Biol Fertil Soils (2000) 32:279–286

ORIGINAL PAPER

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Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase

Received: 29 October 1999

Abstract Extracts were prepared from soil using water, 50 mM citric acid (pH ~2.3) or 0.5 M $NaHCO_3$ (pH 8.5), and were incubated with excess phytase from *Aspergillus niger* to determine the amounts of labile P. Two *A. niger* phytase preparations were used: (1) a purified form which exhibited a narrow substrate specificity and high specific activity against phytate; and (2) a commercial preparation (Sigma) with activity against a broad range of P compounds. A comparatively large proportion (up to 79%, or 5.7 μg g^{-1} soil) of the organic P (P_o) extracted with citric acid was hydrolysed by the commercial phytase, while between 28% and 40% (up to 3.1 μg g^{-1} soil) was hydrolysed using purified phytase. By comparison, only small quantities of the P_o in water and $NaHCO_3$ soil extracts were enzyme labile. While extractable P_o was increased both with increasing concentrations of citric acid (up to 50 mM) and increasing pH (pH 2.3–6.0), enzyme-labile P increased only with citric acid concentration. The labile component of P_o in citric acid extracts from soils with contrasting fertiliser histories indicated that enzyme-labile P_o is a relatively large soil P pool and is potentially an important source of P for plants.

Key words Citric acid · Organic phosphorus · Phosphatase · Phytase · Soil phytate

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Introduction

Although organic P (P_o) is typically a major component of P in soils (e.g. McLaughlin et al. 1990), it remains poorly characterised. More specifically, the contribution of soil P_o compounds to plant P nutrition is not well understood. Generally, it is assumed that plants take up P as inorganic phosphate (P_i) from the soil solution and that phosphatases, either from plant roots or soil microorganisms, must first hydrolyse P_o substrates (Marschner 1995). Thus, the susceptibility of soil P_o to enzyme hydrolysis is potentially a constraint for plant P acquisition.

Phytate (penta- and hexa-phosphate esters of inositol) is the most abundant identifiable P_o compound in soil, comprising up to 50% of total P_o (Anderson 1980), and may be an important source of P for plant nutrition. Quantitative procedures for the determination of phytate in soils require its separation from other soil P compounds, using a combination of extraction, anion exchange chromatography, selective precipitation and re-solubilisation steps (e.g. McKercher and Anderson 1968). The quantity of phytate is then derived from P_i determinations following acid hydrolysis. Alternatively, phytate in soil extracts has been measured by ^{31}P nuclear magnetic resonance (NMR) (Newman and Tate 1980; Tate and Newman 1982). However, such methods only estimate the total quantity of phytate in soils and do not provide any indication of the component that may be available to plants. While Wild and Oke (1966) identified inositol mono-phosphates in $CaCl_2$ extracts of soil, there are no known reports of phytate in soil solution. Indeed, experiments in sterile media using a range of species indicate that plants have only a limited ability to obtain their P requirements from phytate (Hayes et al. 2000), while accounts of the ability of plants to use phytate-P in non-sterile environments are more variable (Kroehler and Linkins 1991; Adams and Pate 1992; Findenegg and Nelemans 1993; Hübel and Beck 1993).

The availability of soil P_o to plants may be characterised by estimating the component that can be converted to P_i with an excess addition of phosphatase. In particular, phytase-labile P_o may be a useful measure: phytases are a class of phosphatase that show activity against phytate. Shand and Smith (1997) and Pant et al. (1994) used phosphatase and a non-specific phytase (from wheat) to show that enzyme-labile P_o was present in water extracts and in soil solution extracted from some Scottish soils. More recently, Otani and Ae (1999) showed that phytase- and phosphatase-labile P_o was extracted from Japanese Andosols only by citric acid.

The objectives of this study were to investigate the amounts of extractable soil P_o that were enzyme labile, using two sources of phytase from *Aspergillus niger*. Phytase-labile P_o was determined in various extracts prepared from two Australian pasture soils with contrasting fertiliser histories.

Materials and methods

Soils

Soil samples were collected to a depth of 10 cm, under permanent pastures (containing perennial grass and annual legume components) located at two sites: (1) Rutherglen Research Institute, Victoria; and (2) Ginninderra Experiment Station, Canberra, ACT. Individual cores (2.5 cm diameter) of soil from each treatment at each site (~30 samples) were bulked as a composite sample which was air-dried, passed through a 2-mm sieve to remove large, particulate matter and stored at room temperature.

Samples from the Rutherglen site were collected in 1993 from treatments of an unreplicated fertiliser trial, the details for which are published in Ridley et al. (1990). Briefly, the trial was established in 1914 on three 1.5-ha fields. Two fields (F_R and $F+L_R$) received approximately 125 kg ha^{-1} of single superphosphate (9% P) each alternate year from 1914, while the third field (U_R) was unfertilised. One of the fertilised fields ($F+L_R$) was also topdressed with lime from 1914 to 1948, receiving nine applications of 1.25 t ha^{-1}.

