Geoderma 305 (2017) 346–353

Contents lists available at ScienceDirect

Geoderma

journal homepage: www.elsevier.com/locate/geoderma

Effect of citrate on *Aspergillus niger* phytase adsorption and catalytic activity in soil

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ARTICLE INFO

Keywords:
Organic anions
Phosphorus
Phytate
Rhizosphere
$InsP_6$

ABSTRACT

Phytase enzymes from bacteria, fungus and plant root exudates are known to hydrolyse organic phosphorus (P_o) to bioavailable inorganic orthophosphate in soil. Exploiting such biochemical functions in agricultural systems, offers the potential for alternative sustainable phosphorus sources. Phytase adsorption to soil particles and phytate metal complexation has been shown to inhibit phytate ($InsP_6$) dephosphorylation. Organic acid anions such as citrate increase phytase catalytic efficiency towards complexed forms of $InsP_6$, but the mechanisms are poorly understood. The aim of this work was to evaluate *Aspergillus niger* phytase inactivation and changes in its catalytic properties upon addition to soil, as well as the effect of citrate on phytase adsorption and activity towards free, precipitated and adsorbed $InsP_6$.

We performed a series of enzyme hydrolysis activity assays when the enzymes were free, in soil solution and adsorbed to soil under varying chemical conditions, with and without citrate. *A. niger* phytase showed a relatively low absorption affinity for the Cambisol test soil. Phytase activity reduced by 37.3% due to adsorption. Citrate had no effect on the rate or total amount of phytase adsorption or soil adsorbed phytase activity thereafter. Free phytases and phytases in soil solution showed optimum activity ($\geq$ 80%) at pH 4.5–5.5. Activity decreased slightly for soil adsorbed enzymes compared to enzymes which were free or in soil solution $>$ pH 5 and $<$ 4 in the pH dependency curve. A decrease in activity was seen only at ionic strengths (NaCl) $>$ 0.6 M in adsorbed and free enzymes, while activity from enzymes in soil solution reduced in all tested ionic strengths. Citrate significantly increased phytase activity towards $InsP_6$ adsorbed to soil when the phytases were free ($p \leq 0.003$) but not in other treatments (Na, Al and Ca-phytate). These results suggest that the effect of citrate on soil $InsP_6$ dephosphorylation is associated with the availability of the substrate ($InsP_6$) rather than its effect on the enzyme *per se*. The ionic strength and pH of soil solution has been shown to impact on phytase activity, suggesting that salinity, quality of irrigation water, wetting/drying cycles and fertilisation will have discrete impacts on the activity of phytases once released in soil and thus the ability to make P_o available for uptake by plants and microbes. The optimum acidic pH of *A. niger* also brings into question its suitability for application in many agricultural soils.

1. Introduction

Phosphorus (P) is an essential element for plant and animal life, thus mined phosphate rock is heavily relied on in agriculture (Smil, 2000; Jasinski, 2014). Although global supply of rock phosphate is predicted to match agricultural demands by an estimated 1.8% annual increase to 2019 (Heffer and Prud'homme, 2015), current application practices are inefficient. Large demands for P fertilisers are a result of limited plant availability in soil. Approximately 80–90% of added P fertiliser becomes unavailable to plants in the first year due to run-off, adsorption onto soil minerals, precipitation with Fe, Al and Ca metals and immobilisation into organic forms by microorganisms (Hinsinger, 2001; Gichangi et al., 2009; Khan and Joergensen, 2009). Global models predict that forecast P demands could be reduced by 50% by utilising soil residual phosphorus pools (Sattari et al., 2012; Stutter et al., 2012, 2015).

