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Covalent immobilization of acid phosphatase on amorphous $AlPO_4$ support

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Abstract

Covalent attachment of acid phosphatase enzyme, AP, on the surface of amorphous $AlPO_4$, used as inorganic support, was studied. Immobilization of the enzyme was carried out by the ε-amino group of lysine residues through an aromatic Schiff's-base (linker A), as well as through an 'azo' linkage to a *p*-OH-benzene group of tyrosine residues of the proteins (linker B). Activation of the supports in both cases was developed through the reaction of appropriate molecules with support surface –OH groups. The enzymatic activities in the 1-naphthyl phosphate hydrolysis of native, the different immobilized AP systems, and the filtrates, were obtained by a spectrophotometric method. According to the results, immobilization through linker A gave $E_{imm} = 99\%$ while the residual activity, E_{res}, at different temperatures was in the range 0.2–0.8%. On the other hand, in the immobilization by linker B, through a diazonium salt, E_{imm} was in the range 35–46%, but residual and specific activity values, E_{res} and E_{spe}, were between 19% and 46%. Thus, instead of linker A was more effective in the enzyme immobilization, the highest enzymatic activity after immobilization was obtained with linker B because with linker A a strong deactivation was developed.

Keywords: Covalent immobilization; Immobilized acid phosphatase; Support-$AlPO_4$; Acid phosphatase; Immobilized enzyme

1. Introduction

The immobilization of biologically active species such as enzymes is an important goal in fine chemical, pharmaceutical and food industries as well as in clinical and chemical analysis because it can offer several advantages including enzyme reuse, ease in product separation, improvement in enzyme stability and continuous operation in reactors. Thus, extensive studies have been made in the last three decades to obtain immobilized enzymes [1]. Immobilization fixes the enzyme onto an inert support such as agarose, alginate or polyacrylamide either by covalent bonding, physical adsorption or encapsulation. In general, adsorption techniques are easy to perform, but the bonding of the enzyme is often weak and such biocatalysts generally lack the degree of stabilization achieved by covalent attachment [2]. Covalent immobilization on the external surface of a support material has also been proposed to decrease mass transfer limitations associated with several immobilization techniques, such as entrapment or adsorption in gels. Thus, covalent binding to an

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insoluble support is the most interesting enzyme immobilization methodology because it combines the high selectivity of enzymatic reactions with the chemical and mechanical properties of the support [3,4]. In this respect, inorganic matrices have a number of advantages over organic ones: no swelling and porosity changes occur with pH; there is excellent storage stability of enzymes and they are not subject to microbial attack [5].

On the other hand, the enzyme immobilization on the external surface of a support is severely limited by a reduced available surface area. Besides, some difficulties arise when trying to obtain an inorganic solid which has surface organic molecules able to attach themselves to some biomolecules [6]. Inorganic oxides such as silica gel have received great attention due to the ability of immobilizing organic functional groups onto its surface by using some silane coupling agent, which reacts with the silanols on the solid surface [7]. Support material, which plays an important role in the utility of an immobilized enzyme, should be readily available and non-toxic, and also should provide a large surface area suitable for enzyme reactions, and substrate and product transport with the least diffusional restrictions possible as well as a high enough number of surface active sites to link the organic functional groups [8]. One major disadvantage of immobilization, however, is low volume productivity. Indeed, normally less than 5% by weight of an immobilized enzyme is catalysts, the rest is an inert support. Although there has been progress in this field, improved methods of immobilization are needed to address the limitations of presently available options and to promote the wider use of enzymes as economic, large-scale catalysts [9].

In this regard, we have previously reported the use of amorphous $AlPO_4$ as metal support [10,11], as well as heterogeneous catalysts in the field of selective organic synthesis [12–18]. This material, tailored by a controlled sol–gel method that allows us to obtain a high surface area as well as a high number of surface –OH groups, is a very adequate support component for the covalent attachment of enzymes, according to the results obtained in the immobilization of lipases and glucose oxidase enzymes [19–21].

