fact, in the range of pH values

Table 2. Properties of the Free and Immobilized Alkaline Phosphatase

parameter	free phosphatase	phosphatase−polyresorcinol complex
V_{max} (μmol *p*NP min^{-1})	1.33	1.02
K_m (mM)	1.07	2.44
$(R^2)^a$	0.9906	0.9978
K_i (mM)	0.069	0.423
$(R^2)^b$	0.9990	0.9840
$IC_{50}{}^c$ (mM)	2.8	4.0

[a] Regression coefficient from a Lineweaver−Burk plot. [b] Regression coefficient from replots of data taken from the reciprocal plot (K_{mapp} versus inhibitor concentration). [c] Inhibitor concentration needed for 50% reduction in enzyme activity.

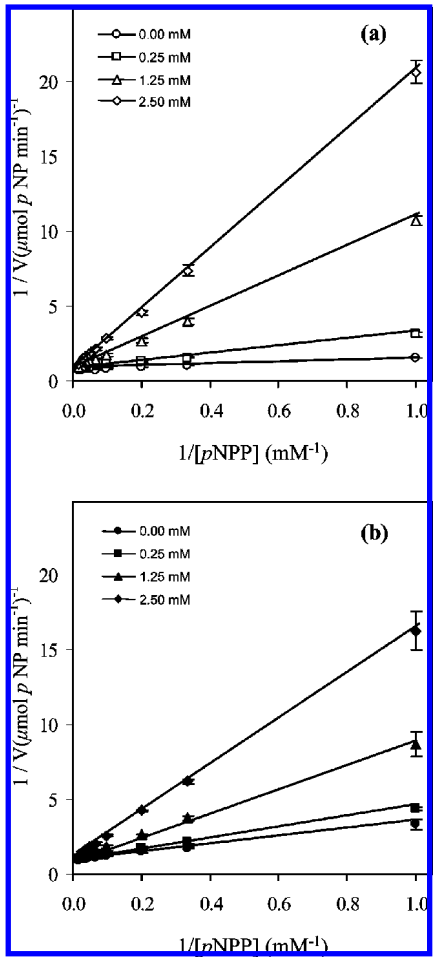

Figure 2. Lineweaver−Burk plot for free (**a**) and immobilized phosphatase (**b**) at different trisodium phosphate concentration. Reaction conditions: 0.1 M 2A2M1P buffer, pH 10.5; incubation temperature, 37 °C; reaction time, 60 min; *p*NPP as the substrate.

where the peroxidase exhibits high activity (from pH 5 to 8) (*24*), the activity of the copolymer obtained was about 85−68% lower than that of the initial enzyme added.

To determine the effect of the amount of added enzyme in the polymerization process, the reaction mixture contained different phosphatase activity ranging from 0.030 to 1.095 U mL^{-1}. The activity of the phosphatase−polyresorcinol complex was assayed, and the results are presented in **Table 1**. Activity retention was optimal (74%) at 0.05 U mL^{-1} enzyme load, and the activity began to decrease with increasing concentrations of added enzyme. Enzyme molecules added during the polymerization process are entrapped in the matrix or chemically bonded to the aromatic moiety (*10, 25*); alternatively, the enzyme may be adsorbed onto the performed phenolic polymer

