PERGAMON

Soil Biology & Biochemistry 32 (2000) 1007–1014

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Interaction of acid phosphatase with clays, organic molecules and organo-mineral complexes: kinetics and stability

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Accepted 22 December 1999

Abstract

The properties of synthetic active enzymatic complexes, simulating those usually present in soil environment, were investigated. Complexes were formed by the interaction of acid phosphatase with clays (montmorillonite and Al hydroxide), tannic acid and organo-mineral aggregates, obtained by mixing tannate, OH-Al species and/or montmorillonite. Immobilized acid phosphatase showed catalytic features quite different from those of the free enzyme. The presence of OH-Al species in the matrix generally resulted in an improvement of some enzymatic properties. A gain in activity of about 45 and 55% was observed for the complexes acid phosphatase–tannate–OH-Al species after thermal deactivation at 60°C and 2 h of exposure to proteinase K. High residual activities ranging from 17 to 61% and from 28 to 57% of the initial one were measured for complexes of the enzyme with inorganic and organic/organo-mineral matrices, respectively. In contrast, the association with a pure constituent such as montmorillonite and/or tannic acid gave rise to an immobilized enzyme, displaying a completely different catalytic behaviour. Compared to the free enzyme, acid phosphatase–montmorillonite and acid phosphatase–tannate complexes had a different pH-activity dependence and a higher and lower sensitivity to temperature and proteolysis, respectively.

Keywords: Enzyme; Clay; Organo-mineral complexes; Kinetics; Stability

1. Introduction

In soil, enzymatic reactions occur in a heterogeneous rather than homogeneous environment (Nannipieri and Gianfreda, 1998). Intracellular enzymes exert their catalytic action within the cell, a restricted and compartmentalized system, while extracellular enzymes primarily function associated with soil colloids, short-lived when acting free in soil solution. Consequently, properties and kinetic behaviour of such enzymes very likely will differ from those of the corresponding protein from animal, microbial, or plant origins (Burns, 1978; Tabatabai, 1982; Ladd, 1985).

A great number of studies have dealt with the properties and the kinetics of enzymes in soils (McLaren and Packer, 1970; McLaren, 1978; Nannipieri and Gianfreda, 1998 and references herein). Several investigations have demonstrated that soil enzymes follow Michaelis–Menten kinetics, despite soil being considered as a discontinuous, structured, and heterogeneous system (Nannipieri and Gianfreda, 1998). Enzyme activities in soil frequently display changed kinetic parameters (usually lower V_{max} and higher K_m values) and exhibit an enhanced stability toward denaturation by different agents such as temperature, proteolysis, chemicals etc. Much of this information has been achieved by studies performed on whole soil samples, and mainly on synthetic enzyme-associations, obtained by in vitro interactions of purified enzymes and natural or synthetic supports.

The enzyme-complexes mostly investigated are those attained by adsorption or interaction of enzyme mol-

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0038-0717/00/$ - see front matter
PII: S0038-0717(00)00010-9

ecules with pure clays, humus materials or humus-like compounds (Theng, 1979; Boyd and Mortland, 1990; Ladd and Butler, 1975). Conversely, a limited number of studies have been dedicated to study the properties and the kinetics of enzymes acting in more complex associations, involving dirty clays and/or organic supports along with aluminium or iron derivatives. The latter ones are abundant in soil and may act as natural bridges between soil supports and enzymatic proteins (Gianfreda et al., 1993; 1995a, 1995b; Rao et al., 1996).

The object of this study is to characterize the kinetics and the stability of synthetic complexes obtained by interaction between acid phosphatase from plant origin and inorganic and organic soil colloids, with and without aluminium species. Montmorillonite and tannic acid have been used as representative of inorganic and organic soil colloids, respectively.

Montmorillonite is one of the most representative clays in soil, and tannic acid is an organic compound very similar to humic precursors and is involved in the formation of humic materials. Moreover, as demonstrated in a previous study (Rao et al., 1996), the OH-Al species may favour the immobilization of more active enzyme molecules on inorganic and organic matrices.