Samples from Ginninderra Experiment Station (Wallaroo 3 Paddock) were collected in October 1996. Three P fertiliser treatments were imposed across the trial on its establishment in 1994. Plots were either unfertilised (U_G) or received three autumn applications of triple superphosphate (20.7% P), totalling 416 kg ha^{-1} ($F1_G$) or 675 kg ha^{-1} ($F2_G$).

Properties of the two soils are presented in Table 1. Total P_i and P_o were obtained by the ignition-extraction (0.5 M H_2SO_4) procedure (Olsen and Sommers 1982) and Colwell P by a 16-h extraction with 0.5 M $NaHCO_3$ (Colwell 1963), followed by determination of P_i using the molybdate-blue colour reaction (Murphy and Riley 1962). Organic C was determined by the modified Mebius procedure (Nelson and Sommers 1982) and soil pH was measured in $CaCl_2$.

Substrate specificities of phytase preparations

The substrate specificities of commercial preparations of wheat-germ acid phosphatase (EC 3.1.3.2; Sigma), *A. niger* phytase (EC 3.1.3.8; Sigma) and a purified preparation of the *A. niger* NRRL 3135 phytase (kindly provided by Dr Markus Wyss; F. Hoffmann-La Roche, Switzerland; refer to Wyss et al. 1999a, 1999b) were determined using a range of P_o compounds. The specific activities of the three enzyme preparations were tested at 27 C, in 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) buffer (pH 5.5) containing 1 mM ethylenediaminetetraacetate (EDTA), against the following substrates: *myo*-inositol hexaphosphoric acid (dodecasodium salt; IHP), α-D-glucose 1-phosphate (disodium salt; G1P), ribonucleic acid (type VI; RNA), adenosine-5′-triphosphate (ATP), D(–)3-phosphoglyceric acid (trisodium salt), *p*-nitrophenyl phosphate (disodium salt; *p*NPP), and *bis-p*NPP (Na salt). With the exception of ATP (Boehringer Mannheim), all substrates were obtained from Sigma. Assays were performed in 1-ml volumes, using 50 μg acid phosphatase ml^{-1}, 1.14 μg commercial phytase ml^{-1} or 0.45 μg purified phytase ml^{-1}. These amounts of enzyme were chosen on the basis of the reported specific activities of the preparations, against either IHP or *p*NPP. Enzyme assays were conducted over 30 min at a range of substrate concentrations between 2.4 mM and 4.8 mM P, and were replicated 3 times. Activities were measured against *p*-nitrophenol standards (Bessey et al. 1946) for the *p*NPP- and *bis-p*NPP-containing samples, and for all other samples by the release of P_i as determined using the malachite-green reaction (Irving and McLaughlin 1990).

Measures of enzyme-labile soil P

Soil extraction and total P, P_o and P_i determinations

Samples of air-dried soil (2–10 g) were extracted in 50-ml polypropylene tubes using two volumes of sterile extractant solution, usually deionised water, 50 mM (~1.0%) citric acid (pH ~2.3) or 0.5 M $NaHCO_3$ (pH 8.5). Other extractants included 0.025 M HCl

Table 1 Properties of two pasture soils (0–10 cm depth) from Ginninderra Experiment Station (Canberra, ACT); and Rutherglen Research Institute (north-east Victoria). *P_i* Inorganic P, *P_o* organic P

Site	Soil type (Stace et al. 1968)	Fertiliser treatment[a]	P (mg kg^{-1} soil)			pH ($CaCl_2$)	Organic C (%)	Colwell P (mg kg^{-1} soil)
			Total P	P_i	P_o			
Ginninderra Experiment Station	Yellow podzolic	U_G (unfertilised)	203	53	150	4.67	1.88	11.3
		$F1_G$ (86 kg P ha^{-1})	242	79	163	4.57	1.91	25.0
		$F2_G$ (140 kg P ha^{-1})	254	97	157	4.57	1.95	43.7
Rutherglen Research Institute	Grey-brown to yellow podzolic	U_R (unfertilised)	154	30	123	4.60	1.99	7.8
		F_R (~450 kg P ha^{-1})	312	113	199	4.21	3.07	38.8
		$F+L_R$ (~450 kg P ha^{-1} and 11.25 t lime ha^{-1})	287	95	192	4.55	2.57	26.4

[a] Total amount of fertiliser applied since inception of trial

and 0.1 M HCl, and 0.01 M $CaCl_2$ (pH 5.5). The soils were extracted at 22 ± 2 C for 30 min on a reciprocal shaker (300 rpm), followed by centrifugation (10,300 *g*) for 15 min. Soil extracts were decanted from the pelleted material and stored at 4 C prior to enzyme analyses.

The P contents of soil extracts were determined on 1-ml subsamples of each solution. P_i was determined by measuring the P_i content of solutions with malachite-green reagent (Irving and McLaughlin 1990). In order to determine total P, samples were autoclaved (120 kPa, 121 C; 40 min) in the presence of 0.6 M H_2SO_4 and 3.3% ammonium persulphate (Schoenau and Huang 1991), and were similarly analysed for P_i content. Corrections for volume loss during autoclaving were made as necessary, based on gravimetric analyses. P_o in the extracts was calculated by deduction of P_i from total P.

