Distribution of exogenous phytase activity in soil solid–liquid phases and their effect on soil organic P hydrolysis

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Abstract

The bioavailability and stability of organic phosphorus (P) in the soil may be affected by exogenous phytase (EPase) activity and distribution, but remain poorly understood. The distribution of EPase activity and hydrolysis ability of EPase on organic P in soil solid-liquid phases was investigated. The EPase addition to soil suspension (1:20, w/v) from three soil types (red soil, brown soil, and cinnamon soil) under three treatments (untreated soil, removing clay from soil, and removing organic matter from soil) with different characters in the solution and solid phases was assayed. The results showed that the disappearance pattern of EPase activity from solution was similar for all soils, whereas the enzyme activity on the solid phase was dependent on soil types and treatments with the greatest in red soil and untreated soil. When EPase was added to soils, the adsorptive ratio of organic matter and clay was 10 to 25% and 3 to 7%, respectively, with sorption capacity of organic matter being significantly \( p < 0.05 \) stronger than that of clay. Additionally, soil dehydrogenase activity, which is the indicator of overall soil microbial activities, increased after EPase addition and the two enzymes showed significant negative relation in the soil suspension and solution. At the same time, the organic P decreased significantly \( p < 0.05 \) after the addition of EPase in the soil solid, which had a varied rate under ~40% after incubating 192 h, whereas organic P in the solution phase increased significantly \( p < 0.05 \). This study demonstrated that organic matter had a strong protective and adsorptive effect on EPase effectiveness and microbes might directly affect EPase longevity and decay. This finding suggests that EPase activity in the solid phase played a more important role in organic P hydrolysis.

Key words: exogenous phytase / soil enzyme / clay / organic matter / organic P

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1 Introduction

Phytate was the predominant form of organic phosphorus (P) in most soils, accounting for 50% or more of soil total organic P content (Turner et al., 2002, 2003). Phytase could be available for organism uptake only under the hydrolysis of phytate (Hayes et al., 2000a; Büinemann, 2008). The phytase deriving from exogenous microbes secreting in soil was determined to be 60% more efficient in the hydrolysis of phytate than soil phytase (pH 5.4 at 30°C for 24 h) (Tarafdar et al., 2002). EPase had both free (i.e., in solution) and adsorptive (i.e., in solid form) types in soil environment (George et al., 2005). The free phytase had high activity and affinity toward a given substrate, but was easily deactivated by soil protease or microorganism which could not be acted upon for an extended period. The adsorptive phytase could be partly reserved by adsorption of soil solid constituents, but its activity was relatively limited (Quiquampoix and Mousain et al., 2005). Consequently, the distribution of phytase activity in the solution and solid phase of soil would directly affect the stability and effectiveness of phytase as well as soil phytate dynamics and P pools (George et al., 2005; Nannipieri et al., 2011). Therefore, understanding the distribution behavior of phytase activity in these phases and complex soils was of strategic importance for improving organism P nutrition (Menezes-Blackburn et al., 2011).

Phytase activity could be affected by many soil properties such as soil pH, clay content, and organic matter. For example, George et al. (2005) reported that soil pH affected the solid–liquid distribution of phytase, while at the same time, EPase could be adsorbed by the solid soil phase quickly when soil pH was close to the phytase isoelectric point (pI). Moreover, Rao et al. (2000) and Giaveno et al. (2010) observed that minerals exhibited a high affinity and strong adsorption to phytase. Phytase activity decreased quickly after adding a mineral suspension, demonstrating the significant inhibitory effect of minerals on phytase (Tang et al., 2006; Giaveno et al., 2010). Indeed, soil organic matter could affect the stability of phytase activity (George et al., 2005; Tang et al., 2006; Giaveno et al., 2010), but soil mineral (clay) and organic matter, which played a major role in phytase activity adsorption, is unclear. In addition, EPase might be degraded in soil by proteases (Sevinc and Demirkan, 2011), but protease was an extracellular enzyme which could be fixed and protected by the external environment so that experimental...
The hydrolysis ability of EPase on organic P would directly affect the biological effectiveness of organic P. However, little information regarding the relationship between soil organic P and EPase activities in the soil liquid–solid phases is available. This study chose a comparison of three typical soils (the red soil, brown soil, and cinnamon soil) varying in soil pH, organic matter, and P content followed by the removal of clay and organic matter from soils. The distribution of EPase from Aspergillus niger-derived was investigated in the soil solution and solid phases. The objective of the research was to (1) explore the sorptive and protective effect of soil clay and organic matter on the distribution of EPase activity in the soil solid–liquid phases and (2) detect the effects of EPase activity in the soil solid–liquid on soil organic P hydrolysis.