Acid phosphatases (EC 3.1.3.2), the enzymes that nonspecifically hydrolyze low and high molecular phosphomonoesters in an acid environment, are widely distributed in mammalian body fluids and tissues and in plants and microorganisms [22,23]. Since the hydrolysis of phosphate esters can be achieved by chemical methods and the application of phosphatase enzymes is only advantageous if the substrate is susceptible to decomposition, the enzymatic hydrolysis of phosphate-esters has found only a limited number of applications [24]. Thus, the employment of phosphatase in order to separate *cis/trans* mixtures of alicyclic alcohols and racemates of D,L-threonine by stereoselective hydrolysis of their phosphoric esters has been described [25]. However, during the last decade several complex biosensors with phosphatasic activity have been described for inorganic phosphate ion detection [26]. This determination is especially important in the field of environmental and food analysis as well as in enzyme label immunoassay. These biosensors are analytical devices containing acid (or alkaline) phosphatase coimmobilized with glucose oxidase, and sometimes even more enzymes [26], as well as some conducting material used as electrode, such as carbon, and/or Pt, Ag, Au, etc. Polymer membranes are in most cases used as enzyme carriers [27].

Comparatively, the applications of immobilized AP in different uses other than biosensors are very limited and, at the present time, no reference to any inorganic support is known in the literature. In this connection, controlled pore glass has been used as support where immobilization is carried out by activation with 3-aminopropyltriethoxysilane and glutaraldehyde as covalent linker [28]. Data on the immobilization of alkaline phosphatase by physical gel entrapment within colloidal particles, produced by inverse microemulsion polymerization, indi-

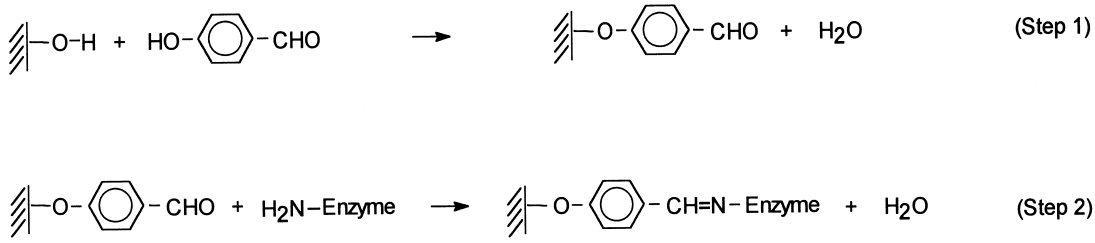

Fig. 1. General scheme for covalent immobilization of the enzyme through the ε-amino group of lysine residues, 'linker A'. Activation of $AlPO_4$ support by microwave heating with 4-hydroxy-benzaldehyde (step 1) before covalent immobilization of the enzyme through the lysine residues (step 2).

cated that 50% of the initial enzyme was immobilized within the latex, keeping essentially the original properties of the native enzyme [29]. Immobilization of acid phosphatase (from wheat germ), and alkaline phosphatase (from calf intestine) onto hydrolyzed poly(2-hydroxyethyl methacrylate)-g-*co*-polyethylene using hydroxyl and carboxyl activating agents has been studied and has led to values of immobilized enzymes ranging between 10 and 110 mg of protein per 1 g of copolymer, with E_{res} values in the range 2–26% [30].

In this study, attempts are made to find a suitable procedure to obtain a covalent attachment of acid phosphatase from potato, AP, to an inorganic support material, such as amorphous $AlPO_4$, previously activated by two different functionalized organic chains anchored to the support surface. In one case, according to the general scheme in Fig. 1, immobilization of the enzyme is carried out by using the ε-amino group of lysine residues through an aromatic Schiff's-base, 'linker A'. This must comparatively be more stable than those usually obtained by using glutaraldehyde or other aliphatic aldehydes [19,20]. On the other hand, according to the scheme in Fig. 2, immobilization of the enzyme is carried out through an 'azo' linkage to a *p*-OH-benzene group of tyrosine residues of the proteins, 'linker B' [19]. Activation of the