Incubation of soil extracts with enzyme

The standard assay procedure involved incubation of 1 ml soil extract in the presence of excess enzyme: either 0.25 nkat commercial phytase (i.e. 0.50 nkat g^{-1} soil; a katal of enzyme activity is equivalent to one mole of product released per second), as determined against IHP substrate for the incubation conditions specified herein, or 1.14 nkat purified phytase (2.28 nkat g^{-1} soil). These amounts of enzyme also displayed 1.56 nkat ml^{-1} (3.12 nkat g^{-1} soil) and 0.03 nkat ml^{-1} (0.05 nkat g^{-1} soil) acid phosphatase activity against *p*NPP, for the commercial and purified preparations, respectively. Samples were adjusted to pH ~5.5 with either HCl or NaOH, 300 µl of 250 mM MES buffer (pH 5.5; containing 5 mM EDTA) was added, and the solutions were made up to a final volume of 1.5 ml with deionised water. Immediately on addition of the enzyme to each reaction, an aliquot was removed and mixed with a one-fifth volume of 25% trichloroacetic acid (TCA). Remaining solutions were incubated at 27 C for 6 h, after which TCA was added to terminate the reactions. The TCA-treated samples were centrifuged in an Eppendorf microfuge (6000 *g*, 10 min) prior to analysis for P_i using malachite-green reagent. Determinations of P_i were made at a fixed time (within 1 h) following addition of the colour reagent, and control treatments were routinely included whereby either soil extract or enzyme were omitted.

Statistics

Enzyme assays and other soil measures were replicated 3 times and the data were analysed to investigate variation associated with soil extraction and incubation procedures, using one- and two-way ANOVAs. Where *F*-ratios were significant ($P<0.05$), treatment means were compared by least significant difference (LSD).

Results

Conditions for the determination of enzyme-labile P in extracts of soils

Substrate specificities and specific activities of phytase preparations

The specific activities of the three enzyme preparations for IHP ranged from 1.0 nkat mg^{-1} protein (wheat-germ acid phosphatase) to 560.9 nkat mg^{-1} (purified *A. niger* phytase; Table 2). The purified *A. niger* phytase preparation had a 12-fold higher specific activity for IHP than the commercial phytase. Moreover, purified phytase showed a narrow substrate specificity, with specific activities for a range of P_o substrates that were 10% or less than the specific activity for IHP. The commercial *A. niger* phytase preparation was less substrate specific, with highest activity observed against *p*NPP. The activity profile of the commercial phytase preparation was similar to that of wheat-germ acid phosphatase. It is likely that the commercial *Aspergillus* phytase preparation contained a considerable amount of contaminant phosphatase activity, which was not specific for phytate. This was removed from the more purified preparation. The wheat-germ acid phosphatase was not used in subsequent experiments because of its similarity to commercial phytase.

P_i and P_o contents of extracts from unfertilised Ginninderra soil

U_G soil from Ginninderra Experiment Station was selected initially for P measurements because of its low P status (Table 1). There was a requirement for soil extracts to contain sufficient levels of P_o, so that any change upon incubation with enzyme would be detectable. Various extracts of field-moist U_G soil were prepared, in which recoverable P_o was determined. Water, 0.01 M $CaCl_2$, 0.025 M HCl and 0.1 M HCl extracted <1.0 µg P_o g^{-1} soil. By contrast, up to 22.5 µg P_o g^{-1} soil was extracted using citric acid and 0.5 M $NaHCO_3$.

Table 2 Substrate specificities of commercial preparations of wheat-germ acid phosphatase (Sigma) and *Aspergillus niger* phytase (Sigma) and a purified preparation of the *A. niger* phytase. Inositol hexaphosphate and other organic P substrates were used at concentrations of between 2.4 mM and 4.8 mM. *LSD* Least significant difference

Specific activity (nkat mg^{-1} protein)[a]			
Substrate	Acid phosphatase	Sigma phytase	Purified phytase
myo-Inositol hexaphosphate	1.0	46.9	560.9
Glucose 1-phosphate	0.4	84.8	0.1
Ribonucleic acid	0.4	38.5	0.3
Adenosine triphosphate	4.2	55.2	59.4
Phosphoglyceric acid	3.6	78.7	54.5
p-Nitrophenyl phosphate	7.0	293.0	13.1
bis(*p*-Nitrophenyl) phosphate	0.2	11.5	35.5
LSD ($P=0.05$)	0.3	14.5	51.7

[a] A katal of enzyme activity is equivalent to one mole of product released per second

$NaHCO_3$, citric acid and water were thus selected as extractants for further analysis. A solution-to-soil ratio of 2:1 extracted higher concentrations of P_o (as compared to a 5:1 ratio) and was adopted for the subsequent incubation experiments.

