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Interaction of phytases with minerals and availability of substrate affect the hydrolysis of inositol phosphates

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Abstract
The stability and activity of phytases in the soil environment may be affected by their sorption on soil particle surfaces and by substrate availability with important consequences for P cycling and nutrient bioavailability. This work evaluated the interaction of phytases with goethite, haematite, kaolinite, montmorillonite and two oxisol clays and investigated how this interaction is affected when *myo*-inositol hexakisphosphate (InsP_6) was sorbed on the mineral surfaces. *phyA* histidine acid phosphatases of fungal origin were used and their ability to release orthophosphate from the InsP_6 saturated minerals was evaluated.
The phytases showed a high affinity for the mineral surfaces, with a loss of enzyme activity generally being observed over 24 hours (up to 95% of the initially added activity). The loss of phytase activity was dependent on the type of mineral, with kaolinite and montmorillonite showing the greatest effect. Retention of enzyme activity was higher with the two oxisol clays, suggesting that the heterogeneous nature of clay surfaces and the presence of endogenous organic matter may limit the inhibition caused by interaction with minerals.
In the presence of mineral surfaces saturated with InsP_6, the partitioning of enzyme activity between the solution and the solid phase was shifted more towards the solution phase, presumably due to the mineral surfaces being occupied by the substrate. However, phytases were not able to release any orthophosphate directly from InsP_6- saturated goethite and haematite, and hydrolysed InsP_6 that was desorbed from haematite. Conversely, in the case of kaolinite and of the oxisol clays, where desorption was limited, phytases appeared to be able to hydrolyse also a small fraction of the InsP_6 adsorbed on the surfaces. These findings suggest that the bioavailability of P from inositol phosphates is governed to a large extent by the mineral composition of soil and by competitive effects for sorption on reactive surfaces among inositol phosphates and phytases.

Key words: Inositol hexakisphosphate, phytases, phosphorus, oxisols, hydrolysis

1. Introduction
Phytases catalyse the hydrolysis of inositol phosphates and are potentially important in the soil environment for their role in the phosphorus cycle, by allowing the release of orthophosphate which is the form of P taken up by plants and microorganisms. The stability and activity of phytases in soil may be affected by their sorption on soil particle surfaces which may reduce the potential for

interaction with substrates. However this may also provide longer-term advantages for their persistence and function in soils (Nannipieri et al., 1996; Naidja et al., 2000). When fungal phytases were added to soil suspensions, a rapid loss of activity from soil solution and a significant recovery in the solid phase was observed (George et al., 2005), indicating a rapid immobilization of the enzyme on soil particles. Such reaction is likely to affect the effectiveness of phytases for reaction with inositol phosphates in soil (Quiquampoix and Mousain, 2005). On the other hand, the persistence of phytase activity on the solid phase of soil has been shown to provide stability against microbial degradation (George et al. 2007).

The differential partitioning of enzymes between the solid phase and the soil solution may be related to the physicochemical characteristics of the phytase proteins, in particular their isoelectric point (Naidja et al., 2000). As the range of pI for phytases is large, from pH 3.6 to 7.3 (Vats and Banerjee, 2004), different enzymes may be more or less adsorbed over a wide range of soil pH.

The behaviour of phytases in soil also depends on the soil type, as the nature of soil components can affect the retention and persistence of enzyme activity (George et al., 2005; Tang et al., 2006). For instance, Al hydroxides and clays are reported to be more inhibitory than organic matter towards phosphatases (Rao et al., 2000) and clay minerals with interlaminar spaces reduced phosphatase activity more than 1:1 phyllosilicates (Nannipieri et al., 1996). Furthermore, when the minerals are present in more complex systems, either through association with each other or in the presence of organic matter, adsorption is reported to be less inhibitory to enzyme activity (Gianfreda et al., 1993; Rao et al., 2000).

The activity of phytases in soil is further influenced by substrate availability. Inositol phosphates show a strong affinity for soil colloids with a large surface area and anion retention capacity (Celi and Barberis, 2007). Among different soil components, iron and aluminum oxides have a higher capacity to retain inositol phosphates compared with phyllosilicates (Shang et al., 1990; Ognalaga et al., 1994; Celi et al., 1999). The extent of retention has been reported to be significantly enhanced by the presence of poorly crystalline oxides associated with phyllosilicates (Celi et al., 2003).

It is commonly reported that the strong stabilization offered by these abiotic reactions hampers biological degradation of inositol phosphates and thus contributes to their presence or accumulation in soil (Magid et al., 1996; Turner et al., 2002). However, few studies have specifically addressed the role of pure minerals on the extent and processes which govern inositol phosphate availability to phytases. Tang et al. (2006) and Lung and Lim (2006) showed that phytases were able to hydrolyse only soluble forms of Ca^{2+}- and Mg^{2+}- inositol phosphates, but were ineffective on Al^{3+}-, Fe^{3+}-, Cu^{2+}-, and Zn^{2+}- inositol phosphates or on inositol phosphates adsorbed to sand. By comparison, the interaction of phytases with other minerals that commonly occur in soils and with the most reactive soil fraction, i.e, clay, as well as the extent to which phytases can interact with inositol phosphates adsorbed to mineral surfaces, have received little attention.

Thus, whilst it is known that the inositol phosphate content in soils, along with total organic P content, may be highly variable (Turner, 2007), factors that govern their bioavailability and then their cycling in soil still remain unclear. Recently, Turner et al. (2007) and McDowell et al. (2007) reported that the amount of inositol phosphates in soils changes with soil evolution with relatively lower amounts occurring in highly weathered soils, which may be associated with the extent of mineral weathering and susceptibility to higher rates of microbial decomposition.

Therefore, in this work we investigated the interaction of fungal phytases with pure minerals and soil clays both in the absence and presence of sorbed inositol phosphates. The specific aims were to evaluate (i) the interaction of the phytases with iron oxides (goethite and haematite) and phyllosilicates (kaolinite and montmorillonite); (ii) how this interaction is affected when inositol phosphates are sorbed to these soil components and the capacity of the phytases to hydrolyse the sorbed substrate; and (iii) the behaviour of the phytases on clays extracted from highly weathered soils.

2. Materials and methods

2.1 Soil minerals

Goethite (Gt) was prepared according to the method of Schwertmann and Cornell (1991) by dissolving $Fe(NO_3)_3 \cdot 9H_2O$ in deionised water and then adding 5M KOH rapidly and stirring. The suspension was immediately diluted with deionised water and kept at 70°C for 60 h. The suspension was then centrifuged, washed with deionised water and freeze-dried.

Haematite (Hm) was prepared by dissolving 20 mM $Fe(NO_3)_3 \cdot 9H_2O$ in 2 mM HNO_3 previously heated at 98°C (Schwertmann and Cornell, 1991). The solution was then maintained for 7 days at 98°C, after which the suspension was centrifuged, dialysed against deionised water and then freeze-dried.