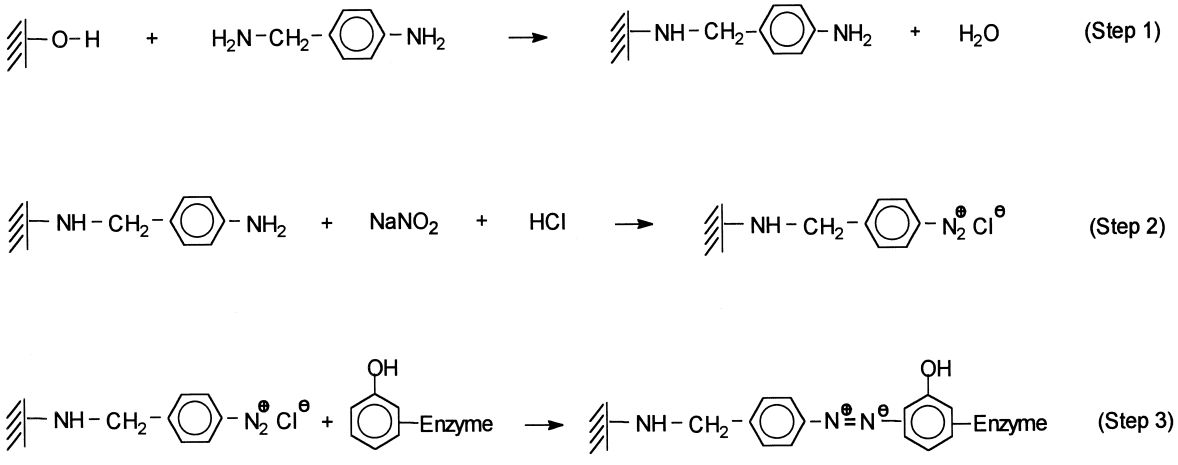

Fig. 2. General scheme for covalent immobilization of the enzyme through an 'azo' linkage to the *p*-OH-benzene group of tyrosine residues, 'linker B'. Activation of $AlPO_4$ support by microwave heating with 4-aminobenzylamine (step 1), and, then converting the aniline residue of the composite to a diazonium salt in the usual way with sodium nitrite, (step 2) before covalent immobilization of the enzyme through the tyrosine residues (step 3).

supports in both cases is developed by anchoring a linker through the reaction of appropriate molecules with support surface –OH groups of amorphous $AlPO_4$ used as support.

2. Experimental

2.1. Support synthesis and support activation

Amorphous $AlPO_4$, used as support, was obtained by precipitation from the corresponding $AlCl_3 \cdot 6H_2O$ and H_3PO_4 aqueous solutions by addition of ammonium hydroxide, according to a sol–gel method previously described [10–18]. In the present case, after filtration, washing with isopropyl alcohol and drying at 120°C, the solid was calcined by heating at 350°C for 3 h and then screened to a particle size < 0.149 mm. The procedure for the textural and acid–base properties of these systems has been published elsewhere [10–13]. The textural properties of the support, determined by nitrogen adsorption at liquid nitrogen temperature from the B.E.T. method, were as follows: surface area, $S_{BET} = 211\ m^2\ g^{-1}$; pore volume, $V = 0.72\ cm^3\ g^{-1}$; mean pore diameter, $d = 12.8$ nm. The surface acidity obtained by titration with pyridine ($pK_a = 5.25$) was $PY = 200\ \mu mol\ g^{-1}$, and with 2,6-dimethylpyridine ($pK_a = 6.99$), $DMPY = 249\ \mu mol\ g^{-1}$. Surface basicity obtained by titration with benzoic acid ($pK_a = 4.19$) was $BA = 352\ \mu mol\ g^{-1}$. These values were determined by a spectrophotometric method described elsewhere [10,11] that allows titration of the amount of irreversible adsorbed titrant molecules.

Two different functionalized organic chains were attached to the $AlPO_4$ support. Support activation was initiated by the reaction of the two functionalized organic chains with the $AlPO_4$ surface –OH groups [19–21]. Both linkers, according to schemes in Figs. 1 and 2, were obtained by a microwave heating reaction (15 min at 380 W) of the support (20 g) and *p*-hydroxy-benzaldehyde (2 g), 'linker A'; or 4-aminobenzylamine (4 g) 'linker B' and, then converting the aniline residue of the composite to a diazonium salt by the usual procedure in the same reaction vessel. Thus, the activated solid turns a permanent red color when ice, 20 g, acetone, 20 ml, and 1.3 g sodium nitrite are introduced. The efficiency of these steps are confirmed by visible–ultraviolet/diffuse reflectance experiments shown in Figs. 3 and 4. Spectra were recorded on a UV–Visible diffuse reflectance Varian Carey 1E Spectrophotometer.

