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Differential interaction of *Aspergillus niger* and *Peniophora lycii* phytases with soil particles affects the hydrolysis of inositol phosphates

Timothy S. George[a,b], Richard J. Simpson[a], Peter J. Gregory[b], Alan E. Richardson[a,*]

[a]*CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia*
[b]*Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA Scotland, UK*

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Abstract

The effect of the soil environment on the mobility, stability and catalytic activity of phytase from two sources was compared, as these factors have important implications for the efficacy of enzyme function in soil. Phytase from an ascomycete fungus (*Aspergillus niger*) and a basidiomycete fungus (*Peniophora lycii*) was added to soil suspensions from three contrasting soils and activities in the solution and solid phase were monitored. The two enzymes were compared because the *P. lycii* phytase was known to have greater specific activity and a more acidic isoelectric point (pI) than *A. niger* and therefore predicted to have different adsorption characteristics. When added to soil suspensions buffered at pH 7.5, both phytases remained in solution in all of the soils. In contrast at near natural soil pH (pH 5.5), only the *P. lycii* phytase remained in solution, while the *A. niger* phytase was rapidly adsorbed to the soil solid phase. The extent of this adsorption was reduced, however, in a soil-dependent manner by prior addition of bovine serum albumin (BSA) to the soil suspensions. At the natural pH of the soil, the stability of the *P. lycii* phytase in soil solution was improved under sterile conditions, whereas degradation of the *A. niger* phytase was unaffected. Subsequently, *P. lycii* phytase was shown to be more effective at hydrolysing *myo*-inositol hexakisphosphate added to the soil. Moreover, the *P. lycii* phytase also hydrolysed more organic phosphate that was endogenous to a range of soils. This research indicates that the physicochemical properties of fungal phytases affect their mobility and temporal stability and their capacity to hydrolyse inositol phosphates in soil environments.

Keywords: Alfisol; Organic phosphorus; Oxisol; Phosphatase; Phytate; Rhizosphere; Spodosol

1. Introduction

Phosphorus (P) is a major limiting nutrient for biological systems in the terrestrial environment. Paradoxically, many soils contain a relatively large amount of P which, if totally available, would exceed requirements for growth of both plants and soil microorganisms. However, much of this soil P is in forms that are not directly available for use. Up to 80% occurs in organic forms (Richardson et al., 2005), of which inositol phosphates constitute the largest (~50%) fraction (Anderson, 1980; Turner et al., 2002).

The biological availability of inositol phosphates depends on hydrolysis by phytases (Irving, 1980). Three distinct classes of phytase have been identified based on protein sequence and the order of dephosphorylation of phosphate moieties (*myo*-inositol-hexaphosphate phosphohydrolases; EC 3.1.3.8, EC 3.1.3.26 and EC 3.1.3.72), occurring at the three, six and five phosphate positions, respectively. These phytases sequentially hydrolyse inositol hexakisphosphates to various lower order esters including inositol monoesters. Given the lack of extracellular phytases of plant origin (Hayes et al., 1999) and relative abundance from soil microorganisms (Vats and Banerjee, 2004), it appears that microorganisms are key to the cycling of P from inositol phosphate in soil. Indeed, extracellular phytases from both bacteria and fungi can improve the P nutrition of plants when present in the rhizosphere (Findenegg and Nelemans, 1993; Idriss et al., 2002; Richardson et al., 2001b).

Transgenic plants that express microbial phytase genes have been produced, with the specific aim of improving

*Corresponding author. Tel.: +61 2 6246 5189; fax: +61 2 6246 5000.
E-mail addresses: Tim.George@scri.ac.uk (T.S. George), alan.richardson@csiro.au (A.E. Richardson).

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plant P-nutrition (George et al., 2004, 2005a; Lung et al., 2005; Richardson et al., 2001a; Zimmermann et al., 2003). These transgenic plants have improved ability to acquire P from *myo*-inositol hexakisphosphate when grown in sterile agar, but show limited responses when grown in soil with endogenous substrates or soil to which *myo*-inositol hexakisphosphate was added (George et al., 2004, 2005a). While the availability of inositol phosphates to phytases for mineralisation is likely to be a key factor regulating their utilisation in soil (George et al., 2005c, 2006), the stability and activity of phytase exuded to the extracellular environment may also be important (George et al., 2005b).

Phytase collected from roots of plants that expressed a heterologous *Aspergillus niger phyA* gene has been shown to be rapidly adsorbed to the soil solid phase (George et al., 2005b). Maximal adsorption of the phytase occurred near the isoelectric point (pI) of the protein, which coincided with the pH of the soils (~pH 5.5) used in the experiments. Adsorption of microbial phytases to the soil solid phase may reduce their affinity for inositol phosphates and thus reduce their effective activity (Quiquampoix and Mousain, 2005). However, adsorption may also be necessary for the long-term persistence of phytase in soils (George et al., 2005b; Naidja et al., 2000; Nannipieri et al., 1996).