Enzyme-labile P in extracts from fertilised Rutherglen soil

F_R soil from Rutherglen was used to determine the appropriate conditions to be employed for enzyme incubation studies with all soil-extractant combinations. The hydrolysis of P_o from extracts of soil F_R was measured for up to 8 h in the presence of three concentrations of commercial or purified phytase, or in the absence of enzyme. The release of P_i from incubated citric acid soil extracts is shown in Fig. 1. Additions of 0.50 nkat commercial phytase g^{-1} soil or 2.28 nkat purified phytase g^{-1} soil were required for reactions to approach completion within 8 h. With lower amounts of enzyme, reactions proceeded more slowly and did not reach completion during the incubation period. For all subsequent measures of labile P, including various soils containing lower P_o concentrations, soil extracts were incubated for 6 h in the presence of 0.50 or 2.28 nkat phytase g^{-1} soil, using commercial or purified preparations, respectively.

To determine the contribution of soil microbe-derived enzyme activities to the observed rates of hydrolysis in amended soil extracts, duplicate samples were passed through 0.45-μm filters (Millex-HA; Millipore). There were no differences in either the rate or total quantity of P_i liberated from citric acid, water or $NaHCO_3$ extracts of soil F_R, between filtered and unfiltered samples (data not shown). Therefore, over the 6-h incubation period, microbial enzyme activity did not contribute to the observed rates of hydrolysis. A low, and insignificant, level of hydrolysis was measured when no enzyme was added to the extracts (Fig. 1).

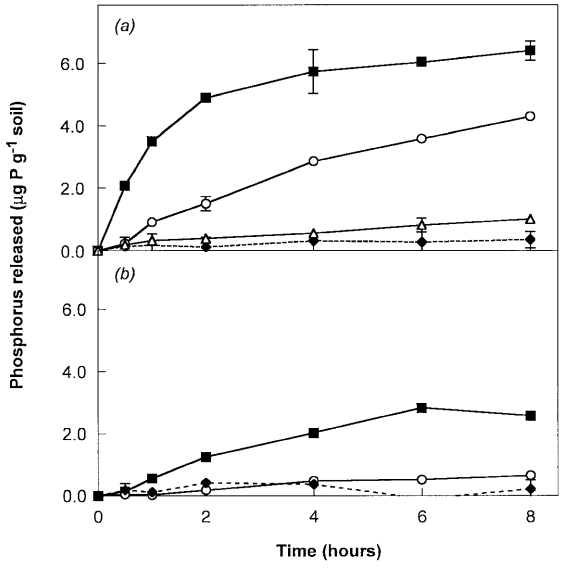

Fig. 1 Release of P from 50 mM citric acid extracts of fertilised Rutherglen soil (F_R), during 8-h incubation with **a** 0.005 (△), 0.05 (○), and 0.50 (■) nkat commercial phytase g^{-1} soil (a katal of enzyme activity is equivalent to one mole of product released per second), and **b** 0.23 (○). and 2.28 (■) nkat purified phytase g^{-1} soil. In each experiment, control incubations with no added enzyme (◆) were also included. *Each point* represents the mean of three observations. *Bars* indicate SE and, where not shown, were smaller than the symbol

Effects of citric acid concentration and pH on phytase-labile P from F_R soil

Citric acid concentration

Extractable P_o from soil F_R increased significantly with increasing concentrations of citric acid to 50 mM ($P < 0.05$; Fig. 2). The amounts of P_o that were hydrolysed by the commercial and purified phytases, and the proportion of the total P_o which was enzyme labile, also increased with citric acid concentration. For the commercial phytase preparation, labile P represented 13.5% and 82% of the P_o in water and 50 mM citric acid soil extracts, respectively. Purified phytase-labile P_o was approximately half of that hydrolysed by the commercial enzyme preparation, representing between 1.8% and 44.7% of the total P_o over the same range of citric acid concentrations.

Effect of pH of citric acid

Soil extractions were prepared using 50 mM citric acid solutions adjusted to pH 2.3–6.0. Extractable P_o increased with pH, from 7.5 μg P g^{-1} soil at pH 2.3 to 25.3 μg P g^{-1} soil at pH 6.0 (Fig. 3). However, enzyme-

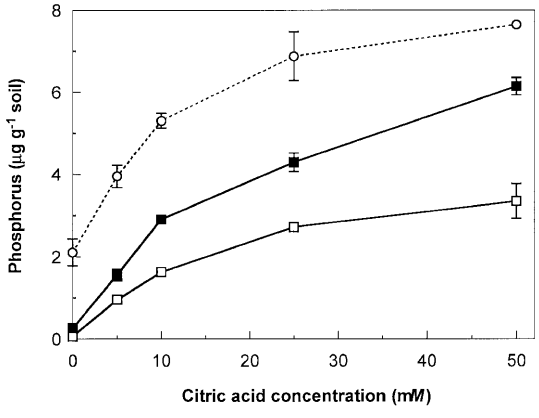

Fig. 2 Commercial (■) and purified (□) phytase-labile P (μg g^{-1} soil) in citric acid extracts of fertilised Rutherglen soil (F_R), using between 0 mM and 50 mM citric acid concentration. Extractable organic P (○; μg g^{-1} soil) is also shown. *Each point* represents the mean of three observations. *Bars* indicate SE and, where not shown, were smaller than the symbol