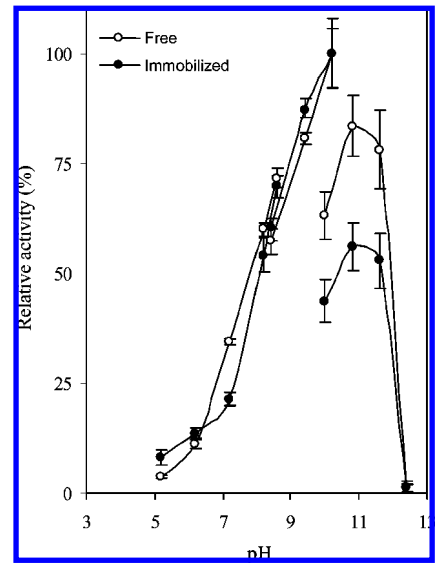

Figure 3. pH/activity curves for free and immobilized alkaline phosphatase. Reaction conditions: 0.1 M Tris-maleate (pH 5.2−8.6), 0.1 M borate (pH 8.4−10.2), and 0.1 M glycine (pH 10−12.4); incubation temperature, 37 °C; reaction time, 60 min; 25 mM *p*NPP. Relative activity was expressed as a percentage of maximum activity under experimental conditions.

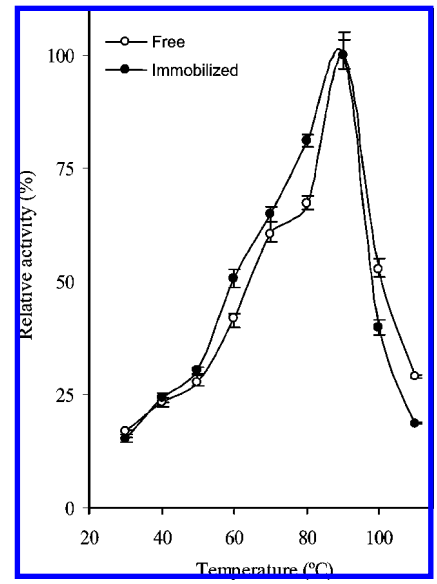

Figure 4. Effect of temperature on the activity of free and immobilized alkaline phosphatase. Reaction conditions: 0.1 M 2A2M1P buffer, pH 10.5; incubation temperature ranging from 30 to 110 °C; reaction time, 60 min; 25 mM *p*NPP.

(*26*). When the active sites of the carriers were saturated, the enzyme molecules could not efficiently perform the catalysis because of substrate diffusion limitation.

The phosphatase−polyresorcinol complex synthesized had 25% lower enzyme activity than the soluble alkaline phosphatase. It is probable that the quinones or the residual aromatic monomers caused varying degrees of inactivation during the formation of the copolymer. Furthermore, the chemical structure and degree of polymerization of the final product will affect the expression of enzyme activity (*10*). The exact mechanism of enzyme attachment to the phenolic molecules is unclear. Thus, Rowell et al. (*11*) suggested that the association between enzyme and polyresorcinol fundamentally took place through covalent and hydrogen bonds, while the ionic bonds were

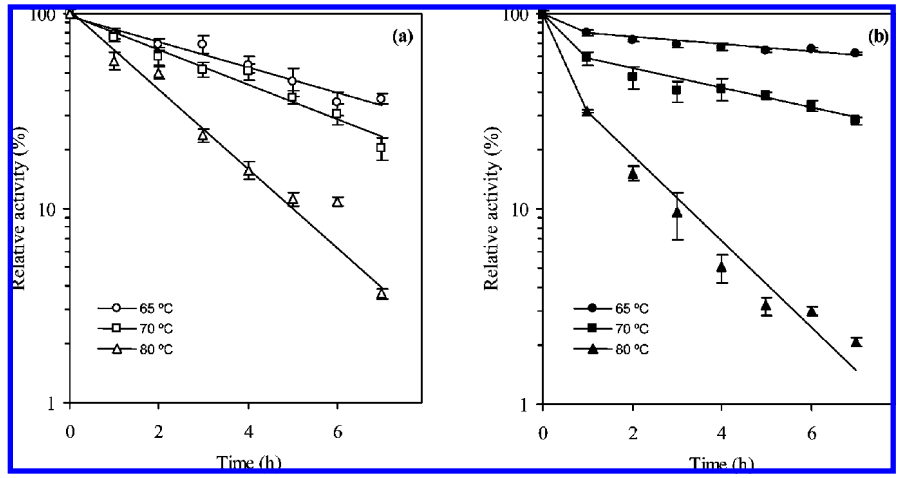

Figure 5. Semilogarithmic plots of the remaining activity of free (**a**) and immobilized (**b**) alkaline phosphatase as a function of time of incubation at different temperatures. Enzyme samples were preincubated at the indicated temperatures for different times. At the time indicated, aliquots were removed and assayed for activity at 37 °C, with *p*NPP as the substrate. Initial activity corresponds to 100%.