In soil, phosphatases, extracellularly secreted by plants and microorganisms, play a key role in the phosphorus cycle, allowing the formation of inorganic phosphorus, the only phosphate-form taken up by plants and microorganisms. These enzymes are usually not free in solution but associated with soil constituents. Indeed, they have been localized within structured soil particles, by means of experimental probes utilizing microscopic observations of soil sections (Ladd et al., 1996).

2. Materials and methods

2.1. Chemicals

Acid phosphatase (P) (EC 3.1.3.2, from potato, MW $\sim$ 100 kDa, Type I, 60 U mg^{-1}) was purchased from Boehringer Mannheim, Germany and proteinase K (EC 3.4.21.14, about 20 Anson U g^{-1}) was purchased from Sigma, St Louis, MO, USA. Tannic acid (MW 1701.23) was a reagent from Fluka AG, CH. All the other chemicals were reagent grade from Analar, BDH, Poole, UK.

2.2. Phosphatase complexes

Phosphatase–clay complexes [phosphatase–montmorillonite (P-M), phosphatase–$Al(OH)_{18}$–montmorillonite (P-AM), phosphatase–aluminium hydroxide (P-AL)] and phosphatase–organo and organo–mineral complexes [phosphatase–tannic acid (P-T), phosphatase–OH-Al–tannic acid (P-AT), and phosphatase–OH-Al–tannic acid–montmorillonite (P-ATM)] were prepared in 0.1 M Na-acetate buffer solution at pH 5.0, at 10 and 30°C, respectively, as described in detail by Rao et al. (1996). A higher temperature was used in the preparation of complexes with organic and organo-mineral matrices to accelerate the complexation process (Rao et al., 1996). The complexes, resuspended in 0.1 M Na-acetate buffer solution at pH 5.0, were stored at 10°C and their residual activity periodically measured. When the enzymatic activity of complexes was reduced by more than 20% compared to complexes freshly prepared, new complexes were prepared. All the data were normalized on the same residual activity.

2.3. Activity assay

The activity of free and immobilized phosphatase was usually measured with 1 ml of 6 mM *p*-nitrophenylphosphate (*p*NPP) in 0.1 M Na-acetate buffer at pH 5.0 (standard conditions). As explained in detail by Rao et al. (1996), temperature of 10 and 30°C was utilized for phosphatase–clay minerals and phosphatase-organo and organo-mineral complexes, respectively. The utilization of assay temperatures equal to those employed in the preparation of complexes avoided any disturbance to the equilibrium reached in the preparation process. After addition of 1 M NaOH, the concentration of *p*-nitrophenate was directly determined by the adsorbance at 405 nm in a spectrophotometer (Perkin Elmer Lambda 3B).

One enzymatic unit was defined as the μmoles of *p*-nitrophenol produced by 1 ml of free and immobilized enzyme solution over 1 min at 10 or 30°C and at pH 5.0. The specific activity was expressed as the units measured per mg of protein.

The influence of pH on the activity of both free and immobilized phosphatase was assayed at 10 and 30°C with 6 mM *p*NPP in the pH range 4.0–8.0, by using 0.1 M acetate (pH 4.0–5.5) and 0.1 M phosphate (pH 5.6–8.0) buffers.

The activity–temperature profile was obtained by activity assays with 6 mM *p*-nitrophenylphosphate (*p*NPP) in 0.1 M Na-acetate buffer at pH 5.0 and temperature ranging from 10 to 60°C. To minimize deactivation effects, the incubation time was progressively shortened with increasing temperature. The activation energy E_a was evaluated by plotting the log of activities of free and immobilized phosphatase vs. $1/T$ (K), according to the Arrhenius equation. The value of activation energy was obtained by a computed linear regression analysis of the experimental data.

The enthalpies of activation ΔH_a were evaluated

from the slopes of the curves obtained by plotting log activity/T (K) vs. $1/T$, according to the equation reported by Segel (1975).