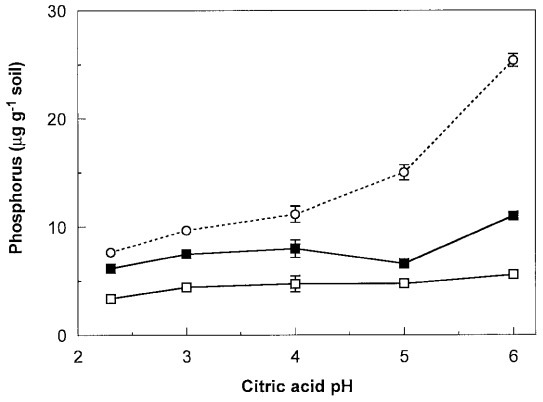

Fig. 3 Commercial (■) and purified (□) phytase-labile P (μg g^{-1} soil) in 50 mM citric acid extracts of fertilised Rutherglen soil (F_R), using citric acid solutions adjusted to between pH 2.3 and pH 6.0. Extractable organic P (○; μg g^{-1} soil) is also shown. *Each point* represents the mean of three observations. *Bars* indicate SE and, where not shown, were smaller than the symbol

labile P was similar across the entire pH range, with ~7.8 μg P g^{-1} soil and ~4.5 μg P g^{-1} soil hydrolysed by the commercial phytase preparation and purified phytase, respectively (Fig. 3).

Comparison between citric acid and HCl as extractants

Total P_o, and commercial and purified phytase-labile P_o in water, citric acid and HCl extracts from soil F_R are presented in Table 3. Two HCl extractants were used: one at the same molar concentration as citric acid (50 mM) and the other at the same pH (pH 2.3, at ~5 mM). When used at an equivalent molar concentration, HCl extracted only 13% of the quantity of P_o extracted with citric acid, and the enzyme-labile P component was negligible relative to that of citric acid extracts. When the concentration of HCl was adjusted for a solution of pH 2.3, the amount of extractable P_o was increased, but was still only 27% of that extracted by citric acid. Likewise, the enzyme-labile P_o component was <3% of that extracted by citric acid. While commercial and purified phytase-labile P_o in 50 mM citric acid extracts represented 80% and 44% of the extractable P_o, respectively, less than 13% and 4% of P_o in the other extracts were hydrolysed by the two enzyme preparations (Table 3).

Extractable and phytase-labile P_o from soils with different fertiliser histories

The total P and P_o contents of water, 50 mM citric acid (pH ~2.3), and 0.5 M $NaHCO_3$ (pH 8.5) extracts of soil from P fertiliser trials at two sites are illustrated in Fig. 4, along with the fractions of extractable P_o that were hydrolysed by commercial and purified phytase during separate incubations.

While $NaHCO_3$ extracted more P_o than the other solutions (12–30 μg g^{-1} soil; Fig. 4e,f), citric acid extracted the most enzyme-labile P_o (up to 5.7 μg P g^{-1} soil; Fig. 4c,d), with between 56% and 79% of the P_o in citric acid extracts being hydrolysed by the commercial phytase preparation. In contrast, only 7–17% and 2–9% of the P_o in water and $NaHCO_3$ extracts, respectively, was hydrolysed by the commercial phytase preparation (Fig. 4a,b; Fig. 4e,f). A smaller component of the P_o was hydrolysed by purified phytase. In citric acid extracts, 28–40% of the P_o was purified phytase labile (Fig. 4c,d), while only 3–8% and 1–2% of the P_o in water and $NaHCO_3$ extracts, respectively, was hydrolysed (Fig. 4a,b; Fig. 4e,f).

P_o, P_i and enzyme-labile P extracted by citric acid were significantly higher ($P<0.05$) for the fertilised soils F_R and $F+L_R$ than for soil U_R (Fig. 4c). In addition, both total extractable P_o and the component which was phytase labile, were greater for F_R soil than soil which had received both fertiliser and lime ($F+L_R$). While P_o and P_i extracted by $NaHCO_3$ were significantly greater ($P<0.05$) for both F_R and $F+L_R$ soils, only the treatment which had received fertiliser alone (F_R) contained a higher level of extractable, commercial phytase-labile P than the unfertilised control (U_R) (Fig. 4e). No differences in fertiliser treatment were evident for purified phytase-labile P.

For the soil from Ginninderra, which had received only recent applications of P fertiliser, there were no differences in either extractable P_o or enzyme-labile P across fertiliser treatments (Fig. 4). However, the extractable P_i component increased significantly ($P<0.05$) with P fertility, in both citric acid and $NaHCO_3$ extracts (Fig. 4d,f).