Kaolinite (KGa2) and Montmorillonite (SWy-2) were obtained from the Clay Minerals Society (Wyoming, USA). For these phyllosilicates, the <2 µm fraction was separated by dispersion at pH 8.5, followed by repeated sedimentation and decantation. This fraction was K-saturated by shaking three times with 1M KCl and then dialysed against deionised water until the water was Cl^- free. The suspensions were kept as such for the adsorption experiments.

Two clays (A and B) were extracted from two different highly weathered soils (Oxisols), located in the Mato Grosso do Sul State (Brazil). The clay fractions were separated by the pipette method after soil dispersion in water and then freeze-dried (EMBRAPA, 1997). A more detailed description of site area and clay characteristics is reported in Giaveno et al. (2008). Organic C was 15.0 and 6.01 g kg^{-1} and total P was 401 and 250 mg kg^{-1} in clay A and B, respectively. The mineral composition and surface properties of the two clays and the pure minerals used in the study are summarised in Table 1.

2.2 Phytases

Phytases from two different fungal sources were used: a basidiomycete (*Peniophora lycii*) and an ascomycete (*Aspergillus niger*). Both enzymes are classified as *phyA* histidine acid phosphatases (Ullah and Sethumadhavan, 2003), but differ in their point of cleavage initiation in the dephosphorylation of *myo*-inositol hexakisphosphate, with the *P. lycii* enzyme being a 6-phytase (EC 3.1.3.26), and the *A. niger* enzyme a 3-phytase (EC 3.1.3.8). The isoelectric point (pI) of the two phytases also differed, being 3.6 and 5.0 for the *P. lycii* and *A. niger* proteins, respectively. Both phytases were from commercially available preparations, the *P. lycii* being a Ronozyme® phytase (Hoffman-La Roche) and the *A. niger* being a Natuphos® phytase (BASF). Details of reaction kinetics of the enzymes against *myo*-inositol hexakisphosphate and other organic P substrates are as previously reported (George et al., 2007). Stock solutions of the two enzymes were prepared in 15mM 2-morpholinoethanesulfonic acid (MES) buffered at pH 5.5, filter sterilised (0.22 µm) and stored at 4°C prior to use.

Phytase activity of the stock solutions was determined using 2 mM *myo*-inositol hexakisphosphate dipotassium salt (InsP_6), a substrate concentration in excess (~10-fold) of the reported K_m for *PhyA*, as described by Richardson et al. (2000). Phosphate released during the assay was quantified spectrophotometrically after reaction with malachite green (Irving and McLaughlin, 1990). The stock solutions were then diluted in order to obtain an activity of 1 nkat ml^{-1} in subsequent assays.

2.3 Addition of phytases to soil minerals

Prior to adsorption experiments, different suspensions containing 6 mg ml^{-1} of goethite, haematite, and the oxisol clay fractions A and B, and 12 mg ml^{-1} of kaolinite and montmorillonite, were shaken for 24 hours at room temperature with 15 mM MES at pH 5.5. Higher amounts of the latter minerals were used to obtain conditions appropriate for enzyme and InsP_6 adsorption (Celi et al., 1999).

The effect of minerals on phytases was tested by adding 0.75 ml of each suspension to 0.75 ml of phytase solution (final activity of 1 nkat ml^{-1}) and the samples were put on an orbital shaker for up to 48 hours with a slow rotation to maintain uniform suspensions.

Sub-samples (500 µl) were then taken for measurements of phytase activity after 1 and 24 hours (described below). For each sub-sample, an aliquot was used to measure the total enzyme activity in the mineral suspension, while the remainder was centrifuged at 13000 *g* for 4 min and the supernatant taken for measurement of phytase activity in the solution phase.

Phytase activities in the mineral and clay suspensions and collected supernatants were measured at a sample:buffer ratio of 1:1. Assays were routinely done in micro-titre plates, using a final reaction volume of 300 µl, by addition of InsP_6 to a final concentration of 2mM and buffered to pH 5.5 with 15mM MES and incubated for 60 minutes at 37°C. The InsP_6 stock solution (20mM) was first acidified to pH 5.5 with 10M HCl and filter sterilised (0.22 µm) prior to use (Richardson et al., 2000). Reactions were stopped with an equal volume of 10% trichloroacetic acid (TCA). The samples were centrifuged at 3800 *g* for 5 min prior to determination of P concentration in the supernatant using malachite green. Phytase activity was calculated as the P released during the 60 min assay. Activity of phytase retained on the solid phase was derived as the difference between the total mineral suspension and solution phytase activities.

In order to determine the exact activity of the phytase added and its stability over time, blanks in the absence of the minerals were run, with 0.75 ml of 15mM MES instead of the suspension. After 24 h the percentage of phytases retained under these conditions was generally greater than 90% of the initial activity.

The possibility that the presence of minerals may affect the recovery of inorganic P released by the enzymatic hydrolysis during the assay was also checked by addition and subsequent recovery of P in spiked samples. Consistent with observations by George et al. (2007), this was shown to be insignificant due to both the wide solution to mineral ratio used in the final assay and the presence of an excess of InsP_6 in the suspensions.

Specific assays with oxisol clays were also run to evaluate the release of orthophosphate from endogenous sources following phytase activity, which was ruled out as no P was detected in the suspensions.

2.5 Formation of InsP_6-mineral complexes

Suspensions containing the different minerals were similarly prepared and shaken for 24 hours at room temperature with 15 mM MES at pH 5.5. An equal volume of 2mM InsP_6 was then added (prepared in 15mM MES at pH 5.5) to saturate the minerals and the suspensions were shaken for a further 24 hours at room temperature. The suspensions were then re-centrifuged at 1600 *g* for 15 min, the supernatant removed and the sediment washed with deionised water to remove the excess of InsP_6. The suspensions were centrifuged and washed a further three times and then taken back to the original volume by adding 15mM MES. In order to determine the exact quantity of P adsorbed on the mineral surfaces, the supernatant and the washing solutions were collected quantitatively and aliquots were digested with concentrated $HClO_4$-H_2SO_4 to hydrolyse InsP_6 to orthophosphate (Martin et al., 1999).

2.6 Addition of phytases to InsP_6-mineral complexes

Enzymatic assays were carried out on the InsP_6-mineral complex suspensions, as described above. The ability of phytases to hydrolyse InsP_6 adsorbed on the mineral surfaces was determined over both 1 and 24 h by detection of orthophosphate in the solution phase of aliquots of each sample that were collected by centrifuging the suspensions at 13000 *g* for 4 min. Appropriate blanks were also run with enzymes in the absence of the minerals, and with minerals in the absence of enzymes. In the latter, the potential desorption of InsP_6 from the mineral surfaces was also measured by taking aliquots of the suspensions at 0, 1 and 24 h; these were centrifuged, the supernatant collected and digested with concentrated $HClO_4$-H_2SO_4 for determination of orthophosphate.