Table 3. Half-Lives ($t_{1/2}$) and Inactivation Rate Constant (k_d) of the Free and Immobilized Alkaline Phosphatase

	free phosphatase		phosphatase−polyresorcinol complex	
T (°C)	k_d (min^{-1})	$t_{1/2}$ (min)	k_d (min^{-1})	$t_{1/2}$ (min)
65	2.5×10^{-3}	277	3.2×10^{-4}	1118
70	3.4×10^{-3}	204	1.7×10^{-3}	408
80	7.8×10^{-3}	89	7.5×10^{-3}	92

insignificant. In contrast, Garzillo et al. (*12*) suggested that secondary interactions (hydrogen bonding and hydrophilic reactions) were more feasible in this polymerization process.

Kinetic Properties of Free and Immobilized Phosphatase. The Michaelis−Menten constant (K_m) and the maximum reaction rate (V_{max}) for the free and immobilized phosphatase were calculated from the reciprocal of Lineweaver−Burk plot (**Table 2**). Immobilization decreased in V_{max} value from 1.33 to 1.02 μmol *p*NP min^{-1}. The K_m value of the phosphatase−polyresorcinol complex (2.44 mM) was 2.3 times higher than that of free phosphatase (1.07 mM), which means the immobilized phosphatase had lower affinity toward the substrate, in agreement with other investigators reporting significant decreases in affinity in immobilized catalysts (*27*). The increase in K_m might be caused by the steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding, or diffusion limitation of the substrate and products because of the nonporous nature of the support (*28*). This behavior, increase in K_m and decrease in V_{max}, is a common characteristic of immobilized enzymes whether immobilized on soil components or synthetic support (*26*).

The inhibition of both free and immobilized phosphatase by phosphate ions was studied. The Lineweaver−Burk graphs (**Figure 2**) showed that phosphate could competitively inhibit the alkaline phosphatase native and immobilized with inhibition constants of 0.069 and 0.423 mM, respectively (**Table 2**). Higher K_i values for the phosphatase−polyresorcinol complex showed lower propensity to product inhibition. The lower IC_{50} (the inhibitor concentration needed for 50% reduction in the enzyme activity) value of the free enzyme (**Table 2**) confirmed this observation. Krajewska et al. (*29*) reported that the immobilization of urease in chitosan weakened the inhibitory effect of three competitive inhibitors as measured by the inhibition constants. The increase in their values was accounted for by environmental effects generated by the heterogeneity of the immobilized system: (i) mass transfer limitations imposed on the substrate and reaction product in the external solution and (ii) the change pH in the local environment of the immobilized enzyme produced by both the enzymatic reaction and the electric charge of the support.

Effect of pH on Enzyme Activity. The effect of pH on the activities of free and immobilized phosphatase when using tris-maleate, borate, and glycine buffers at different pH values