2 Material and methods

2.1 Sampling site and soil collection

In late August 2012, soil samples were collected from three places: (I) the Yingtan National Agroecosystem Field Experiment Station (YT) (28°15′N, 116°55′E, elevation 40 m asl), Jiangxi province, where the annual precipitation and mean annual temperature in this area were 1795 mm and 17.6°C, respectively, and the soil type was red soil, or Ferralsol; (II) the National Field Research Station of Shenyang Agroecosystems (SY) (41°31′N, 123°24′E, elevation 41 m asl), Liaoning Province, where the annual precipitation and mean annual temperature in this area were 675 mm and 7.5°C, respectively, and the soil type was brown soil, or Alfisol; (III) the Cinnamon Soil Long–Term Trial Site of Chaoyang Agroecosystems (CY) (41°41′N, 120°33′E, elevation 173 m asl), Liaoning province, where the annual precipitation and mean annual temperature in the area were 500 mm and 10°C, respectively, and the soil type was cinnamon soil, or Semi-Luvisol. The land-use was farmland and the crop was maize among all three sites.

Soil samples (0–20 cm depth) were taken from each site over an area of 1 ha by collecting 50 to 60 subsamples to mix into a composite sample. After mixing, soils were sieved through a 2-mm sieve to remove stones and coarse roots, and then were air-dried and stored at room temperature for chemical analysis.

2.2 Soil preparation for microcosm construction

To make the effect of clay and organic matter on the EPase activity clear, the soils were treated in three different ways for the next EPase addition experiment: untreated soil, taking clay off soil (OFF-CLAY) which was dissolved in deionized water and separated clay by ultrasonication for 15 min (361 W) and centrifugation for 5 min (682 RPM), 5 min (476 RPM), and 30 min (400 RPM), respectively (Gama-Rodrigues et al., 2010), and taking organic matter off soil (OFF-SOM) which was saturated with 6% H₂O₂ for 30 days to remove organic matter (Pérez-Novo et al., 2008).

2.3 EPase addition experiment

The EPase (3-phytase) used in this study secreted from Aspergillus niger with an enzymatic activity of 5,000 U g⁻¹ (Beijing Winovazyme), which was commercialized as feed additives. The exogenous phytase additive was dissolved by deionized water and stirred for 30 minutes by vortex mixer at 0°C. The phytase solution was centrifuged at 10,000 × g for 10 min, and the supernatant was used in this experiment.

The experimental procedure of EPase addition was modified from the methods described in George et al. (2005). Briefly, 1-g samples of the three different treated soils were weighed into 50-ml screw-capped polyethylene tubes with three replicates. 20 mL of phytase solution (16.67 nKat g⁻¹ soil) was added and the tubes were shaken horizontally at room temperature (~ 22°C) on a flat bed shaker (75 oscillations min⁻¹). Two sub-samples (500 µL) were taken for measurement of phytase activity at 0, 1, 3, 5, 10, 15, and 30 min; 1, 2, 4, 8, 16, 24, 32, 48, 72, 96, 120, 144, 168, and 192 h. One sub-sample was directly used to determine the suspension EPase activity which included solution and solid enzyme activity, another one was centrifuged at 13,000 × g for 4 min, and the supernatant taken for measurement of EPase activity in the solution phase. Furthermore, the EPase activity in the solid phase was derived as the difference between the total soil suspension and soil solution EPase activity.