2.2. Acid phosphatase immobilization and enzymatic activity

Immobilization of the enzyme was carried out at room temperature by thoroughly mixing (24 h) the activated solid (2.5 g) and AP, acid phosphatase (EC 3.1.3.2), from potatoes (Sigma Type II), (0.5 g) in 0.05 M citrate buffer, pH = 5.6 (18 ml). Finally, the immobilized AP was collected by centrifugation and the resulting filtrate was separated to obtain the activity of supernatant AP.

The enzymatic activities of native and different immobilized AP systems and filtrates were

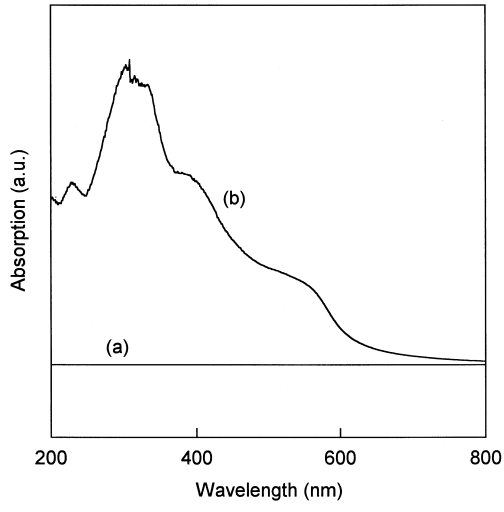

Fig. 3. Visible–ultraviolet/diffuse reflectance spectra of different samples obtained in different steps in Fig. 1: (a) $AlPO_4$ support and (b) 4-hydroxy-benzaldehyde on support after microwave heating (step 1).

obtained at different temperatures by a continuous-monitoring spectrophotometric method where the enzymatic hydrolysis of 1-naphthyl phosphate at pH 5.6 liberates 1-naphthol which is spectrophotometrically determined by reaction with Fast Red TR, presents in the incubation mixture to form a red dye [31]. With the aid of a Beckman mod. DU-62 Spectrophotometer, the appearance of the dye was monitored continuously (every 5 min) at 410 nm. In every assay, 0.05 g of different enzymatic systems (native, immobilized or filtrate) was added to 5 ml of citrate buffer containing substrate 1-naphtyl phosphate (0.5 ml of a 0.003 M solution) and Fast Red TR (0.5 ml of a 0.8×10^{-3} M solution) to obtain reaction rates of native enzymes, r_{nat}, filtrates of supernatant AP, r_{fil}, and with immobilized AP systems, r_{imm}. Reaction rates, r, (in μmol min^{-1}) for 0.5 g of AP enzyme, native or immobilized in 2.5 of support, were determined by taking the slope of the linear plot of absorbances vs. time. Diffusional limitations in r_{imm} values are avoided by the sufficiently small particle size of the support

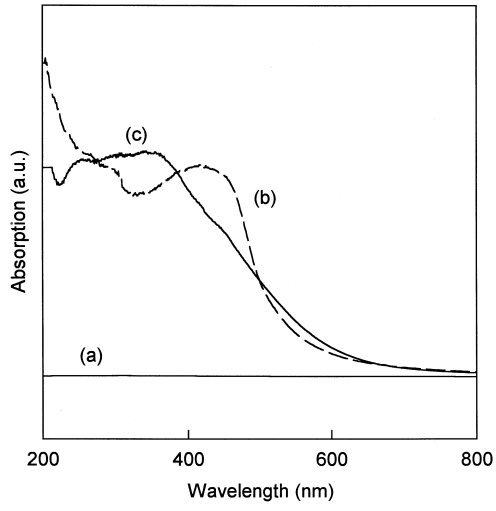

Fig. 4. Visible–ultraviolet/diffuse reflectance spectra of different samples obtained in different steps in Fig. 2: (a) $AlPO_4$ support, (b) 4-aminobenzylamine on support after microwave heating (step 1) and (c) diazonium salt obtained by the usual way with sodium nitrite (step 2).