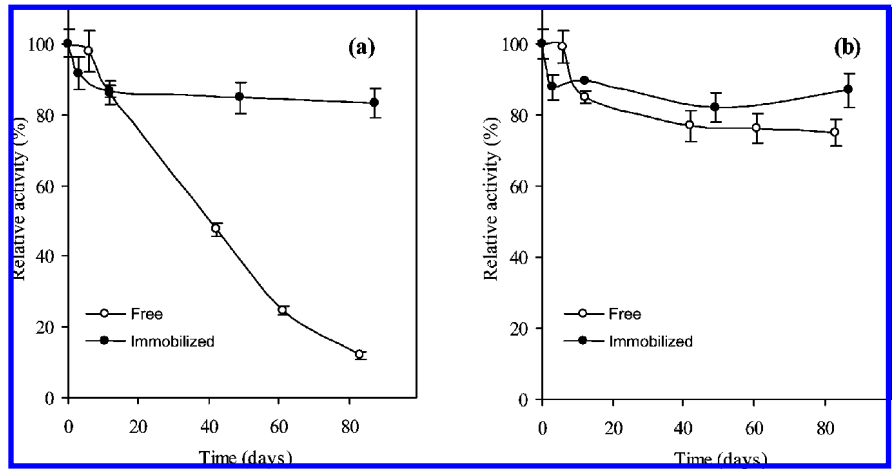

Figure 6. Stability of free and immobilized alkaline phosphatase on storage at 4 °C (**a**) and −20 °C (**b**).

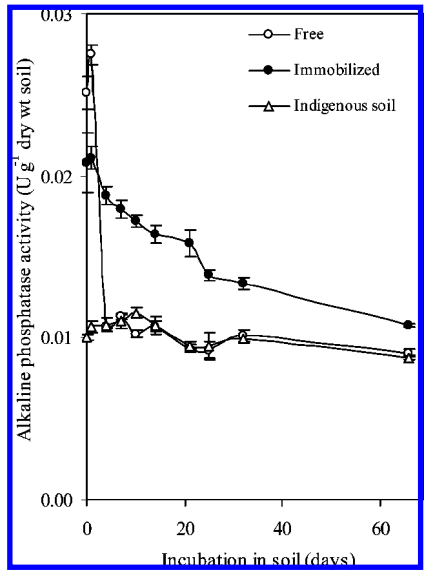

Figure 7. Stability of the free and immobilized alkaline phosphatases in fresh soil. Experimental conditions: free enzyme, 0.025 U g^{-1} soil; phosphatase−polyresorcinol complex, 0.01 g g^{-1} soil.

between 5 and 12.4 is plotted in **Figure 3**. Both the immobilized and free enzyme exhibited maximum activity at pH 10.2 (borate buffer) and a comparable behavior in the pH range studied. Garzillo et al. (*12*) reported that the optimal pH of the acid phosphatase−polyresorcinol copolymer was similar to that of the native enzyme. In contrast, Grego et al. (*25*) observed that the immobilized enzyme of the Pronase−resorcinol copolymers displaced the pH-activity profile to more alkaline regions with respect to that of the free enzyme. Furthermore, both enzymes exhibited lower phosphatase activity in glycine buffer (**Figure 3**). Thus, at pH 10 in glycine buffer the free and immobilized enzymes had lost 37 and 56% of its activity, respectively, in comparison with that in borate buffer at pH 10.2. This lower activity upon immobilization could be expected as a result of a higher concentration of glycine in the vicinity of the support possibly due to ionic interactions between the glycine (zwitterionic species at pH 10) and the hydroxyl and carboxylic groups present in the phosphatase−polyresorcinol complex (*10*).

Effect of Temperature on Enzyme Activity. Figure 4 shows that both the free and immobilized alkaline phosphatases exhibited the highest activity at 90 °C. However, at temperature values between 70 and 90 °C, the immobilized phosphatase showed an activity about 10% higher than that of the free enzyme. The results of the temperature range from 30 °C to optimum temperatures are also used for the calculation of activation energy (from the Arrhenius plot). The plots for both the enzymes were linear, and the calculated values of activation energy were about 27.1 and 29.2 kJ mol^{-1} for the free and immobilized phosphatases, respectively. The fact that the activation energy for the immobilized enzyme was not smaller than that of the free enzyme indicated that no diffusion limitation existed.