2.7 Statistical analysis

Enzyme activities for both *A. niger* and *P. lycii* phytases are presented throughout the paper as the percentage of the initially added activity, recovered after 1 and 24 h, respectively. All data are presented as the mean of three or four replicates and error bars represent one standard error either side of the mean. Significant differences were determined using ANOVA ($p<0.05$).

3. Results

3.1 Interaction of phytases with soil minerals

Activities of the *A. niger* and *P. lycii* phytases after 1 and 24 h of interaction with the various minerals and clays are shown in Figures 1 and 2, respectively.

When *A. niger* phytase was added to goethite and haematite the activity after 1 h was nearly totally retained, whilst after 24 hours it decreased significantly. At both times of incubation the activity was associated completely with the solid phase (Figure 1). By comparison, with kaolinite and montmorillonite a greater loss of activity ($p<0.05$*)* was observed after 1 h, with less than 10% of the activity being retained after 24 h. When added to the oxisol clays, *A. niger* phytase showed a behaviour similar to that with the iron oxides ($p<0.05$). In both cases the activity was associated almost entirely with the solid phase.

When *P. lycii* phytase was used, similar interactions to those with *A. niger* enzyme were observed (Figure 2). However, in the case of goethite and montmorillonite, a variable portion of the total activity was recovered in the solution phase rather than being associated with the solid phase only.

3.2 InsP_6 adsorption to mineral complexes

The amounts of InsP_6 adsorbed on the mineral surfaces at pH 5.5 are shown in Table 2. For comparative purposes the amounts of InsP_6 adsorbed at pH 4.5 are reported (Celi et al., 1999; 2003; Giaveno et al., 2008). The maximum amount of InsP_6 adsorbed on goethite was equivalent at both pHs, while on the two soil clays and on montmorillonite it was lower at pH 5.5 as compared to pH 4.5. By comparison, on haematite and kaolinite InsP_6 adsorption was substantially lower at pH 5.5 relative to pH 4.5.

3.3 Interaction of phytases with InsP_6-mineral complexes

When *A. niger* phytase was added to the minerals saturated with InsP_6 its behaviour was different to that observed in the absence of substrate (Figure 3). On InsP_6 -saturated goethite and clay A there was a significant difference ($p<0.05$) in the partitioning of the *A. niger* phytase activity between the solid and solution phases. With all other minerals the activity was associated completely with the solid phase, similar to that observed with the minerals in the absence of InsP_6 saturation.

In the case of *P. lycii* phytase there was a marked difference in the behaviour of the enzyme on all of the minerals and clays saturated with InsP_6 (Figure 4). With the exception of kaolinite after 24 h, the major portion of enzyme activity was retained in solution and there was only small but significant ($p<0.05$) loss of the total activity over the 24 hour period.

3.3 Release of orthophosphate from InsP_6-saturated minerals

The desorption of InsP_6 from the InsP_6–saturated minerals was measured and in all cases was shown to be negligible after 0 and 1 h (data not shown), whereas after 24 h there was some desorption from all of the minerals (Table 2). Highest desorption occurred from haematite and kaolinite, where 8.3 and 6.0%, respectively, of the total amount of adsorbed P (as InsP_6) was detected in solution. The amount of P desorbed from the other minerals was less than 2%.

The quantities of orthophosphate released by phytases from InsP_6 adsorbed on the various minerals and clay fractions after 24 h are shown in Table 3. In suspensions of goethite and montmorillonite small amounts of P were detected for both *A. niger* and *P. lycii* phytases, but these were not significantly different ($p<0.05$) from those desorbed without enzyme. From haematite, kaolinite and the two soil clays, higher amounts of P were detected in solution. However, in the case of haematite the amount recovered after interaction with either enzymes was again not significantly different

($p<0.05$) from the amount of P desorbed without enzyme. A similar result was observed on kaolinite and on clay A for *P. lycii*, whilst for *A. niger* the amounts of P recovered were significantly greater ($p<0.05$) than those due to desorption. Significant differences were found also on clay B for both enzymes.
Where significant, and after correction for the amount of P desorbed in the absence of enzyme (Table 3), the amounts of P released by *A. niger* interaction with the InsP_6-saturated minerals accounted for 11.8% of the total P adsorbed on kaolinite, and for 2.0% and 5.9% on clays A and B, respectively. With *P. lycii* 4.8% of P was released from the InsP_6-saturated clay B.

4. Discussion

4.1 Interaction of phytases with soil minerals

The two phytases showed differential interaction with the various minerals with a strong partitioning of activity toward the solid phase and a significant loss of activity over time. Soil or mineral surface charge and the isoelectric point (pI) of the enzymes are reported to be the key factors affecting adsorption through the formation of electrostatic forces (Quiquampoix et al., 1993; Staunton and Quiquampoix, 1994; Rao et al., 2000). In this study, minerals with different surface charge were considered and two enzymes with differing pI were used. At the pH of the assays (pH 5.5), goethite and haematite were positive (Table 1), clay B was close to its point of zero charge (pzc), whereas the two phyllosilicates and clay A had a negatively charged surface. At this pH, *A. niger* phytase approaches its pI (pH 5.0) and retention by the solid phase occurred with all pure minerals and soil clays. Electrostatic interaction would be expected to occur between the negative charges of the enzyme and the positive iron oxides; other mechanisms, mostly active at pH close to enzyme pI, may involve hydrogen bonding and physical partitioning between the liquid and the solid phase (Naidja et al., 2000). By comparison, the negatively charged phyllosilicates and clay A should attract the enzyme to a lesser extent. However, it is known that the pH in the microenvironment surrounding an enzyme may be 1 to 2 units lower than the bulk solution (Dick and Tabatabai, 1987; Rao et al., 2000), thus providing a more positively charged enzyme than expected from its pI. This would allow a greater attraction to negatively charged mineral surfaces.
In the case of *P. lycii* phytase the pH of the assay was well above its pI (pH 3.6). Thus, negative charges on the enzyme would lead to an electrostatic interaction with the positively charged goethite and haematite, which should stabilise the enzyme-mineral complex. Conversely, in the case of phyllosilicates the negative charges present on both enzyme and mineral surface should develop repulsive forces and consequently favour its partitioning toward the solution phase. Thus, other mechanisms again appear to be responsible for the retention of the *P. lycii* phytase by the solid phase of the minerals studied. Also on the soil clays, *P. lycii* activity was predominantly found in the solid phase.
The immobilization of the two enzymes on the studied adsorbents had a significant effect on their activities. Adsorption by the positively charged goethite and haematite maintained phytase activity over 1 h but had a significant inhibitory effect over time with enzyme activity more than halved after 24 hours. Attractive forces between the positive charges of oxides and the negative charges of protein may hinder substrate binding or denature the enzyme (Dick and Tabatabai, 1987; Nannipieri et al., 1996; George et al., 2007). This is consistent with the observations for *P. lycii* phytase, which had a higher negative charge than *A. niger* phytase and generally showed greater loss of activity upon sorption on goethite and haematite.
In the presence of kaolinite and montmorillonite, enzyme activity was significantly reduced already after one hour of interaction. This result is in agreement with a number of studies showing that phyllosilicates have a strong inhibitory effect and that the loss of activity is almost entirely due to an inactivation of enzyme by adsorption (Dick and Tabatabai, 1987; Naidja et al., 2000; Rao et al., 1996; 2000). Moreover, on montmorillonite, because of its swelling properties, the enzymes could intercalate in the interlayer spaces, thus restricting substrate to reach the active site (Naidja et al., 2000).