2.4. Kinetic tests

The kinetics of free and immobilized phosphatase was determined by activity assays at 10 and 30°C and pH 5.0 with *p*NPP concentrations ranging from 0 to 6 mM. The kinetic parameters (V_{max} and K_m) were obtained by a computed non-linear regression analysis according to Michaelis–Menten equation (Segel, 1975).

2.5. Stability studies

The proteolytic stability of free and immobilized phosphatase was studied by determining the residual enzymatic activity after 1 h-, 2 h-, and 24 h-exposure to a bacterial proteinase K (500:1 proteinase K-to-acid phosphatase activity units ratio) at 37°C and pH 5.0. Control tests were performed in the absence of proteinase.

Enzyme thermal stability was assessed at 60°C over a 2-h incubation time. At predetermined time intervals, suitable volumes of free phosphatase solution or immobilized enzyme suspension were withdrawn and their residual activity was assayed under standard conditions.

All experiments were carried out in triplicate.

3. Results

Phosphatase reduced its activity to a different extent depending on the nature and type of immobilizing supports.

Enzymatic activity decreased to 20% of the initial one when the enzyme was adsorbed on montmorillonite (Table 1). A reduced decrease of residual activity (45 and 58%) occurred upon adsorption of acid phosphatase, respectively, on a non-crystalline aluminium oxide (P-AL) and an $Al(OH)_x$-montmorillonite complex containing 18 meq Al g^{-1} clay (P-AM).

When acid phosphatase was complexed with an organic matrix such as tannic acid, a recovery of 33% of enzymatic activity was detected. More active tannic acid–phosphatase complexes were obtained when OH-Al species, and, even more montmorillonite, were present during the complexation process. Residual activities increased up to 48 and 55% for P-AT and P-ATM complexes, respectively (Table 1).

The pH-dependence of phosphatase free and complexed with inorganic, organic, and organo-mineral supports is shown in Fig. 1.

The difference of temperature of the activity assays

Table 1
Residual activities (%) of inorganic, organic, and organo-mineral phosphatase complexes

Complexes[a]	Residual activity (%)[b]	Number of experiments
Inorganic		
P-M	20 ± 2.5[c]	12
P-AL	45 ± 3.3	12
P-AM	58 ± 2.9	12
Organic		
P-T	33 ± 2.7	11
Organo-mineral		
P-AT	48 ± 2.6	11
P-ATM	55 ± 1.4	11

[a] Symbols indicate: P = acid phosphatase, M = montmorillonite, AL = aluminium hydroxide, AM = $AL(OH)_{18}$–montmorillonite complex, T = tannic acid, A = OH-AL species.
[b] As percentage of the initial units added.
[c] Standard deviation.