Table 3 Extractable P_o, and enzyme-labile P (μg g^{-1} soil) using two sources of phytase from *A. niger*, in water, citric acid and HCl extracts of fertilised Rutherglen soil (F_R). *n.d.* Not detectable; for other abbreviations, see Tables 1 and 2

Extractant	P_o (μg g^{-1} soil)	Enzyme-labile P_o (μg g^{-1} soil)			
		Commercial phytase	(% P_o)	Purified phytase	(% P_o)
Water	2.10	0.26	(12.4)	0.07	(3.3)
50 mM citric acid (pH 2.3)	7.65	6.15	(80.4)	3.35	(43.8)
50 mM HCl (pH 1.45)	1.01	0.09	(8.9)	n.d.	–
5 mM HCl (pH 2.3)	2.09	0.17	(8.1)	0.07	(3.3)
LSD ($P=0.05$)	0.54	0.30		0.57	

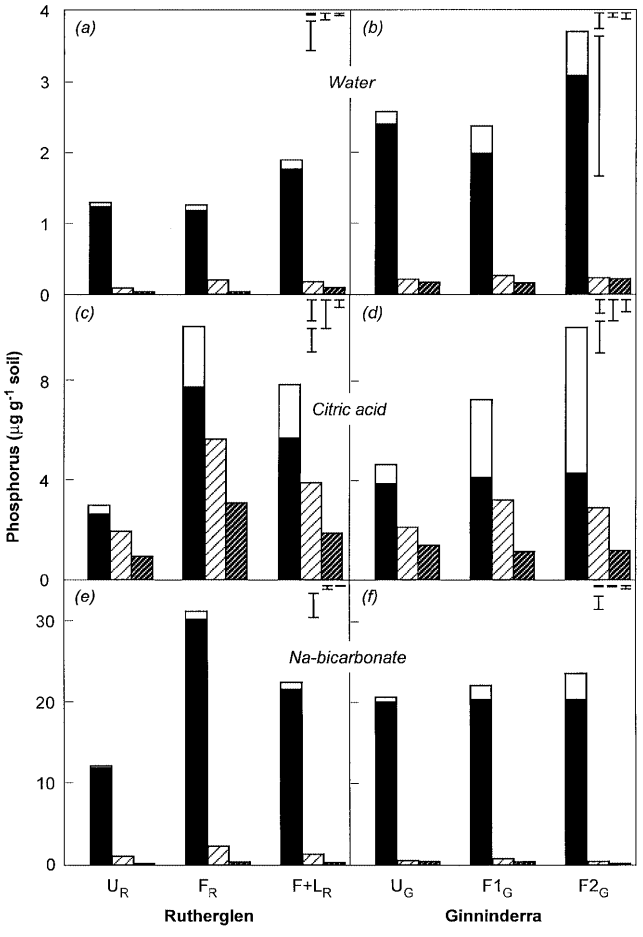

Fig. 4 Extractable organic (■) and inorganic (□) P (μg g^{-1} soil) of soils from Rutherglen Research Institute (**a**, **c**, **e**) and Ginninderra Experiment Station (**b**, **d**, **f**). Water (**a**, **b**); 50 mM citric acid (**c**, **d**); and 0.5 M $NaHCO_3$ (**e**, **f**) were used as extractants. Enzyme-labile P, using commercial (*open hatching*) and purified (*dense hatching*) preparations of phytase, is also shown for each extract of soil. *Each bar* represents the mean of three observations. *Bars* showing least significant differences (LSDs) ($P = 0.05$) for each site-extractant combination are presented, and are shown for organic P and inorganic P (stacked), P hydrolysed by commercial phytase, and P hydrolysed by purified phytase, respectively. Where not shown, LSDs were too small to be depicted.

Discussion

Characterising phytase-labile P_o in extracts of soil

Two *A. niger* phytase preparations with markedly different substrate specificities were used to measure the amounts of enzyme-labile P_o present in extracts of soil. A commercial preparation of *A. niger* phytase (from Sigma) showed activity against a range of P_o substrates, with highest activity for *p*NPP. The preparation may also contain contaminating phosphatase activity. Conversely, in accordance with previous findings (Wyss et al. 1999a), a purified form of the *A. niger* phytase was highly specific for phytate. The contrasting substrate specificities of the two phytase preparations were exploited to characterise the enzyme lability of P_o extracted from soils. Material hydrolysed by the purified phytase was considered likely to be indicative of the phytate content of the extracts, whereas hydrolysis by the commercial "phytase" preparation reflected a more general acid phosphatase activity. However, it should be noted that the activities of the two preparations were not entirely mutually exclusive, as shown by their activities against a range of P_o substrates (Table 2).

In water extracts from soils collected from Ginninderra Experiment Station and Rutherglen Research Institute, less than 8% of the P_o present was hydrolysed by the purified phytase and only 7–17% was hydrolysed by the commercial preparation (Fig. 4). Previous measures of enzyme-labile P_o in water extracts of soils have not been made using phytase with a narrow specificity for IHP. The present work suggests that low levels of phytate occur in soil solution (< 0.22 μg P g^{-1} soil), and also that P_o esters accessible to a more general phosphatase (i.e. commercial phytase) activity are present in only small quantities. In contrast, higher amounts of P_o in water extracts (42–70%, Pant et al. 1994) and soil solution (48–62%, Shand and Smith 1997) from Scottish soils were hydrolysed by a phytase (from wheat) which was not specific for phytate.