Interaction of the phytases with the soil clay fractions generally resulted in less inhibition over time than was observed with the pure minerals and occurred irrespective of any differences in the pI of the two enzymes. Both clays were characterised by a high content of kaolinite and crystalline iron oxides (mainly haematite) and exhibited surface areas comparable or even higher than those of pure haematite and goethite (Giaveno et al., 2008). A reduction of the kaolinite effect on enzyme activity cannot be attributed to the presence of crystalline iron oxides (Violante and Gianfreda, 2000), as they seem to form microaggregates among themselves rather than as coatings on kaolinite (Shang and Tiessen, 1998; Giaveno et al., 2008). Thus mineralogical composition of the two clays alone was not sufficient to explain the lesser inhibitory effect that was observed. Organic compounds present in the two clays (organic C was 15.0 and 6.01 g kg^{-1}, for clay A and B, respectively) may compete for the same sorption sites of enzymes and, along with the association among the different clay components, may limit the strength of enzyme interaction and thus exert a lesser inhibitory effect (Gianfreda et al., 1993; 1995; Rao and Gianfreda, 2000). This may be further corroborated by the observation that clay A, which was characterised by a higher content of organic C and a larger external surface (Table 1), exhibited greater retention of enzyme activity on the solid phase and its persistence over time as compared to clay B. The two clays showed a behaviour similar to that observed in soil where a lower inhibition of enzyme activity was found in the soils richer in organic matter (George et al., 2005a). The importance of organic matter is related to its heterogeneity in charge and greater presence of macropores where enzymes may physically lodge and small molecules, such as substrate and reaction products, may diffuse easily (Naidja et al., 2000; Rao et al., 2000).

4.2 Interaction of phytases with InsP_6-mineral complexes

The behaviour of the two phytases in the presence of minerals saturated with *myo*-inositol hexakisphosphate was strongly modified as compared to the pure minerals. Enzyme adsorption may be affected by anion adsorbates directly through competition for surface sites and indirectly through effects of anion adsorption on surface charge (Dynes and Huang, 1997; Huang et al., 2003). Goethite showed a high retention of InsP_6 at both pH 5.5 and pH 4.5. Complete saturation with InsP_6 would result in an increase of the negative surface charge, that is derived from the phosphate groups not involved in the binding (Celi et al., 1999). Thus, repulsive forces between the negatively charged surface and the enzyme may cause the *A. niger* phytase to remain in solution. Competition between phosphate and a number of enzymes has similarly been observed on different minerals (Naidja et al., 1995; Huang et al., 2003).

When *A. niger* phytase was added to the minerals that did not reach the complete saturation of InsP_6 at pH 5.5, the enzyme was adsorbed on the solid phase. Haematite, which retained at pH 5.5 half of the amount of InsP_6 adsorbed at pH 4.5 (Giaveno et al., 2008), would be expected to have a large part of its surface free for the interaction with the enzyme. Furthermore, dispersion caused by InsP_6 retention (Celi and Barberis, 2005) may also modify sorption surfaces and favour enzyme adsorption (Violante and Gianfreda, 2000). As was observed with the non-saturated minerals, the adsorption of *A. niger* phytase on haematite caused a loss of activity over time and the inhibitory effect was stronger than that observed for goethite.

In the case of kaolinite and montmorillonite, *A. niger* phytase activity was also retained on the solid phase, probably related to the low amount of substrate adsorbed on their surfaces (Celi et al., 1999).

The higher retention of InsP_6 by clay A, compared to clay B, due to its larger external surface area (Giaveno et al., 2008), also appeared to limit the sorption of enzyme with a larger proportion remaining in solution. This was likely to be enhanced also by the higher presence of organic matter in clay A. It should also be considered that InsP_6 might compete and displace organic material from the surface (Celi et al., 2003). This provides a complex competitive interaction among endogenous organic matter, phytases and inositol phosphates for the reactive surfaces. Although InsP_6 exerts a strong competitive effect, the heterogeneous nature of soil clays and the presence of organic matter may explain why phytases were observed to remain more active in soils than with pure minerals.

In the case of *P. lycii* phytase, it was observed that the enzyme (with exception of its interaction on kaolinite over 24 h) remained almost entirely in solution after both 1 and 24 h. Thus, the driving force determining the behaviour of the enzyme seemed to be its pI rather than the InsP_6 saturation. These results are consistent with the observations by George et al. (2007) for the behaviour of this enzyme in a range of soils. Interestingly in the present experiments the partitioning of the *P.lycii* phytase to the solution phase also retained higher levels of enzyme activity over time, which is consistent with the behaviour of this enzyme in sterilized soils where opportunity for microbial degradation was limited (George et al. 2007).

4.3 Release of orthophosphate from InsP_6-saturated minerals

The capacity of phytases to release orthophosphate from InsP_6 adsorbed to minerals was generally limited and affected by different processes occurring in the enzyme-InsP_6-mineral suspensions.

For InsP_6 adsorbed to either of the iron oxides (goethite or haematite) or montmorillonite there was no significant difference between the amount of P derived from InsP_6 desorption over 24 h and that measured after interaction with either of the two phytases. As both enzymes remained active over 24 hours of interaction, this suggests that once adsorbed to these minerals the substrate was not accessible for enzyme hydrolysis and that any release of orthophosphate derived from desorbed substrate only.

In the case of kaolinite the amount of orthophosphate detected after interaction with *P. lycii* phytase was also consistent with the amount of InsP_6 desorbed. By contrast with *A. niger* phytase, the amount of orthophosphate present after 24 h was significantly greater in the presence of enzyme compared to desorption alone (~11.8% of sorbed InsP_6 after correction for desorption), suggesting either a direct hydrolysis of InsP_6 substrate adsorbed on the surface or through further desorption.