2.4 Soil properties analysis

Soil pH was determined at a 1:5 soil:deionized water ratio using a glass electrode (Shen et al., 2013). The measurement for organic matter content, cation exchange capacity (CEC), and soil clay content followed the methods, as described in Page et al. (1982). Total P was firstly combusted by a muffle furnace at 550°C for 1 h (Walker and Adams, 1958), then extracting P with 1 M H₂SO₄, and finally determining P in the extraction at 880 nm using the molybdate procedure. Available P was obtained by the molybdenum colorimetric method after extraction by 0.5 M sodium bicarbonate (NaHCO₃) (Ryan et al., 2007). The basic information for sampling site and soil properties are shown in Tab. 1.
2.5 Analysis of phytase, dehydrogenase and protease activities, and soil organic P during EPase addition experiments

Phytase activities of soil solutions and suspensions were measured at a sample to buffer ratio of 1:1. Assays were performed against an InsP6 substrate at 37°C for 60 min at a final concentration of 2 mM and buffered to pH 5.5 with 15 mM 2-morpholinoethanesulfonic acid (MES). InsP6 stock solution (20 mM) was firstly acidified to pH 5.5 with 10 M HCl and the filter was sterilized (0.22 mm) prior to use (George et al., 2005; Giaveno et al., 2010). Reactions were stopped with an equal volume of 10% trichloroacetic acid (TCA) and samples were centrifuged at 3,800×g for 5 min prior to determination of P concentration in the supernatant using malachite green (Irving and McLaughlin, 1990). Phytase activity (nKat g⁻¹ soil) was calculated as the P released during the 60 min assay.

The phytase activity (PA) was calculated as follows:

\[ PA \, (\text{nKat g}^{-1} \, \text{soil}) = \frac{P \times D \times V \times 16.67}{T \times 31}, \]  

where \( P \) is the P concentration (mg L⁻¹), \( D \) is the divide ratio, \( V \) is the volume (mL), and \( T \) is the incubation time (60 min).

After adding EPase activity, soil organic P and dehydrogenase activity were detected. Organic P was determined by the same method as indicated in section 2.2. The dehydrogenase activity of soil suspension was determined by the 2,3,5-Triphenyltetrazolium chloride colorimetric method (TTC) after extraction by methanol (AR) at the 485 nm colorimetric determination wavelength (Tabatabai, 1994). The soil protease activity followed the methods, as described in Watanabe and Hayano (1995).

3 Results

3.1 The distribution of EPase added to soil

EPase activity declined significantly (LSD, \( p < 0.05 \)) in the soil solution phase with incubation time (Fig. 1, I), decreasing in the form of a logarithmic curve (Tab. 2). Activity increased on the solid phase within 16 h in the form of logarithmic curve. Increment was not obvious in cinnamon soil (Tab. 2), though after 16 h, its activity declined (Fig. 1, II). Furthermore, the greatest solid activity was 93% in red soil, while the lowest was 25% in cinnamon soil in the soil solid phase (Fig. 2).

The fastest decreasing trend of EPase activity in the solution phase was observed in untreated soil followed by OFF-CLAY and was slowest in OFF-SOM (Fig. 1, I). However, the least adsorption was in OFF-SOM followed with intermediate adsorption in OFF-CLAY and was the greatest in untreated soil. Adsorption of OFF-CLAY was the greatest in red soil (the biggest up to 25%), intermediate in brown soil (over 10%) and least in cinnamon soil (below 10%). The adsorption of OFF-SOM was below 7% (Fig. 2).

3.2 The effect of EPase addition on soil organic P

In the soil solution, the varied rate of organic P increased significantly \((p < 0.05)\) which varied from 11% to 120% after adding EPase and incubating 192 h (Tab. 3). The greatest rate (120%) was OFF-CLAY in cinnamon soil and the least rate (11%) was OFF-SOM in red soil. Among the three soils, the greatest rate was cinnamon soil (47%) followed by brown soil.
EPase addition significantly increased solution organic P in different treatments, and the varied rate increased as follows: OFF-SOM < untreated soil < OFF-CLAY. The organic P in solid phase decreased significantly (p < 0.05), which had a varied rate under –40% ranging from –66% to –43% after adding EPase and incubating 192 h (Tab. 3). However, the varied rate was different after adding EPase in soils and treatments. Among the three soils, the greatest varied rate was red soil (–43%), then was brown soil (–44%), and the least was cinnamon soil (–53%). The difference between red soil and brown soil was small, whereas cinnamon soil had a bigger change. During the different treatments, the varied rate was the least in OFF-SOM, intermediate in OFF-CLAY and greatest in untreated soil.