Several extractants were used to assess enzyme-labile P_o. $NaHCO_3$ has previously been considered to extract a component of soil P_o which is readily mineralisable (Bowman and Cole 1978). On this basis, sequential soil P_o extraction procedures have been developed that include HCO_3^--extractable P_o as a labile fraction (Hedley et al. 1982; Sharpley 1985). However, the present experiments do not support the supposition that HCO_3^--extractable P_o is labile. While $NaHCO_3$ extracted the largest quantities of P_o relative to other soil extractants (up to 30 μg P g^{-1} soil), only a small proportion (1–9%) was enzyme-labile, even when using phytase with activity against a wide range of phosphate esters (Fig. 4). Otani and Ae (1999) have similarly shown that negligible amounts of P_o were enzyme-labile in $NaHCO_3$ extracts from a range of soils.

In contrast, 50 mM citric acid extracted intermediate amounts of P_o (3.8–7.7 μg P g^{-1} soil), of which up to 79% could be hydrolysed by the commercial phytase preparation and up to 40% was hydrolysed by the purified preparation. Otani and Ae (1999) also used a range of extractants and found that only citrate extracted P_o that was readily accessible to either acid phosphatase or a broad-specificity phytase. From the present work, it is evident that citric acid and $NaHCO_3$ extracts contain different components of the total P_o pool.

Up to 40% of citrate-extractable soil P_o was hydrolysed by the purified phytase preparation (Table 3; Fig. 4), indicating that a considerable amount may occur as phytate. Citrate is an effective chelator of trivalent metal ions such as Fe^{3+} and Al^{3+} (Jones and Dar-

rah 1994). In soils, citrate can release P_i into solution either by anion exchange with Fe- and Al-associated phosphates on soil adsorption surfaces (Gerke 1992), or by chelation of precipitates to form soluble compounds (e.g. Gardner et al. 1983). Phytate undergoes similar adsorption and precipitation reactions in soils to P_i (Ognalaga et al. 1994). It is conceivable that soil phytate is released into solution in the presence of citric acid via similar mechanisms.

Citrate also extracted P_o that was readily accessible to a phytase preparation possessing general acid phosphatase activity (Fig. 4). The purified and commercial phytase preparations showed markedly different specific activities against a range of phosphate esters (Table 2). However, it was evident that they also hydrolysed a common component of the citrate-extractable P_o; when combined, the amounts of extractable P_o hydrolysed by the two enzymes often exceeded the total quantity of extractable soil P_o (Fig. 4). Enzyme-labile P_o in citric acid extracts may represent a component of soil P that can potentially be used by plants. In previous work, we have observed that plants grown in sterile culture were able to use P_o substrates, such as G1P and β-glycerophosphate, essentially as equivalent P sources to P_i for growth, while phytate was a relatively poor source of P (Hayes et al. 2000; Richardson et al. 2000).

Higher concentrations of citric acid extracted more soil P_o and also increased the proportion of P_o that was enzyme labile. By contrast, HCl (used at the same molarity as citric acid) extracted minimal phytase-labile P_o. Consequently, the chelating properties of citric acid, rather than acidification effects, were considered to be largely responsible for the extraction of enzyme-labile P_o. The amount of extractable P_o also increased with the pH of the citric acid extractant. It is likely that this was due to the greater chelation ability of the citrate^{2-}/citrate^{3-} species, which would predominate over citrate/citrate^{-} species at higher pH. Gerke and Meyer (1995) found that citrate adsorption to a humic podzol was higher at pH 6.5 compared to pH 5.5. Concurrently, they observed greater mobilisation of P. In the present work, the amount of enzyme-labile P_o did not increase with higher citric acid pH.

Effect of fertiliser history on extractable, phytase-labile soil P_o

Measures of P in extracts of soils with contrasting fertiliser histories indicated that there were marked differences in the amounts of both total P_o and enzyme-labile P_o. Similar amounts of P_o and hydrolysed P were observed in fertilised and unfertilised soils from plots at a recently established field site (Ginninderra). Only the P_i content of the soil at this site had been increased by the relatively recent P fertiliser applications. By contrast, clear distinctions could be made between fertiliser treatments for soil with a long history of fertiliser application (Rutherglen). Differences between P treatments were especially evident in citric acid extracts, where the extracts from the fertilised Rutherglen soil contained approximately threefold greater amounts of enzyme-labile P_o than extracts from unfertilised soil. Extracts of soil which had received both fertiliser and lime similarly contained higher levels of total P_o and enzyme-labile P_o. These results may indicate that, with extended periods of fertiliser application, a greater proportion of applied P accumulates in soil as labile P_o. This component of labile P_o may make an important contribution to the P nutrition of pastures. Mineralisable fractions of soil P_o have been reported to be important for P cycling within permanent pasture systems (McLaughlin et al. 1990).

In conclusion, we have shown that a only small component of the P_o in water and $NaHCO_3$ extracts of soil can be hydrolysed by phytase and general acid phosphatase activities. This may indicate that only low levels of potentially plant-available P_o occur in soil solution. By contrast, greater amounts of enzyme-labile P_o were extracted using citrate. Citrate is exuded into the rhizosphere by roots of a number of plant species and, in many instances, exudation is enhanced under conditions of P deficiency (e.g. Lipton et al. 1987; Hoffland et al. 1989; Grierson 1992; Keerthisinghe et al. 1998). Citrate exudation by plant roots is considered to be an important mechanism for increasing the acquisition of soil P_i. Our results imply that citrate may also increase the availability to plants of soil P_o, by solubilising a fraction that can be hydrolysed by enzymes.