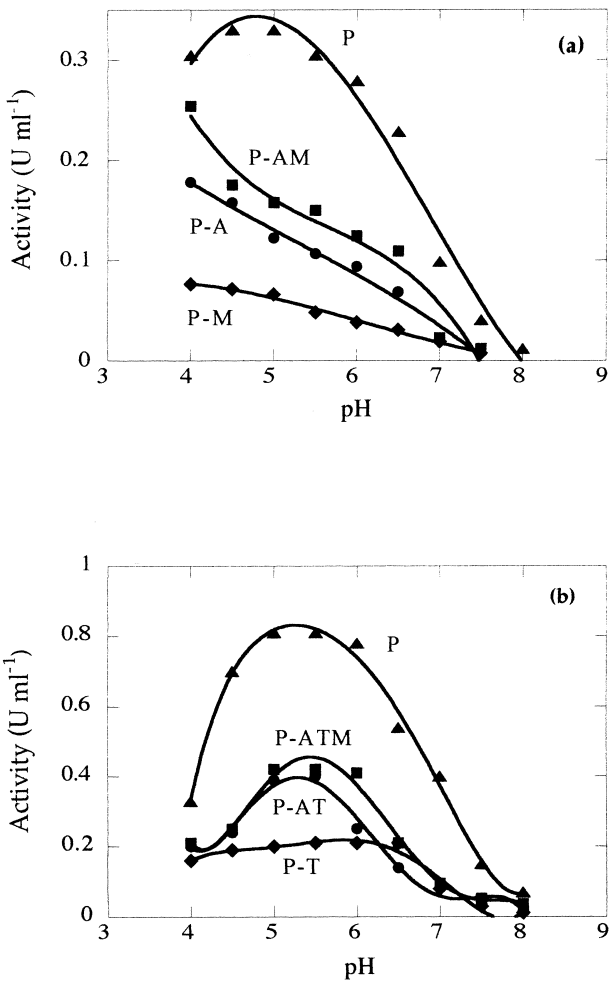

Fig. 1. Activity–pH profiles of free and immobilized acid phosphatase on (a) clay minerals, (b) tannic acid and organo-mineral complexes. Symbols indicate: P = acid phosphatase, M = montmorillonite, AL = aluminium hydroxide, AM = $Al(OH)_{18}$–montmorillonite complex, T = tannic acid, A = OH-Al species.

(see Section 2) accounts for the greater values of activities reported in Fig. 1(b) as well as for the slight shift of the maximum activity pH. At 10°C, free phosphatase exhibited its optimum activity at pH 4.5–5.0, whereas at 30°C the optimum pH shifted slightly upward by 0.5. In the alkaline pH range, the activity of the free enzyme decreased to the same extent at both temperatures. In contrast, at pH 4.0, a sharp reduction more than 60% was measured at 30°C, while at 10°C the activity was reduced by only 10% (Fig. 1(a)).

After adsorption on clays, completely different pH-activity profiles were exhibited by the enzyme (Fig. 1(a)). The activity of the three complexes continuously decreased with increasing pH, identifying a maximum of activity at pH 4.0. However, the decline of activity was much more sharp for phosphatase adsorbed on AM than on AL and M, in the order listed. The increase of one pH unit from 4.0 to 5.0 determined a decrease of activity of more than 40% for P-AM, whereas the activity of P-AL and P-M lowered only by 30 and 13%, respectively (Fig. 1(a)).

When complexed with AT and ATM, the enzyme showed a similar pH-dependence (Fig. 1(b)). The maximum as well as the sensitivity to pH changes of both the complexes were similar to those of the free enzyme. Instead, a more flat curve was observed for the enzyme complexed with tannic acid. The activity resulted quite independent on pH in the range 4.0–6.5, while it suddenly decreased above pH 6.5. A maximum of activity at pH 6.0–6.5 was measured (Fig. 1(b))

The role of the support on the catalytic behaviour of phosphatase was also evident by temperature–activity curves illustrated in Fig. 2. In the temperature-range explored, the activity of the free enzyme increased by increasing temperature up to 50–55°C. No further activity assays were performed at temperatures higher than 60°C, because of the very elevated sensitivity of the enzyme to high temperature (see below).

A smooth behaviour (i.e., less dependence on temperature) was in general exhibited by phosphatase immobilized on inorganic supports. The activity of the enzyme adsorbed on M and AM increased very slowly with increasing temperature. Conversely, phosphatase immobilized on AM displayed a high value of activity throughout a broad range of temperature (40–60°C) (Fig. 2(a)).

The complexes P-T and P-ATM showed similar activity–temperature profiles (Fig. 2(b)). The activities of both the complexes very slightly increased with increasing temperature and exhibited their higher values in the range 50–55°C. At 60°C, the activity of P-T decreased whereas that of P-ATM remained constant. Conversely, the activity of the enzyme immobilized on AT continuously increased with increasing temperature.

The values of the activation energy E_a were determined experimentally by plotting log activities vs. $1/T$ (K). The Arrhenius plots were linear in all cases, indicating the existence of one step rate-limiting at different temperatures. In agreement with the activity-temperature profiles, E_a values were quite low and ranged from a minimum of 13.02 kJ mol^{-1} for the enzyme bound to tannic acid to a maximum of 16.43 kJ mol^{-1} when the enzyme was adsorbed on AM (Table 2). Similar values of E_a were, then, determined for P-T and P-ATM as well as for P-M, P-AT and free P.