The physicochemical properties of microbial phytases vary considerably (Vats and Banerjee, 2004), so it is probable that phytases from different sources behave differently in the soil environment. Previously we showed that the partitioning in soil of activity of phytase from *A. niger* between the soil solution and solid phase was related to the pI of the protein (George et al., 2005b). The objective of the research reported here was to demonstrate whether phytases from different fungal sources (i.e., *A. niger* and *Peniophora lycii*), which differ in key physicochemical properties including pI (Ullah and Sethumadhavan, 2003), behave differently in soil environments with respect to interaction with the soil solid phase. Moreover, we also investigate the affect of differences in physicochemical properties of the phytases on temporal stability and ability to hydrolyse inositol phosphates in the soil environment.

2. Materials and methods

2.1. Soil sampling, preparation and characterisation

Topsoil (0–10 cm depth) was collected from three sites, Tilba and Robertson in New South Wales (NSW) and Hall in the Australian Capital Territory (ACT), Australia. The soils were characterised using the USDA system as a spodosol, oxisol and alfisol (alfisol#1 in Fig. 5), respectively. Each soil was air-dried, mixed and passed through a 2-mm sieve to remove coarse material and vegetative matter and stored at room temperature until required. Detailed characterisation of these soils has been reported previously by George et al. (2005b). Briefly, the soils were acidic (pH 5.2–5.7) and differed markedly in their organic matter content (35–163 g kg^{-1}), clay composition and P-sorption characteristics. These contents and characteristics were least in the spodosol, intermediate in the alfisol and greatest in the oxisol. For most experiments, soils were left unamended or, where indicated, were sterilised by treating 1 g lots at 121 °C and 120 kPa for 20 min in an autoclave. In one experiment soils were also pre-treated with bovine serum albumin (BSA) to test for competition between this model protein and phytase for adsorption sites, where 1 g of soil was shaken for 1 h with solutions (1/10 w/v) containing 0, 2, 4, 6, 8, 12, 16 and 20 mg BSA g^{-1} soil, followed by centrifuging at 5500 rpm for 10 min and removal of the supernatant.

For assessment of the ability of the phytases to mineralise endogenous inositol phosphates in soil, two additional soils were used. These were collected from Goulburn (alfisol #2) and Camden (Vertisol) NSW, Australia, and have also been described previously (George et al., 2005c).

2.2. Sources of phytase

Phytases from two different fungal sources were used; a basidiomycete (*P. lycii*) and an ascomycete (*A. niger*). The *P. lycii* enzyme is a 6-phytase (EC 3.1.3.26), while the *A. niger* enzyme is a 3-phytase (EC 3.1.3.8) (Lassen et al., 2001), although both are classified as *phyA* histidine acid phosphatases (Ullah and Sethumadhavan, 2003). Typical physicochemical characteristics of purified preparations of the two phytases found in the literature are shown in Table 1, with notable differences being the greater specific activity (~6-fold) and a more acidic pI (pH 3.6 compared to pH 5.0) of the *P. lycii* compared to *A. niger* phytase. It should be noted that, due to differences in purification procedure, the specific activity of the phytase preparations used in experimentation was different to that typical of the phytases (Table 1). The purified phytases were also compared with a commercially available crude phytase preparation (Sigma phytase; from *A. ficuum* (*niger*), EC 3.1.3.8; Sigma-Aldrich Corporation St Louis, MI, USA; 0.1 μKat mg^{-1} protein) in order to measure their relative substrate specificity.

The *A. niger* phytase was collected as a heterologous protein from root exudates of *Arabidopsis thaliana* expressing the fungal phytase gene (Richardson et al., 2001a). Approximately 50 surface-sterilised seeds of transgenic *A. thaliana* were germinated and grown for 14 days in 20 ml of a 2-morpholinoethanesulfonic acid (MES) buffered (15 mM MES, pH 5.5) sterile nutrient solution containing 50 μM Na_2PO_4, 4 mM KNO_3, 4 mM $Ca(NO_3)_2$, 1.5 mM $MgSO_4$, 3 mM NH_4Cl, 0.1 mM FeEDTA, micronutrients and 200 mg mL^{-1} sucrose. Plants were grown on an orbital shaker (30 oscillations min^{-1}) with constant light (~200 μE $m^{-2}s^{-1}$) at 21 °C. The plants were then transferred to a sucrose- and P-free equivalent nutrient solution and grown for a further 14 days under the same conditions. Solutions containing root exudates were then collected, filter sterilised (0.22 μm) and the stock solution, with an activity of 4 nKat mL^{-1} (specific activity of 4.6 μKat mg^{-1}