Table 1
Catalytic activity (in μmol min^{-1}) of native, r_{nat}, and immobilized enzyme, r_{imm}, as well as the filtrate, r_{fil}, obtained in the immobilization process (0.5 g of AP enzyme, native or immobilized in 2.5 g of support) under standard experimental conditions and different temperatures, through the ε-amino group of lysine residues ('linker A', Fig. 1), and through an 'azo' linkage to the *p*-OH-benzene group of tyrosine residues ('linker B', Fig. 2)

Temperature (°C)	r_{nat}	Linker A		Linker B	
		r_{imm}	r_{fil}	r_{imm}	r_{fil}
20	6.97	0.013	0.000	2.30	4.15
30	12.10	0.102	0.061	3.67	6.46
40	26.10	0.071	0.012	5.06	16.90
50	10.70	0.048	0.026	4.96	6.92

(< 0.149 mm). Results obtained under different experimental conditions are shown in Table 1.

3. Results and discussion

The properties of immobilized enzymes can easily be stated from experimental results in Table 1 [32]. Thus, protein retention upon immobilization, obtained from the percentage of immobilized enzyme, E_{imm}, was determined by the difference between the catalytic activity of the native enzyme and the activity of the filtrate in the different immobilization processes:

$$E_{imm} = \frac{r_{nat} - r_{fil}}{r_{nat}} \times 100 \quad (1)$$

The residual activity upon immobilization, obtained from the percentage of catalytically active immobilized enzyme, E_{res}, was determined from the relation between the activities of immobilized and native enzymes:

$$E_{res} = \frac{r_{imm}}{r_{nat}} \times 100 \quad (2)$$

The specific activity, E_{spe}, indicating the efficiency of the immobilized enzyme, with respect to the native one, can be obtained from the relation:

$$E_{spe} = \frac{E_{res}}{E_{imm}} \times 100 = \frac{r_{imm}}{r_{nat} - r_{fil}} \times 100 \quad (3)$$

The different values obtained are shown in Table 2. Here we can see that, immobilization through ε-amino groups (linker A) gave E_{imm} = 99% while E_{res} at different temperatures were in the range 0.2–0.8% and the same values for E_{spe}. On the other hand, in the immobilization by 'linker B', through a diazonium salt, E_{imm} was in the range 35–46%, E_{res} between 19–46% and E_{spe}, in the range 46–130%.

According to these results, the percentages of immobilized AP enzymes obtained, E_{imm}, in both immobilization processes, with either linker A or B, were high enough to let us conclude that both procedures of surface activation of the $AlPO_4$ support were effective. In this connection, in Figs. 3 and 4 UV–Visible experiments show the changes obtained in the support surface due to the effects of different reactions carried out in each step of both general schemes for covalent immobilization of the enzyme.

The existence of a high number of –OH surface groups in $AlPO_4$ may be deduced from the great and very close amounts of acid sites titrated with DMPY with respect to those titrated with PY. It is known that DMPY is selectively adsorbed on Brönsted acid sites, but not on Lewis acid sites because of a steric hindrance of the two methyl groups, whereas sterically non-hindered PY is adsorbed on both Brönsted and Lewis acid sites, so that the difference between PY and DMPY adsorption may be considered as a measure of the Lewis acid sites [13–18,33]. According to the results previously obtained [13–18], on increasing calcination temperature of $AlPO_4$, in the range 500–1000°C, the number of acid sites titrated with PY was always clearly higher than that of those titrated with DMPY, indicating the transformation of Brönsted acid sites to Lewis ones by a dehydration reaction of surface P–OH acid sites. The present results, however, indicate that all the surface acid sites in $AlPO_4$ titrated with both amines are in fact, Brönsted ones. It is evident that calcination at a lower temperature, 350°C, not only promotes higher surface areas, but also a higher number of Brönsted acid sites, where the immobilization reaction is carried out. Thus, the number of Brönsted acid sites on the support surface may be tailored by controlling the calcination temperature, after the precipitation step, in the synthesis of $AlPO_4$, so that this amorphous solid may provide an adequate high surface area to chemically bind any functionalized organic chain.