The effect of temperature on the reaction, catalyzed by free and immobilized phosphatase, was also expressed in terms of the temperature coefficient Q_{10}. It is the factor by which the rate constant increases by raising the temperature 10°C (Table 2). The data in

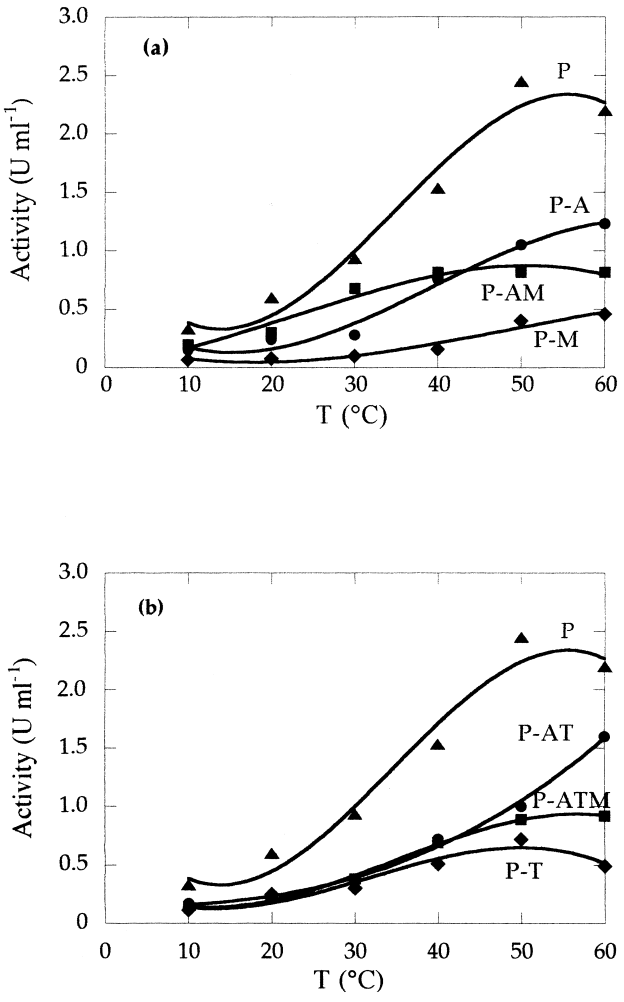

Fig. 2. Activity–temperature profiles of free and immobilized acid phosphatase on (a) clay minerals, (b) tannic acid and organo-mineral complexes. Symbols indicate: P = acid phosphatase, M = montmorillonite, AL = aluminium hydroxide, AM = $Al(OH)_{18}$–montmorillonite complex, T = tannic acid, A = OH-Al species.

Table 2
Energy of activation (E_a), enthalpy of activation (ΔH_a), and thermal coefficient Q_{10} for free and immobilized acid phosphatase

Complexes[a]	E_a (kJ mol^{-1})	r^{2b}	ΔH_a (kJ mol^{-1})	r^{2b}	Q_{10} (20–60)
Free P	14.36	0.98	13.07	0.98	1.51
Inorganic					
P-M	14.63	0.93	13.52	0.95	1.55
P-AL	15.45	0.97	14.35	0.97	1.60
P-AM	16.43	0.98	15.36	0.97	1.40
Organic					
P-T	13.02	0.98	11.92	0.98	1.53
Organo-mineral					
P-AT	14.56	0.99	13.73	0.99	1.54
P-ATM	13.63	0.98	12.52	0.98	1.49

[a] Symbols: see Table 1.
[b] Significant at 0.001 probability level.

Table 2 indicate a mean value of 1.49, which is congruent with low activation energies. Table 2 also reports the values of activation enthalpies ΔH_a, calculated by plotting log activity/T vs. I/T (K). The values lowered by about 1.1 kJ mol^{-1} as compared to the corresponding E_a. The value of ΔH_a is related to the events necessary to the formation of transition state (Segel, 1975; Cornish-Bowdel, 1979). The higher the ΔH_a value, the larger the amount of stretching, squeezing or even breaking of chemical bonds to reach the transition state (Lai and Tabatabai, 1992).