3.3 The relationship among EPase, dehydrogenase and protease

During the incubation period, the dehydrogenase activity increased significantly (LSD, p < 0.05), and the highest value occurred after 96 h with the increment nearly 1.5 activity units, later declined, and finally remained stable after 168 h in all soils (Fig. 3). The dehydrogenase activity of different soils was the highest in cinnamon soil, which was higher by two activity units than red soil and brown soil. Among the different treatments, the highest was in untreated soil, then OFF-CLAY, and the least in OFF-SOM. However, the results showed that the protease activity changed similarly with EPase (Fig. 4).

In terms of the correlation, the EPase activity showed a significant negative correlation with incubation time and dehydrogenase activity in the solid phase, while had a significant negative correlation with protease activity (Tab. 4).

4 Discussion

4.1 The distribution of EPase in soil

This study showed that EPase activity significantly (p < 0.05) decreased in solution irrespective of treatments and soil types, but part of EPase activity (25–93%) returned on the solid phase within 16 h (Fig. 1, II). The results suggested that addition of EPase into soil was adsorbed by soil solid constituents (George et al., 2005). And adsorption by soil solid constituents (clay and organic matter) was an effective way to protect the labile phytase, which had an affinity to substrate in soil solution. This adsorption had a great significance of maintaining the EPase activity and stability, thereby affecting the organic P transformation and supply of P nutrition (George et al., 2007; Giaveno et al., 2010).
stronger adsorption and protective nature of organic matter could be attributed to its greater structurally diversity than clay, with more macropores where enzymes could lodge (Naidja et al., 2000). Additionally, the relatively low retention of enzyme in clay might also be liable to more enzyme hydrolyzed (Tang et al., 2006; Giaveno et al., 2008).

4.2 The effect of EPase addition on soil organic P

The data revealed that organic P significantly ($p < 0.05$) increased in the soil solution after EPase addition (Tab. 3). The increment could be attributed to the following three ways: (1) the dissolution of organic P from the solid phase (Menezes-Blackburn et al., 2014), (2) the immobilization of microorganisms on organic P in the solution phase (Richardson and Simpson, 2011), and (3) EPase activity decreased significantly ($p < 0.05$) in the solution phase which cut down organic P hydrolysis (Menezes-Blackburn et al., 2013).

The decreased and varied content of organic P in solid phases could be due to its small dissolution and large hydrolysis (Menezes-Blackburn et al., 2014). This finding indicated that EPase had a significant effect on improving the mineralization of organic P in the soil solidity, which coincided with the distribution of EPase activity in the solid phase. This was consistent with Menezes-Blackburn et al. (2014) who found that the phytase dose from Escherichia coli and Aspergillus niger was a limiting factor in determining phytase-labile organic P pools in soils. In addition, Hayes et al. (2000b) found that 28% and 40% organic P was hydrolyzed using purified phytase. The least varied rate of soil organic P was
cinnamon soil (−53%), which was with low organic matter; the decreased content of organic P was in the order OFF-SOM > OFF-CLAY > untreated soil. The results demonstrated that the removal of clay and organic matter from soil decreased the adsorption to EPase and strengthened the freedom of EPase with high activity to hydrolyze organic P.

However, EPase could not directly hydrolyze phytate on the saturation but might hydrolyze the desorbed phytate (Giaveno et al., 2010). In this research, the varied rate was under −40%, which suggested that over 40% of organic P was in desorption status. This suggested that a transformable P pool existed in the three soils that could supply labile P for plant. However, adsorption and precipitation (i.e., physical and chemical processes) of EPase were the main mechanisms of phytate fixation in soil and might limit P hydrolysis (Richardson, 2007). Additionally, EPase addition could improve the activity of other phosphatases, such as phosphomonoesterase and phosphodiesterase (Yang et al., 2015), which also enlarged the hydrolysis rate of soil organic P. Nannipieri et al. (2011) also found that other types of phosphatases might be involved in organic P mineralization dynamics and a single type enzyme activity might not be the best approach for P hydrolysis.