On the other hand, according to the results (Table 2) obtained on the immobilization through ε-amino groups (linker A), we can conclude that extensive immobilization (E_{imm} = 99%) is not enough guaranty to obtain appropriate enzyme behavior because, after immobilization, the enzyme became practically inactive E_{res} = 0.2–0.8%. Among other possible causes, this can be due to the fact that the active sites of the enzyme may be involved in its attachment to the support surface or to the influence of steric effects on the immobilized AP preparations using linker A. It should be noticed that linker B provides a longer space arm than linker A,

Table 2
Percentage of immobilized enzyme, E_{imm}, residual activity, E_{res}, and specific activity of immobilized enzyme, E_{spe}, determined from the corresponding values of the catalytic activity of the native enzyme, r_{nat}, the activity of the filtrate in the immobilization process, r_{fil}, and the activities of immobilized enzyme, r_{imm}, according to the results shown in Table 1, corresponding to immobilization through the two different linkers, A and B

Temperature (°C)	Linker A: through lysine residues			Linker B: through tyrosine residues		
	E_{imm} (%)	E_{res} (%)	E_{spe} (%)	E_{imm} (%)	E_{res} (%)	E_{spe} (%)
20	100.0	0.19	0.19	40.4	18.7	46.3
30	99.5	0.84	0.85	46.6	30.4	65.2
40	99.9	0.27	0.27	35.3	19.4	55.0
50	99.8	0.45	0.45	35.5	46.2	130.3

Table 3
Apparent activation energies (E_a, kcal/mol) and Arrhenius constants (ln A, μmol min^{-1}) from results in Table 1, for native and immobilized acid phosphatase enzyme, obtained through an 'azo' linkage to the tyrosine residues ('linker B'), under standard experimental conditions in a temperature range between 20 and 40°C

Native enzyme		Immobilized enzyme through linker B	
E_a	ln A	E_a	ln A
12 ± 1	23 ± 2	13 ± 4	22 ± 6

Uncertainties are determined for a 95% confidence limit.

potentially reducing shielding and steric effects. On the other hand, the good values of E_{res} and E_{spe}, for linker B, also shown in Table 2, indicate little deactivation of AP throughout the immobilization process by an 'azo' linkage to the *p*-OH-benzene group of tyrosine residues. Thus, we can conclude that by using this procedure, no important part of the active sites in the AP enzyme is affected. This behavior seems to be consistent with the close values obtained in E_a and ln A, for native and immobilized AP enzyme, in Table 3.

The highest activity of the AP enzyme, native and immobilized by 'linker B', was always obtained at 40°C. However, in the immobilization by 'linker B' through a diazonium salt, E_{spe}, was in the range 46–65% at temperatures between 20–40°C. However, when it increased to 50°C, the efficiency of the immobilized enzyme, with respect to the native one, went up to 130%. These high values of E_{spe}, point out that immobilization also led to an important increase on the thermal stability of the AP enzyme, increasing its efficiency to a varied degree at the highest temperatures, with respect to the soluble enzyme. This temperature effect is a critical performance indicator of the stability of the immobilized enzyme by the linker B.

Stability of covalently immobilized AP was also high enough to maintain residual activity after three month storage in a refrigerator. The operational stability of re-used immobilized AP was determined after separation and washing of solid biocatalyst. Thus, the solid was easily recovered after filtration and centrifugation in stirred tubes and solid washing with citrate buffer and then reused with fresh substrate mixture. Results in Table 4 indicate that immobilized AP is able to work after being stored for several months and under re-use conditions, while the native enzyme was completely inactivated after several days storage under the same experimental conditions. Here, we can also see how under re-use, residual activity, E_{re}, was very low, but higher with linker A than with linker B, according to the higher values of E_{imm} in the former supported biocatalyst.