For the two soil clays there was also a significantly greater amount of orthophosphate detected in the solutions after interaction with *A. niger* phytase or, for clay B, with *P. lycii* phytase compared to desorption alone. As this additional orthophosphate did not derive from endogenous P present in the soil clays, it is evident that the enzymes were able to hydrolyse a fraction of the adsorbed InsP_6 (2.0% to 5.9%). The heterogeneous surface of the clays, along with the presence of haematite and kaolinite, may favour InsP_6 desorption or its availabity to enzymes. Organic matter may also hinder a perfect arrangement of InsP_6 on the surfaces (Rao et al., 2000) further allowing the enzyme to directly hydrolyse InsP_6. This is supported by the observations of Tang et al. (2006) who showed that different organic acids could enhance the phytase-mediated hydrolysis of inositol phosphates when adsorbed to Al precipitates, presumably by favouring a release of free substrate. Moreover, the lack of P-fixing minerals such as poorly crystalline Fe and Al oxides, which are invoked to account for the relatively low retention of InsP_6 in oxisol clays (Giaveno et al., 2008), may also favour a larger availability of the substrate for enzymatic hydrolysis (Tang et al., 2006), although further work to establish the basis of such interaction is required.

4.4. Conclusions

The release of orthophosphate from inositol phosphates was governed by the activity of phytases and substrate availability, with both being influenced by interaction with soil mineral components. In this work, fungal phytases adsorbed rapidly to the surface of minerals commonly found in soil and, once adsorbed, showed a significant loss of their activity over time. However, this interaction was dependent on the type of mineral, with phyllosilicates generally being more inhibitory than iron oxides. Moreover, the interaction of phytases with the mineral surfaces was further influenced by the presence of adsorbed inositol phosphates which on one hand limited enzyme sorption and then maintained their catalytic activity over time but, on the other hand, made the substrate largely unavailable to enzyme. This depended on the occurrence and mutual influence of different processes such as adsorption, desorption and competition for the reactive surfaces, involving both substrate and enzyme. With iron oxides, such as goethite, which is characterized by high inositol

phosphate retention, there was no hydrolysis of orthophosphate from adsorbed substrate, although there was persistence of phytase activity. Conversely, kaolinite showed a lower capacity for adsorption of inositol phosphate and a more marked effect on the loss of phytase activity, but an appreciable amount of the adsorbed substrate appeared to be hydrolysed by the enzymes. Therefore, the strength and extent of inositol phosphate binding to the mineral, rather than the persistence of phytase activity, seem to control the release of orthophosphate, further reinforcing the view that retention of inositol phosphates by soil minerals may hamper their degradation. This suggests that the release of orthophosphate from inositol phosphates in soil is largely controlled by soil composition. Besides that, our findings with oxisol clays show that the process is further influenced by interaction of the minerals present in the clay fraction and by complex competitive effects for the reactive surfaces among inositol phosphates, phytases, and the presence of endogenous organic matter. Greater desorption of inositol phosphate and enzyme hydrolysis obtained with haematite and kaolinite, as well as with the two oxisol clays, might account for the low accumulation of these compounds observed in some highly weathered soils.

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References

Celi, L., Barberis, E., 2005. Abiotic stabilization of organic phosphorus in the environment. In: Turner, B.L., Frossard, E., Baldwin, D.S. (Eds.), Organic phosphorus in the environment. CAB International, Wallingford, UK, pp. 113-132.

Celi, L., Barberis, E., 2007. Abiotic reactions of inositol phosphates in soil. In: Turner, B.L., Richardson, A.E., Mullaney, E.J. (Eds.), Inositol phosphates, linking agriculture and the environment. CAB International, Wallingford, UK, pp. 207-220.

Celi, L., De Luca, G., Barberis, E., 2003. Effects of interaction of organic and inorganic P with ferrihydrite and kaolinite-iron oxide systems on iron release. Soil Science 168, 479-488.

Celi, L., Presta, M., Ajmone-Marsan, F., Barberis, E., 2001. Effects of pH and electrolyte on inositol hexaphosphate interaction with goethite. Soil Science Society of America Journal 65, 753-760.

Celi, L., Lamacchia, S., Ajmone-Marsan, F., Barberis, E., 1999. Interaction of inositol hexaphosphate on clays: adsorption and charging phenomena. Soil Science 164, 574-585.

Dick, W.A., Tabatabai, M.A., 1987. Kinetics and activities of phosphatase-clay complexes. Soil Science 143(1), 5-15.

Dynes, J.J., Huang, P.M., 1997. Influence of organic acids on selenite sorption by poorly ordered aluminum hydroxides. Soil Science Society of America Journal 61, 772-783.

EMBRAPA, 1997. Manual de mètodos de ànalise do solo, Centro Nacional de Pesquisa de Solos, Rio de Janeiro, Brazil.

George, T.S., Richardson, A.E., Simpson, R.J., 2005. Behaviour of plant-derived extracellular phytase upon addition to soil. Soil Biology and Biochemistry 37, 977-988.

George, T.S., Simpson, R.J., Gregory, P.J., Richardson, A.E., 2007. Differential interaction of Aspergillus niger and Peniophora lycii phytases with soil particles affects the hydrolysis of inositol phosphates. Soil Biology and Biochemistry 39, 793-803.

Gianfreda, L., Rao, M.A., Violante, A., 1993. Interactions of invertase with tannic acid, hydroxy-aluminium (OH-Al) species or montmorillonite. Soil Biology and Biochemistry 25, 671-677.

Gianfreda, L., Sannino, F., Violante A., 1995. Pesticide effects on the activity of free, immobilized and soil invertase. Soil Biology and Biochemistry 27, 1201-1208.

Giaveno, C., Celi, L., Cessa, R.M.A., Prati, M., Bonifacio, E., Barberis, E., 2008. Interaction of organic phosphorus with clays extracted from oxisols. Soil Science 173(10), 694-706.

Huang, Q., Zhao, Z., Chen, W., 2003. Effects of several low-molecular weight organic acids and phosphate on the adsorption of acid phosphatase by soil colloids and minerals. Chemosphere 52, 571-579.

Irving, G.C.J., McLaughlin, M.J., 1990. A rapid and simple field-test for phosphorus in Olsen and Bray No 1 extracts of soil. Communications in Soil Science and Plant Analysis 21, 2245-2255.

Lung, S.-C., Lim B.L., 2006. Assimilation of phytate-phosphorus by the extracellular phytase activity of tobacco (*Nicotiana tabacum*) is affected by the availability of soluble phytate. Plant and Soil 279: 187-199.

Magid, J., Tiessen, H., Condron, L.M., 1996. Dynamics of organic phosphorus in soils under natural and agricultural ecosystems. In: Piccolo, A. (Ed.), Humic substances in terrestrial ecosystems. Elsevier Science, Amsterdam, pp. 429-466.

Martin, M., Celi, L., Barberis, E., 1999. Determination of low concentration of organic phosphorus in soil solution. Commun. Soil Sci. Plant Anal. 30, 1909-1917.