Organic phosphorus (P_o) concentrations in soils vary between 30% and 93% of total P depending on soil type and land use (Turner et al., 2002; Condron and Tiessen, 2005; Stutter et al., 2012). Soil P_o is primarily comprised of orthophosphate monoesters, contributing *c.*

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http://dx.doi.org/10.1016/j.geoderma.2017.06.015
Received 26 July 2016; Received in revised form 15 June 2017; Accepted 16 June 2017
Available online 06 July 2017

60–90% of the total P_o in soils, with large concentrations prevailing in agricultural soils with long term P fertiliser application history (Condron et al., 1985). Inositol phosphates ($InsP_x$, x = 1–6), a family of phosphoric esters of hexahydroxycyclohexane usually constitute the majority of the P_o pool at *c*. 60% (Turner et al., 2002). Of the $InsP_x$ isomers, the *myo* stereoisomer is the most common, usually referred to as phytate, the salt of phytic acid or $InsP_6$ (Cosgrove, 1972; Turner et al., 2002; Shears and Turner, 2007). $InsP_x$ have a large anionic charge, act as strong ligands and have an ability to complex polyvalent cations (Turner et al., 2002; Vohra and Satyanarayana, 2003; Menezes-Blackburn et al., 2013), which determine their recalcitrant nature in soils, sediments and colloidal and particulate matter.

In response to problematic P scenarios, biotechnologies and management strategies aim to exploit crop root exudation of phytases and organic anions to access abundant soil $InsP_x$. Such developments are considered highly desirable, with the potential of reducing requirements for added P fertiliser and environmental nutrient pollution via leachate and run-off (George et al., 2005b; Condron et al., 2013; Giles et al., 2016; Ockenden et al., 2016). Phytases (*myo*-inositol hexakisphosphate phosphohydrolases, E.C. 3.1.3) catalyse the hydrolysis of phytate to *myo*-inositol pentakisphosphate ($InsP_5$) or to less phosphorylated *myo*-inositol phosphates ($InsP_{1–4}$) increasing plant availability. As with acid phosphatases, phytase, and alkaline phosphatase, phosphatases produced by plant roots and/or soil microorganisms show increased abundance and activity in the rhizosphere as compared to bulk soil (Otani and Ae, 1999; Richardson et al., 2011), and have been shown to increase plant availability of P (Vohra and Satyanarayana, 2003; Menezes-Blackburn et al., 2013).

Work with a number of plant species, which have been modified to express heterologous extracellular phytases, has shown increased utilisation of soil P_o, specifically $InsP_6$, in controlled conditions such as sterile agar media (Hayes et al., 2000; Tang et al., 2006; Tang et al., 2014), but have failed to show consistent abilities to utilise P_o in soil (George et al., 2004, 2006; Giles et al., 2016). Extracellular phytase activity can be short-lived when free in soil solution and often inactive when adsorbed onto soil particles (George et al., 2004, 2007b). Numerous studies (Rao and Gianfreda, 2000; Huang et al., 2003; Dao, 2004; George et al., 2007b; Tang et al., 2006; Giaveno et al., 2010; Rao et al., 2010; Menezes-Blackburn et al., 2011) detailed the loss of phytase activity associated with adsorption and conformational changes of phytase enzymes with pure clays, humus materials, humus like compounds and towards mineral complexes (Tang et al., 2006). The six reactive groups of $InsP_6$ make it a strong chelating agent that readily binds to cations such as Ca^{2+}, Mg^{2+} and Fe^{2+} in soil (Vohra and Satyanarayana, 2003). Strong adsorption of the high charge density molecule and ligand exchange reactions can form inner-sphere complexes, which are heavily affected by pH, ligand competition and P saturation (Menezes-Blackburn et al., 2013). This propensity to form chelates highlights the importance of examining complex substrates to better understand phytase catalytic efficiency and fully capitalise on plant P benefits from phytase labile P hydrolysis.

Organic anions have long been associated with increased nutrient availability and are common in the soil-root interface. Several studies have reported the promotion of substrate solubilisation by organic anions such as citrate, malate and oxalate via competition for binding sites on mineral surfaces and/or metal ions in complexes with phytate (Huang et al., 2003; Tang et al., 2006; Dao, 2007; Giles et al., 2014). However, less is known regarding the direct effect of organic anions on enzyme catalytic function.