The results obtained in the stability studies are illustrated in Fig. 3, as log activity vs. deactivation time. No results referring to P-M are illustrated because of the high sensitivity of the complex to temperature. After few minutes (<5) of exposure to 60°C, a sudden decrease ($>95\%$) of activity was measured.

The double-slope pattern shown in Fig. 3 indicates

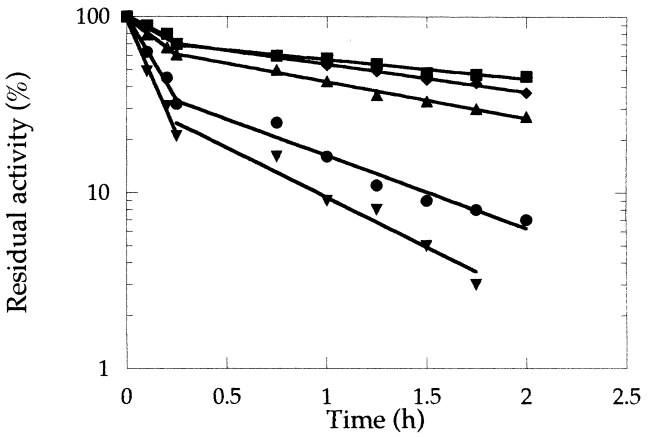

Fig. 3. Thermal stability (60°C) of free and immobilized acid phosphatase on (a) clay minerals, (b) tannic acid and organo-mineral complexes. Semilog plot of residual activity, expressed in percentage of the initial activity, vs. time. Symbols indicate: P = acid phosphatase, M = montmorillonite, AL = aluminium hydroxide, AM = $Al(OH)_{18}$–montmorillonite complex, T = tannic acid, A = OH-Al species.

that a complex deactivation mechanism is followed by both free and immobilized phosphatase. As exhaustively demonstrated by Gianfreda et al. (1984, 1985), this behaviour may be described by a two-step, series scheme N → (X) → D, where N is the native enzyme, (X) indicates, a collective distribution of intermediate forms mutually at equilibrium, and D is a final, inactive conformation of the protein. By assuming the first-order and irreversible kinetics for both steps, enzyme deactivation may be quantitatively described by a relationship including four parameters, related to the activity of N and X and to the kinetic constants of the two steps. According to this mathematical model, the curves reported in Fig. 3 were obtained by a non-linear regression routine.

When immobilized on the organo-mineral supports AT and ATM, phosphatase was much more stable than free in solution. In contrast, the enzyme immobilized on AM and/or T showed a higher sensitivity to thermal deactivation than free phosphatase.

The immobilization on the inorganic, and much more on the organic and organo-mineral supports increased the long-term stability of the enzyme to proteolysis. Free phosphatase showed a low sensitivity to proteolysis within 2 h-exposure, but it was completely deactivated after 24 h of contact with proteinase (Table 3). According to the results obtained in thermal stability studies, the enzyme complexed with ATM resulted very stable and its residual activity decreased only by 13 and 20% after 1 and 2 h exposure to the proteolytic protein, respectively. After 24 h, the immobilized enzyme still showed about 50% of its initial activity (Table 3).

The kinetics of free and immobilized phosphatase is illustrated in Fig. 4. All curves show a typical Michaelis–Menten behaviour.

The maximum velocities of complexes were lower than that of the free enzyme (Table 1). In contrast, the values of Michaelis–Menten constant K_m for the im-

Table 3
Stability of free and immobilized phosphatase to proteloysis exposure

Complexes[a]	Residual activity (%) after exposure proteinase K[b]		
	1h	2h	24h
Free P	75 ± 3.2[c]	55 ± 2.8	0
Inorganic			
P-M	14 ± 1.6	0	0
P-AL	41 ± 3.1	24 ± 3.4	4 ± 2.3
P-AM	41 ± 4.2	25 ± 2.4	1 ± 1.6
Organic			
P-T	73 ± 3.2	51 ± 5.1	16 ± 2.0
Organo-mineral			
P-AT	86 ± 5.2	80 ± 3.3	44 ± 2.7
P-ATM	87 ± 2.2	80 ± 3.9	48 ± 3.2

[a] Symbols: see Table 1.
[b] Proteinase K/acid phosphatase ratio 500:1.
[c] Standard deviation.