McDowell, R.W., Cade-Menun, B., Stewart, I., 2007. Organic phosphorus speciation and pedogenesis: analysis by solution ^{31}P nuclear magnetic resonance spectroscopy. European Journal of Soil Science 58, 1348-1357.

Naidja, A., Huang, P.M., Bollag, J.M., 2000. Enzyme-clay interactions and their impact on transformations of natural and anthropogenic organic compounds in soil. Journal of Environmental Quality 29, 677-691.

Naidja, A., Violante, A., Huang, P.M., 1995. Adsorption of tyrosinase onto montmorillonite as influenced by hydroxy-aluminum coatings. Clays and Clay Minerals 43, 647-655.

Nannipieri, P., Sequi, P., Fusi, P., 1996. Humus and enzyme activity. In: Piccolo, A. (Ed.), Humic Substances in Terrestrial Ecosystems. Elsevier, Amsterdam, pp. 293-328.

Ognalaga, M., Frossard, E., Thomas, F., 1994. Glucose-1-phosphate and myo-inositol hexaphosphate adsorption mechanisms on goethite. Soil Science Society of America Journal 58, 332-337.

Quiquampoix, H., Mousain, D., 2005. Enzymatic hydrolysis of organic phosphorus. In: Turner, B. L., Frossard, E., Baldwin D. S. (Eds.), Organic phosphorus in the environment. CAB International, Wallingford, UK, pp. 89-112.

Quiquampoix, H., Staunton, S., Baron, M.-H., Ratcliffe, R.G., 1993. Interpretation of the pH dependence of protein adsorption on clay mineral surfaces and its relevance to the understanding of extracellular enzyme activity in soil. Colloid Surface A 75, 85-93.

Rao, M.A., Gianfreda, L., 2000. Properties of acid phosphatase-tannic acid complexes formed in the presence of Fe and Mn. Soil Biology and Biochemistry 32, 1921-1926.

Rao, M.A., Violante, A., Gianfreda. L., 2000. Interaction of acid phosphatase with clays, organic molecules and organo-mineral complexes: kinetics and stability. Soil Biology and Biochemistry 32, 1007-1014.

Rao, M.A., Gianfreda, L., Palmiero, F., Violante, A., 1996. Interaction of acid phosphatase with clays, organic molecules and organo-mineral complexes. Soil Science 161, 751-760.

Richardson, A.E., Hadobas, P.A., Hayes, J.E., 2000. Acid phosphomonoesterase and phytase activities of wheat (Triticum aestivum L.) roots and utilization of organic phosphorus substrates by seedlings grown in sterile culture. Plant, Cell and Environment 23, 397-405.

Schwertmann, U., Cornell, R.M., 1991. Iron oxides in the laboratory. Preparation and characterization. VCH Verlagsgesellschaft mbH, D-6940 Weinheim.

Shang, C., Tiessen, H., 1998. Organic matter stabilization in two semiarid tropical soils: size, density and magnetic separations. Soil Sci. Soc. Am. J. 62(5), 1247-1257.

Shang, C., Huang, P.M., Stewart, J.W.B., 1990. Kinetics of adsorption of organic and inorganic phosphates by short-range ordered precipitate of aluminium. Canadian Journal of Soil Science 70, 461-470.

Staunton, S., Quiquampoix, H., 1994. Adsorption and conformation of Bovine Serum Albumin on montmorillonite: modification of the balance between hydrophobic and electrostatic interactions by protein methylation and pH variation. Journal of Colloid and Interface Science 166, 89-94.

Tang, J., Leung, A., Leung, C., Lim, B.L., 2006. Hydrolysis of precipitated phytate by three distinct families of phytases. Soil Biology and Biochemistry 38, 1316-1324.

Turner, B.L., 2007. Inositol phosphates in soil: amounts, forms and significance of the phosphorylated inositol stereoisomers. In: Turner, B.L., Richardson, A.E., Mullaney, E.J. (Eds.), Inositol phosphates, linking agriculture and the environment. CAB International, Wallingford, UK, pp. 186-206.

Turner, B.L., Condron, L.M., Richardson, S.J., Peltzer, D.A., Allison, V.J., 2007. Soil organic phosphorus transformations during pedogenesis. Ecosystems 10, 1166-1181.

Turner, B.L., Paphazy, M.J., Haygarth, P.M., McKelvie, I.D., 2002. Inositol phosphates in the environment. Phil. Trans. R. Soc. Lond. B 357, 449-469.

Ullah, A.H.J., Sethumadhavan, K., 2003. PhyA gene products for Aspergillus ficuum and Peniophora lycii produces dissimilar phytases. Biochemical and Biophysical Research Communications 303, 463-468.

Vats, P., Banerjee, U.C., 2004. Production studies and catalytic properties of phytases (myo-inositol hexakisphosphate phosphatases) an overview. Enzyme and Microbial Technology 35, 3-14.

Violante, A., Gianfreda, L., 2000. Role of biomolecules in the formation and reactivity toward nutrients and organics of variable charge minerals and organo mineral complexes in soil environments. In: Bollag, J.-M., Stotsky, G. (Eds.), Soil Biochemistry, Vol. 10. Marcel Dekker, New York, pp. 107-270.

Table 1: Characteristics of pure minerals and oxisol clays: x-ray composition (X-RAY), Fe extractable in oxalate (Fe_O) and in dithionite-citrate-bicarbonate (Fe_{DCB}) ratio, specific surface area (SSA), external surface area (S_{EXT}), and point of zero charge (pzc).

	X-RAY	Fe_O/Fe_{DCB}	*SSA*	S_{EXT}	*pzc*
			($m^2\ g^{-1}$)		pH
Clay A*	haematite, kaolinite, maghemite, gibbsite	0.023	66.0	54.5	3.9
Clay B*	haematite, kaolinite, maghemite	0.011	67.0	44.6	5.3
Goethite[#]			40.0	n.d.	8.0
Haematite*			38.4	24.2	7.0
Kaolinite[§]			18.0	18.0	5.0
Montmorillonite			509	n.d.	n.d.