An increased understanding of factors that determine the behaviour of phytases in soil may inform recent (Giles et al., 2016) and future plant growth trials in soil which would allow for the better design and application of agronomic developments. This research aims to evaluate the effect of (a) citrate on *A. niger* phytase adsorption, adsorbed activity and catalytic behaviour; (b) phytase adsorption onto soil particles and presence in soil solution, on the biochemical properties of the enzyme; and (c) citrate on the hydrolytic performance of free or soil-adsorbed phytases towards free, precipitated and adsorbed forms of phytate.

We hypothesise that citrate will decrease phytase adsorption to soil thereby increasing catalytic efficiency and subsequent $InsP_6$ hydrolysis. Following phytase adsorption to soil we hypothesise that with the addition of citrate, hydrolysis of $InsP_6$, when free (Na-phytate), precipitated (Al-phytate, Ca-phytate, Fe-phytate) or soil adsorbed phytate, will be greater than without citrate.

2. Materials and methods

2.1. Soil characteristics

The soil used was a top soil (0–10 cm depth) collected from Tayport, Fife, near The James Hutton Institute in Dundee, Scotland (56°25′22.94″N–2°53′15.29″W) and was previously sown with winter barley. The soil is classified as a Cambisol (FoFAO, 2014) and is derived from Old Red Sandstone sediments and lava with some metamorphic rocks (Bell, 1990). The chemical properties of the soil have been previously reported by Stutter et al. (2015). The soil is slightly acidic (pH in $CaCl_2$ of 5.95) and contains 1475 mg kg^{-1} total P, with relatively high concentrations of Olsen P (84.5 mg kg^{-1}), 765 mg kg^{-1} of P_i, and 410 mg kg^{-1} orthophosphate monoester P (determined by ^{31}P NMR). The organic component of the soil contains 20 g of organic carbon kg^{-1}, 0.3 mg kg^{-1} of soil microbial P, 6.3 mg kg^{-1} of water-extractable inorganic P (WEP_i), 0.5 mg kg^{-1} of water-extractable P_o (WEP_o), 8.7 mg kg^{-1} of soil citrate-extractable P_o (CEP_o), and a degree of P saturation of 50% based on ammonium-oxalate extraction. The soil had previously been air-dried, mixed and sieved (4 mm) and was stored at room temperature. Soil dry weight, obtained by drying a 5 g sub sample for 24 h at 60 °C, was 0.87 g g^{-1}.

2.2. Chemical sources

Phytic acid sodium salt hydrate ($InsP_6$: $C_6H_{18}O_{24}P_6{\cdot}xNa^{+}{\cdot}yH_2O$; P0109) and sodium citrate dehydrate ($HOC(COONa)(CH_2COONa)_2{\cdot}2H_2O$; W302600) were purchased from Sigma-Aldrich, UK.

2.3. Preparation of soil suspension

A soil suspension was prepared by shaking a 1:10 soil to water mixture for 1 min by hand. The silt and clay fractions were decanted off following a 30 s settling period. The sand was discarded. The clay fraction was used for the suspensions representing the chemically significant component of the soil. The smaller particle size also allows for a more homogenous working suspension and promotes consistent samples, in addition pipetting large particles (e.g.: sand) can be problematic with such small samples. The suspension was then dried and brought to a 2 g L^{-1} concentration with Milli-Q water, hereby referred to as the working soil suspension.

2.4. Enzyme solution preparation

The source of *A. niger* phytase E.C.3.1.3.8., (Greiner and Konietzny, 2006) was a solid formulation of Natuphos® (BASF, Germany) with a reported isoelectric point of 4.17–5.25 (Wyss et al., 1999). The enzyme formulation has been found to contain other phosphatases and inorganic elements such as calcium, however due to the large dilutions used in the assays; such measures were considered not important. The enzyme working solution was prepared by dissolving 2 g of the enzyme powder in 20 mL of high purity water (Milli-Q), the suspension was gently shaken and left for 2 h. The suspension was then centrifuged twice for 10 min at 150*g*, and the supernatant removed and diluted to a 2 mg mL^{-1} protein concentration, referred to hereafter as the enzyme solution. The standard protein assay determination method of Bradford