Thermal Stability. Thermal inactivation of the native and immobilized phosphatases obtained experimentally at three constant temperatures (65, 70, and 80 °C) can be seen in **Figure 5**. At 65 °C, the free and immobilized enzymes retained 37% and 62% of their activity, respectively, after 7 h of treatment. At higher temperatures, their activities decayed more than 70% in 5 or 3 h at 70 or 80 °C, respectively. Moreover, **Figure 5** suggests that the native enzyme was heat-inactivated with first order kinetics. However, the immobilized phosphatase presents

Figure 8. Barley seedling growing in agar with 0.1% phenolphthalein phosphate. Seed untreated (**a**) and seed encapsulated in calcium hollow beads with the phosphatase−polyresorcinol complex (**b**).

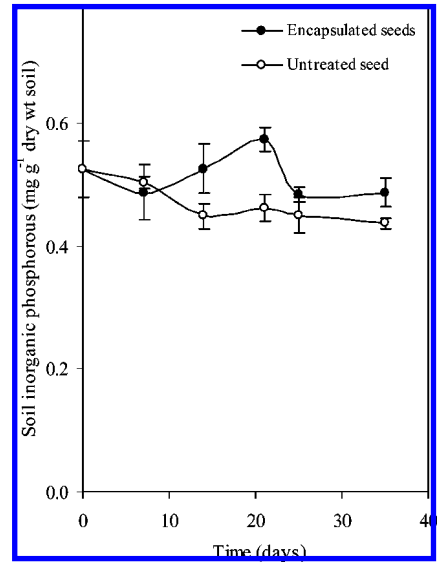

Figure 9. Effect of seed coating with the phosphatase−polyresorcinol complex on soil inorganic phosphorus.

biphasic behavior, i.e., the rate of phosphatase activity decrease could be divided into two periods. The first period was characterized as a thermolabile fraction, which was inactivated too quickly to calculate the decline of activity. The second period can be defined as the thermo-resistant fraction of the enzyme, and the inactivation can be explained as a single exponential decay, indicating that inactivation followed first-order kinetics. The inactivation rate constants can be determined from the slope of such semilog-plots (*30*).

The thermal inactivation rate constants (k_d) and the half-life values ($t_{1/2}$) for the free and immobilized enzymes are presented in **Table 3**. The stabilization factor (the relationship between the half-lives of the immobilized form and that of the native enzyme) obtained at 65 and 70 °C were 4 and 2, respectively, while at 80 °C, the half-life of both enzymes was about 1.5 h.

Table 4. Influence of the Seeds Coated with the Phosphatase−Polyresorcinol Complex on the Biomass, Length, and P_i Concentration of the Shoot[a]

	untreated seeds			encapsulated seeds		
days aftergermination	shoot biomass (mg)	shoot length (cm)	shoot $[P_i]^b$ (mg g^{-1})	shoot biomass(mg)	shoot length (cm)	shoot $[P_i]^b$ (mg g^{-1})
7	13.78 ± 0.82	15.44 ± 1.34	7.26 ± 0.46	14.56 ± 1.60	14.70 ± 1.23	7.10 ± 0.75
14	17.20 ± 0.60	19.05 ± 2.05	7.12 ± 0.59	22.02 ± 0.73	18.34 ± 1.27	6.20 ± 0.48
21	29.84 ± 2.60	24.58 ± 1.43	4.75 ± 0.31	34.63 ± 3.20	25.98 ± 3.44	4.73 ± 0.10
28	43.93 ± 4.97	26.50 ± 1.39	3.44 ± 0.26	40.70 ± 6.90	23.83 ± 1.93	4.47 ± 0.32
35	46.33 ± 3.35	26.22 ± 1.49	2.93 ± 0.26	50.50 ± 1.88	26.23 ± 1.70	3.40 ± 0.12

[a] Results are the average values of five replicates. [b] Inorganic phosphorus concentration of the shoot.

Similar results have previously been reported for the acid phosphatase−polyresorcinol complex (*12*) and other immobilized enzymes (*31*). It is often found that the immobilized enzyme has a higher thermal stability than the free enzyme because of the restriction of conformational flexibility in the immobilized enzyme.