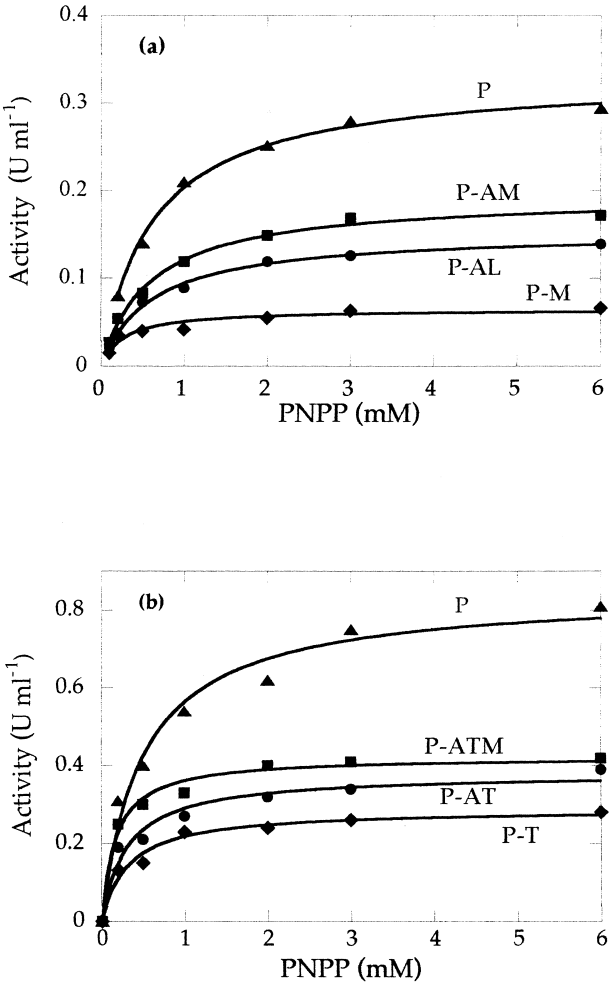

Fig. 4. Michaelis–Menten plots of free and immobilized acid phosphatase on (a) clay minerals, (b) tannic acid and organo-mineral complexes. Symbols indicate: P = acid phosphatase, M = montmorillonite, AL = aluminium hydroxide, AM = $Al(OH)_{18}$–montmorillonite complex, T = tannic acid, A = OH-Al species.

mobilized enzyme were generally lower than that of the free enzyme, indicating that phosphatase had a higher affinity for the substrate after immobilization on the supports. K_m value decreased from 0.5 mM (at 30°C) and 0.6 mM (at 10°C) for the free enzyme to about 0.3 mM for P-M, P-T and P-AT. A very low value (0.17 mM) was measured for the P-ATM complex.

4. Discussion

The results obtained in this study suggest that the catalytic behaviour of phosphatase may change after immobilization depending strongly on the nature of the immobilizing support. In particular, an improvement in the catalytic features of the immobilized enzyme is achieved when phosphatase is complexed with an inorganic and/or organic support in the presence of OH-Al species.

In a previous paper (Rao et al., 1996), we have shown that the activity levels of phosphatase adsorbed on clays increased from 21 to 54% with increasing OH-Al covering from 3 to 18 meq Al g^{-1} montmorillonite. Similar results were obtained by Naidja et al. (1997), who demonstrated that the residual specific activity of tyrosinase immobilized on montmorillonite coated by $Al(OH)_x$ species increased from about 24 to 62% of that of the free enzyme, with increasing the level of Al coating from 1.0 to 5.0 mmol Al g^{-1} clay.