From these results we can conclude that, through lysine residues to obtain an aromatic Schiff's-base, covalent immobilization by linker

Table 4
Percentage of residual activity, E_{re}, of immobilized acid phosphatase enzyme, after three month storage in a refrigerator, filtration and solid washing, determined from the relation between the corresponding values of the catalytic activity of immobilized enzyme, r_{imm}, shown in Table 1, and those obtained in the reuse, r_{reuse}, under the same standard operating conditions at different temperatures

Temperature (°C)	Linker A: through lysine residues			Linker B: through tyrosine residues		
	r_{imm} (μmol min^{-1})	r_{reuse} (μmol min^{-1})	E_{re} (%)	r_{imm} (μmol min^{-1})	r_{reuse} (μmol min^{-1})	E_{re} (%)
20	0.013	0.000	0.0	1.30	0.147	11.3
30	0.102	0.025	24.5	3.67	0.520	14.2
40	0.071	0.053	77.7	5.06	0.355	7.0
50	0.048	0.019	39.6	4.96	0.085	1.7

A, is comparatively stronger and more stable than covalent immobilization using linker B, through the coupling of phenolic groups of tyrosine residues to a diazonium linkage. However, according to the low values of E_{res} and E_{spe}, in Table 2, the former procedure, by linker A, promotes an extensive deactivation of immobilized enzyme because the active sites in the AP enzyme probably include some lysine residues, that are involved in its attachment to the support surface. From the values of E_{res} and E_{spe}, also in Table 2, in the immobilization process by an 'azo' linkage through linker B, we must conclude that no tyrosine residue in the active sites of AP enzymes can be involved in the attachment to the support.

These results are consistent with those obtained in the immobilization of porcine pancreatic lipase [20] and glucose oxidase [21], where enzymes were also covalently immobilized through the formation of an aromatic Schiff's-base. In both enzymes, E_{imm} was in the range 50–90% but contrary to AP enzyme, E_{res} was also high, near 90%, so that in these enzymes little deactivation was obtained throughout the immobilization process. Thus, their active sites probably do not include any lysine residue involved in the attachment to the support surface.

4. Conclusions

The results obtained here, as compared to those described in the literature [33], lead us to conclude that AP covalently immobilized on an inorganic support such as amorphous $AlPO_4$ could be advantageously used in most previously described applications because, according to results in Table 2, the values of immobilized AP under the present experimental conditions are per gram of support: 200 mg with linker A and 70–93 mg with linker B, with E_{res} values in the range 19–46% with linker B, while linker A promotes an important deactivation of immobilized AP, as previously indicated.

For covalent attaching of some organic functionality onto the surface of SiO_2, the most common inorganic support, a previous treatment of surface activation by the reaction with some functional organosilane always is needed, which then reacts with the silanols on the solid surface [7]. Subsequent reactions can expand the organic covalent chain to attach the desired complex-forming group in the main chain or in a side chain [34]. However, when $AlPO_4$ is used as inorganic support, surface functionalization may be carried without using any silane coupling reagent. Thus, we can directly obtain the functionalization of $AlPO_4$ surfaces, by the reaction of Brönsted acid sites (surface –OH groups) with suitable functionalized molecules (*p*-hydroxy-benzaldehyde, 'linker A' or 4-aminobenzylamine, 'linker B') under microwave heating. Within the same general schemes currently used with silanized silica, these functionalized molecules attached on $AlPO_4$ surfaces can be modified by some reactions such as diazotization for 'linker B' or condensation to give a new 'spacer' of higher length through an aromatic Schiff's-base [19–21].

In this way, due to the easy activation of the surface –OH groups, the support here studied opens many possibilities associated with the facility for obtaining organic 'linkers' of variable length, attached to the $AlPO_4$ surface, within the general schemes for covalent immobilization of enzymes on silanized SiO_2 [23,34]. Thus, tailor-made hybridized organic/$AlPO_4$–inorganic composites, could be appropriately functionalized not only by linking some aliphatic or aromatic aldehyde or by diazotization but also through several activation reactions with carbodiimides, thiophosgene, thionyl chloride, *N*-hydrosuccinimides or transition metal salts such as titanium chloride, such as in silanized SiO_2. The activated support, via direct reaction with available organic functions in proteins, such as amino, thiol or phenol, could then covalently incorporate a variety of biocatalysts and enzymes, with higher efficiency and yield.

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