Storage Stability. In general, enzymes are not stable during storage in solution, and their activities are gradually reduced or lost through time. The storage stability of the free and immobilized phosphatase was determined under 2A2M1P buffer (0.1 M, pH 10.5) at 4 and −20 °C and periodically sampled to determine their residual activity (**Figure 6**). At 4 °C, the phosphatase−polyresorcinol copolymer preserved 90% of its activity after 4 months with respect to its original activity, while the free enzyme lost all its activity within this period. These results indicate that copolymerization with the resorcinol of phosphatase provided a stable environment and prevented the loss of activity that occurs when the enzyme is stored at refrigerated temperature. In contrast, at −20 °C the immobilized enzyme was slightly more stable than the free enzyme.

Stability in Soil. The stability of free and immobilized alkaline phosphatases incorporated into soil was assessed (**Figure 7**). The degradation of free enzyme started 24 h after its addition to soil and lasted for 4 days. Loss of activity may be due to adsorption, chemical denaturation, or proteolysis. In contrast, after 7 days the immobilized enzyme lost 36% of its initial activity and retained about 18% for a period of 66 days. Therefore, the present investigation showed that the phosphatase−polyresorcinol complex was fairly stable in soil, i.e., the immobilized enzyme was more resistant to proteolysis or to inactivation, through adsorption and abiological denaturation. This result contrasts with that previously reported by Garzillo et al. (*12*), who indicated that soil stability was similar for the acid phosphatase−polyresorcinol complex and the free enzyme probably because of the adsorption of the free phosphatase on the soil clay−humic colloids is stronger than the immobilized one so that the free enzyme forms in soil active complexes resembling those synthesized in vitro between resorcinol and enzyme.

Effect of Seed Coating on Phosphate Uptake and Plant Growth. Barley seeds were encapsulated in calcium alginate hollow beads with the characterized phosphatase−polyresorcinol complex. The percentage of germination of the coated seeds was about 84%. The effect of seed coating on rhizosphere phosphatase activity is shown in **Figure 8**. It can be seen that the seedling grown from coated seeds showed a more intense pink coloration around the roots than the control, which indicated a higher phosphatase activity. The enzyme activity observed in the controls was associated with acid phosphatase activity released by roots. Likewise, as the barley seedlings were ground under unsterilized conditions, some of the phosphatase activity observed could be released from microorganisms attached to the root surface (*21*). In any case, in view of the results obtained it is obvious that the seed coating with phosphatase remarkably increased rhizosphere phosphatase activity.

The effect of seed coating with the phosphatase−polyresorcinol complex on phosphate uptake and plant growth was assayed under pot culture conditions in a controlled environment chamber. A persistent increase in the soil inorganic P was detected with the treated seeds against the controls (**Figure 9**). Thus, at 21 days after sowing the soil P concentration varied from 0.526 mg g^{-1} to 0.575 mg g^{-1} (seed coated) and 0.463 mg g^{-1} (seed uncoated). After 35 days of growth, the level of soil inorganic P was higher in soil cultivated with the seed coated with immobilized phosphatase. In addition, a positive influence on biomass and inorganic P concentration of the shoot was observed (**Table 4**). Thus, a significant improvement in plant P concentration due to seed coating occurred from 28 days of culture.

In conclusion, the copolymerization of alkaline phosphatase with resorcinol resulted in an increase in some stability parameters such as heat resistence, stabiliy during storage and stability in soil. Moreover, the seeds coated with the immobilized system showed high rhizosphere phosphatase activity as a positive effect on the phosphate uptake and plant growth. However, it is a preliminary study, and it is necessary check out other methods of seed encapsulation. Nevertheless, the application of immobilized enzymes for seed coating can lead to an increase in soil biochemical activities, which could be used as natural fertilizers more convenient for sustainable agriculture.

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