A beneficial effect of OH-Al species on the formation of more active tannate–enzymatic complexes have been demonstrated with urease and invertase as well (Gianfreda et al.,1993, 1995a). The activities of urease- and invertase–OH–Al–tannate complexes were 66 and 77% higher than those obtained without OH–Al species, respectively. A further gain of enzymatic activity was observed in the presence of montmorillonite. The activity of an invertase–OH–Al–tannate–montmorillonite complex was 84%, much more higher than that of the complex prepared in the absence of montmorillonite (Gianfreda et al., 1993).

The highest value at acidic pHs shown by the enzyme immobilized on AL and AM (Fig. 1a) is consistent with the fact that enzyme–substrate interactions occur on a positively charged support. According to the comprehensive analysis of the microenvironmental effect on the behaviour of immobilized enzymes (Katchalski et al., 1971) a positively charged carrier (such as AL or AM) will attract hydroxyl ions (OH^-) to its surface and the concentration of these latter will be higher than that of the bulk solution. Consequently, a shift toward more acid pHs is expected (Fig. 1(a)).

The opposite shift towards more alkaline pHs observed for P-T (Fig. 1(b)) is still in agreement with the presence of a microenvironmental effect by the

support (in this case tannate molecules negatively charged) on the activity of immobilized phosphatase. In this case, the microenvironment surrounding the enzyme is more acid (lower pH) than the bulk and a more alkaline pH is necessary to reach the enzymatic optimum-pH value. When OH-Al species, tannic acid and montmorillonite, showing positive and negative charges, respectively, are simultaneously present on the support, a balance between the two effects probably occurs and no significant changes in the pH-activity profile will result (Fig. 1(b)).

A different theoretical approach was used by Quiquampoix (1987) and Staunton and Quiquampoix (1994) to explain the effect of pH on the activity of proteins adsorbed on clay minerals. The authors hypothesize that the forces of interaction occurring between proteins and mineral surfaces give rise to structural modifications or orientational effects of the adsorbed proteins with a subsequent variation of the enzymatic activity.

The most beneficial effect deriving from the coexistence of OH-Al species, tannic acid, and montmorillonite reflected on the enhanced thermal and proteolytic stability shown by P-AT and P-ATM (Fig. 3 and Table 3). Evidently, considerable restriction to conformational changes of the enzyme structure as well as to the attack of proteinase arose after entrapment and/or adsorption of the enzyme within and/or on the organo-mineral complex.

The kinetic findings shown in Fig. 4 are unusual. Generally, the Michaelis–Menten constant of immobilized enzymes increases, because of a reduced affinity for the substrate, achieved by enzymes upon immobilization on solid supports. However, some exceptions to these findings have been demonstrated. As compared to free enzymes, lower K_m values were determined for α-amylase, protease, alkaline phosphatase and urease when adsorbed on various clays (Kobayashi and Aomine, 1967; Makboul and Ottow, 1979; Gianfreda et al., 1992).

The lower K_m values measured for P-AT and P-ATM (Fig. 4) could be explained by reduced conformational changes undergone by enzyme upon immobilization. In previous studies (Gianfreda et al., 1993; 1995b), we found that the presence of OH-Al species during the complexation of urease and invertase with tannic acid positively affected the residual activities of the immobilized enzymes and their catalytic behaviour. We suggested that the structure of such complexes could have a reticulated tridimensional organisation with large pores. This structure probably enables a free diffusion of small molecules such as substrate and reaction products, but markedly hinders the accessibility to large size molecules such as proteinase. Similarly, temperature-promoted conformational changes of phosphatase molecules, embedded in these structures, could be prevented and an increased thermal stability may result (Fig. 3).

In conclusion, phosphatase immobilized on clays, organic and organo-mineral complexes showed catalytic features quite different from those of the free enzyme. These findings confirm that catalysis by an immobilized enzyme may differ from that of the free form because of chemical or conformational changes of enzyme structure, occurring upon immobilization; modification of the microenvironment surrounding enzyme due to the physical and chemical nature of the immobilizing support; and steric hindrance effects on substrate accessibility to the active site. Some or all these factors may concurrently affect the catalytic behaviour of the immobilized enzyme (McLaren and Packer, 1970; Nannipieri and Gianfreda, 1998).

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