* data from Giaveno et al. (2008)

[#] data from Celi et al. (1999)

[§] data from Celi et al. (2003)

n.d. = not determined

Table 2. Maximum adsorption capacity (X_{max}) at pH 4.5 and pH 5.5, and desorption of InsP_6 after 24 h (expressed in mg P l^{-1} and as a percentage of the total amount of P adsorbed) from the different minerals (goethite, haematite, kaolinite, montmorillonite, clays A and B) loaded with InsP_6 at pH 5.5.

	adsorption			*desorption*	
	X_{max} *pH 4.5*	X_{max} *pH 5.5*	$X_{max\ pH5.5}$ / $X_{max\ pH4.5}$	InsP_6 *desorbed at pH 5.5*	*Desorbed P* / $X_{max\ pH5.5}$
	(µmol P m^{-2})		%	(mg P l^{-1})	%
Goethite	3.80[#]	3.80	100	0.27	1.9
Haematite	1.98*	1.11	56	0.32	8.3
Kaolinite	1.60[§]	0.63	39	0.13	6.0
Montmorillonite	0.08	0.06	75	0.02	0.8
Clay A	4.02*	2.78	69	0.22	1.3
Clay B	2.94*	2.15	73	0.05	0.4

[#] data from Celi et al. (2001)

[§] data from Celi et al. (2003)

* data from Giaveno et al. (2008)

Table 3. Amount of P released from different minerals (goethite, haematite, kaolinite, montmorillonite, clays A and B) loaded with InsP_6 at pH 5.5 and incubated for 24 h with *A. niger* and *P. lycii* phytases. The P release was expressed as the amount of orthophosphate (P) detected in solution after enzymatic reaction and as the percentage of the amount of P adsorbed as InsP_6 (from Table 2) after subtraction of the quantity of P desorbed in the absence of phytases.

	P release with A. niger phytase		*P release with P. lycii phytase*	
	(mg P l^{-1})	% of adsorbed InsP_6[§]	(mg P l^{-1})	% of adsorbed InsP_6[§]
Goethite	0.05 ± 0.00	ns	< 0.01[#]	ns
Haematite	0.31 ± 0.03	ns	0.31 ± 0.01	ns
Kaolinite	0.38 ± 0.00	11.8	0.09 ± 0.02	ns
Montmorillonite	< 0.01	ns	0.03 ± 0.02	ns
Clay A	0.56 ± 0.01	2.0	0.33 ± 0.02	ns
Clay B	0.84 ± 0.05	5.9	0.69 ± 0.01	4.8

[#] sensitivity of detection was determined to be 0.01 mg P l^{-1}

[§] only significant values ($p<0.05$) are shown and ns indicates no significant hydrolysis of the adsorbed InsP_6.

Figure 1. Activity (% of initial activity added) of *A. niger* phytase in the solution (open bars) and in the solid phase (filled bars) of suspensions of goethite (Gt), haematite (Hm), kaolinite (KGa2), montmorillonite (Mt) and the oxisol clays A and B, after 1 and 24 h of incubation. Data are the mean of three or four replicates, with error bars representing one standard error either side of the mean.

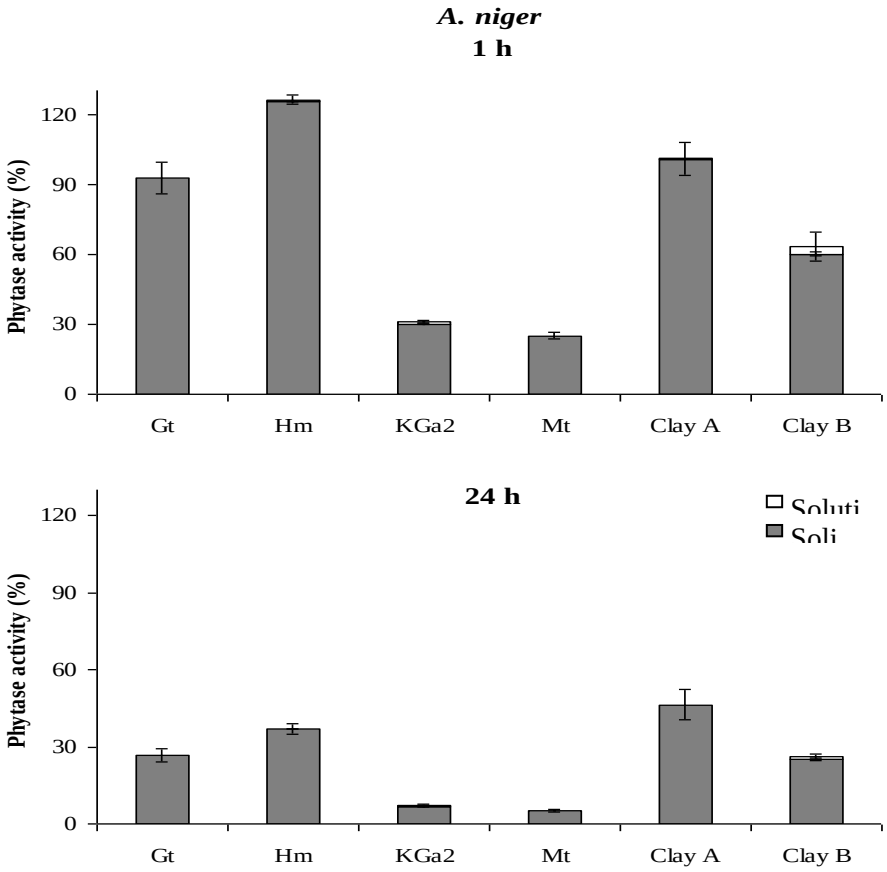

Figure 2. Activity (% of initial added activity) of *P. lycii* phytase in the solution (open bars) and in the solid phase (filled bars) of suspensions of goethite (Gt), haematite (Hm), kaolinite (KGa2), montmorillonite (Mt) and the oxisol clays A and B, after 1 and 24 h of incubation. Data are the mean of three or four replicates, with error bars representing one standard error either side of the mean.