At the same time, the hydrolyzed P composition might not be solely due to the mineralization of phytate, which was not the only labile organic P available to the used enzymes (Me-

Table 3: The varied rate of organic P in the soil solution and solid phases after adding EPase to soils.

<table>
<thead>
<tr>
<th>Solution Phase</th>
<th>Solid Phase</th>
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<tbody>
<tr>
<td>Soil</td>
<td>Treatments</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Red Soil</td>
<td>Untreated Soil</td>
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<tr>
<td></td>
<td>OFF-CLAY</td>
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<tr>
<td></td>
<td>OFF-SOM</td>
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<tr>
<td>Brown Soil</td>
<td>Untreated Soil</td>
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<td></td>
<td>OFF-CLAY</td>
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<td></td>
<td>OFF-SOM</td>
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<tr>
<td>Cinnamon Soil</td>
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<td>OFF-CLAY</td>
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<td>OFF-SOM</td>
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</table>

³Values were the content of organic P contained with samples at 0 and 192 h in the soil solution and solid phases.
²Values were the changed content of organic P.
°Values were the changed rate of organic P followed by different lowercase letters within a column indicating differences (p < 0.05) among the three soils and treatments.
However, Quiquampoix (2000) found that the hydrolysis rate of organic P was the greatest by phytase (55–12%), while acid phosphatase was 20–6% and alkaline phosphatase was 28–8%. In addition, dry weight and total P contents of the transgenic phytase gene of Arabidopsis were 3.1- to 4.0-fold and 4.1- to 5.5-fold higher than general Arabidopsis, respectively (Xiao et al., 2005). Therefore, the hydrolysis rate of phytase to organic P was stronger than other phosphatases and the EPase had great potential for improving plant phosphorus acquisition and growth.

4.3 The effect of dehydrogenase on decay of EPase

Dehydrogenase and Epase had a significant negative correlation in the suspension and solution soils but a significant positive correlation in the solid soils, which indicated that microbes mainly existed in the solution soils with the role of...
consumption on EPase. However, microbes were weak in hydrolysis on the solid EPase activity, which might result in saving EPase in a solid status. This could be liable for the solid EPase hydrolyzing phytate when the environment was suitable (Weintraub, 2011). Additionally, an organism that required a functional exogenous enzyme in soil environments was kept in the way of the retention of obstructed activity of enzymes upon adsorption (Quiquampoix, 2000).

Phytase, similar to any other enzyme, is degraded in soil by proteases (Sevinc and Demirkan, 2011). However, the results showed that protease activity decreased significantly \( p < 0.05 \) in three soils and treatments (Fig. 4). This indicated that protease and EPase were in a coupling relationship in which their variation trend was consistent. Meanwhile, dehydrogenase activity increased significantly as dehydrogenase was a type of an endoenzyme and was associated with the microbial respiration (Sebiomo et al., 2013). The amount of microorganism expanded with the dehydrogenase activity increasing (Kussainova et al., 2013). This was an implication for microbial degradation and consumption on EPase activity (George et al., 2007). In addition, microorganisms that could resist EPase would thereby increase their biomass after adding EPase to soils. Furthermore, the P status in soil had a significant effect on microbial community structure, whereas soil microorganisms were explicitly involved in the availability of P to plants (George et al., 2009). Microorganisms affected the efficacy and longevity of EPase, which hydrolyzed soil organic P for plant growth.

5 Conclusion

After adding EPase to three typical farmland soils treated differently, EPase activity significantly decreased in the solution, but part of the EPase activity recovered on the solid phase within 16 h. During the cultivation trial, the organic P of the solid phase was hydrolyzed over 40% in all soils and treatments. The study has shown that adding EPase is a promising strategy for improving P mobilization in soil. The decay reasons of EPase could be attributed to clay and organic matter adsorption and dehydrogenase decomposition. Therefore, the adsorption of soil clay, organic matter, and consumption of microbes determined the stability and biological effectiveness of EPase on organic P. This would be helpful in investigating the distribution, activity and mechanisms of the EPase action.

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