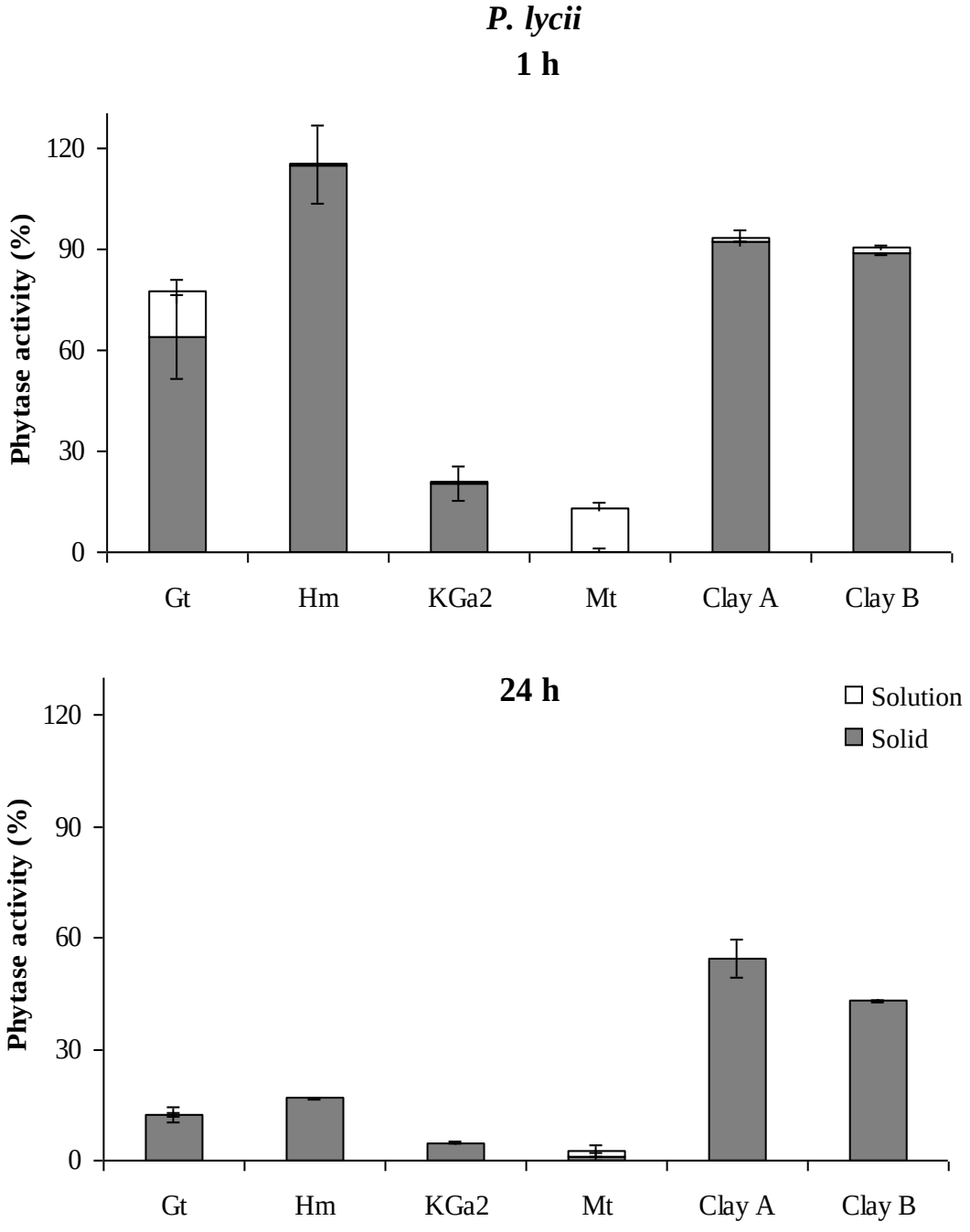

Figure 3: Activity (% of initial added activity) of *A. niger* phytase in the solution (open bars) and in the solid phase (filled bars) of suspensions of InsP_6–saturated goethite (Gt), haematite (Hm), kaolinite (KGa2), montmorillonite (Mt) and oxisol clays A and B after 1 and 24 h of incubation. Data are the mean of three or four replicates, with error bars representing one standard error either side of the mean.

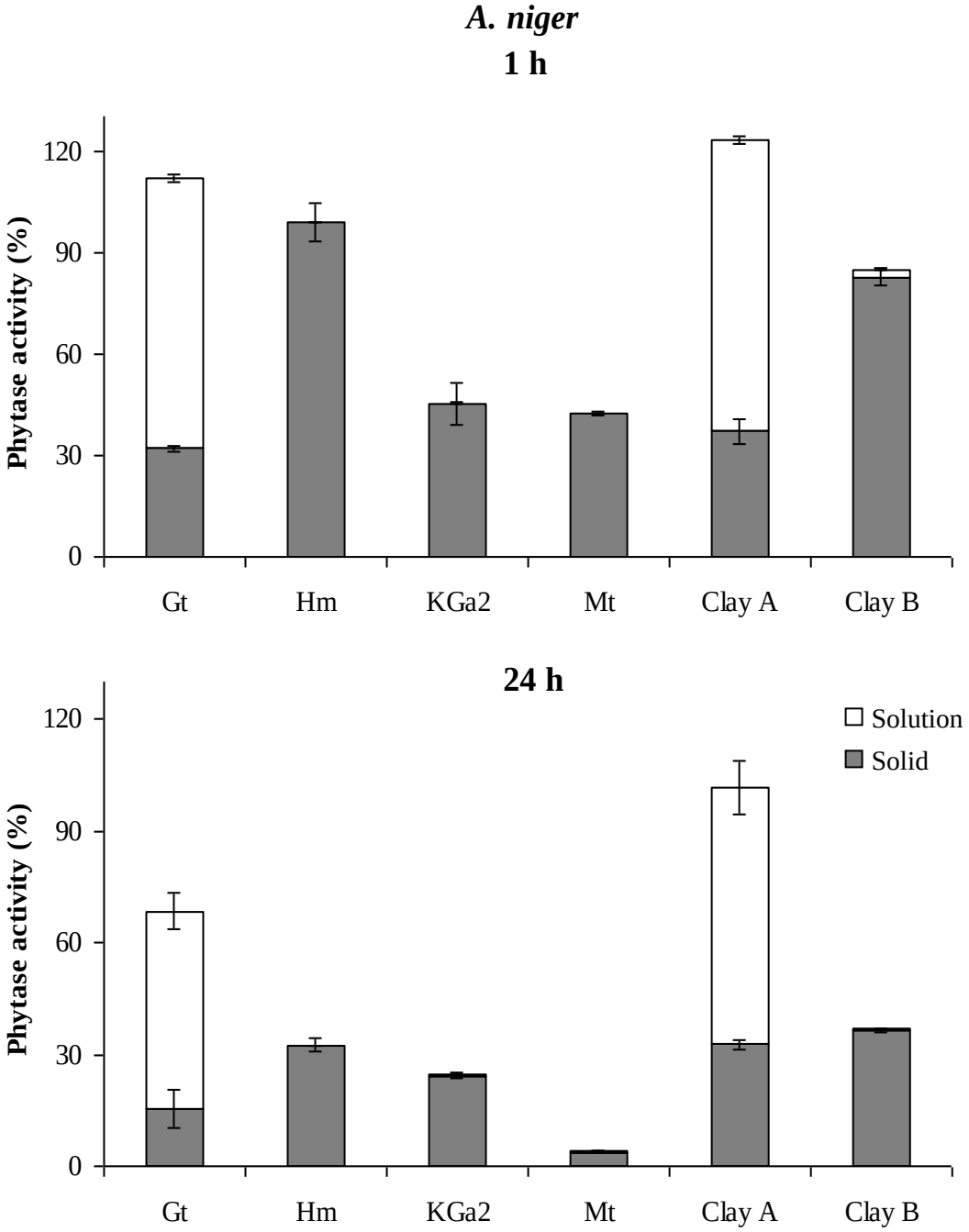

Figure 4: Activity (% of initial added activity) of *P. lycii* phytase in the solution (open bars) and in the solid phase (filled bars) of suspensions of InsP_6–saturated goethite (Gt), haematite (Hm), kaolinite (KGa2), montmorillonite (Mt) and oxisol clay A and B after 1 and 24 h of incubation. Data are the mean of three or four replicates, with error bars representing one standard error either side of the mean