Enzyme-labile soil P_o was also influenced by soil fertiliser history. While P_i was the dominant labile fraction in soils which had received recent applications of fertiliser, in soils of low fertility (e.g. soil U_G) or with a long history of fertiliser application (Rutherglen soil), the enzyme-labile component of citrate-extractable P_o was equivalent to, or exceeded, the quantity of extractable P_i.

Acknowledgements This work forms part of a project which is supported by Australian wool growers and the Australian Government through The Woolmark Company. J.E.H. is a recipient of an Australian Wool Research and Promotions Postgraduate Study Scholarship. The authors are grateful to Markus Wyss, F. Hoffmann-La Roche, for kindly providing the purified *A. niger* phytase preparation, and to the Rutherglen Research Institute, Victoria, for permission to obtain soil samples from plots of the long-term field trial.

References

Adams MA, Pate JS (1992) Availability of organic and inorganic forms of phosphorus to lupins (*Lupinus* spp.). Plant Soil 145:107–113

Anderson G (1980) Assessing organic phosphorus in soils. In: Khasawneh FE, Sample EC, Kamprath EJ (eds) The role of phosphorus in agriculture. American Society of Agronomy, Madison, Wis., pp 411–431

Bessey OA, Lowry OH, Brock MJ (1946) A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J Biol Chem 164:321–329

Bowman RA, Cole CV (1978) Transformations of organic phosphorus substrates in soils as evaluated by $NaHCO_3$ extraction. Soil Sci 125:49–54

Colwell JD (1963) The estimation of the phosphorus fertilizer requirements of wheat in southern New South Wales by soil analysis. Aust J Exp Agric 3:190–198

Findenegg GR, Nelemans JA (1993) The effect of phytase on the availability of P from *myo*-inositol hexaphosphate (phytate) for maize roots. Plant Soil 154:189–196

Gardner WK, Barber DA, Parbery DG (1983) The acquisition of phosphorus by *Lupinus albus* L. III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. Plant Soil 70:107–124

Gerke J (1992) Phosphate, aluminium and iron in the soil solution of three different soils in relation to varying concentrations of citric acid. Z Pflanzenernaehr Bodenkd 155:339–343

Gerke J, Meyer U (1995) Phosphate acquisition by red clover and black mustard on a humic podzol. J Plant Nutr 18:2409–2429

Grierson PF (1992) Organic acids in the rhizosphere of *Banksia integrifolia* L.f. Plant Soil 144:259–265

Hayes JE, Simpson RJ, Richardson AE (2000) The growth and phosphorus utilisation on of plants in sterile media when supplied with inositol hexaphosphate, glucose 1-phosphate or inorganic phosphate. Plant Soil 220:165–174

Hedley MJ, Stewart JWB, Chauhan BS (1982) Changes in inorganic and organic soil phosphorus fractions induced by cultivation practices and by laboratory incubations. Soil Sci Soc Am J 46:970–976

Hoffland E, Findenegg GR, Nelemans JA (1989) Solubilization of rock phosphate by rape. II. Local root exudation of organic acids as a response to P-starvation. Plant Soil 113:161–165

Hübel F, Beck E (1993) In-situ determination of the P-relations around the primary root of maize with respect to inorganic and phytate-P. Plant Soil 157:1–9

Irving GCJ, McLaughlin MJ (1990) A rapid and simple field test for phosphorus in Olsen and Bray No. 1 extracts of soil. Commun Soil Sci Plant Anal 21:2245–2255

Jones DL, Darrah PR (1994) Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. Plant Soil 166:247–257

Keerthisinghe G, Hocking PJ, Ryan PR, Delhaize E (1998) Effect of phosphorus supply on the formation and function of proteoid roots of white lupin (*Lupinus albus* L.). Plant Cell Environ 21:467–478

Kroehler CJ, Linkins AE (1991) The absorption of inorganic phosphate from ^{32}P-labeled inositol hexaphosphate by *Eriophorum vaginatum*. Oecologia 85:424–428

Lipton DS, Blanchar RW, Blevins DG (1987) Citrate, malate and succinate concentration in exudates from P-sufficient and P-stressed *Medicago sativa* L. seedlings. Plant Physiol 85:315–317

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press, London

McKercher RB, Anderson G (1968) Content of inositol penta- and hexaphosphates in some Canadian soils. J Soil Sci 19:47–55

McLaughlin MJ, Baker TG, James TR, Rundle JA (1990) Distribution and forms of phosphorus and aluminium in acidic topsoils under pastures in south-eastern Australia. Aust J Soil Res 28:371–385

Murphy J, Riley JP (1962) A modified solution method for the determination of phosphate in natural waters. Anal Chim Acta 27:31–36

Nelson DW, Sommers LE (1982) Total carbon, organic carbon and organic matter. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis, part 2. Chemical and microbiological properties, vol 2. ASA-SSSA, Madison, Wis., pp 561–573

Newman RH, Tate KR (1980) Soil phosphorus characterisation by ^{31}P nuclear magnetic resonance. Commun Soil Sci Plant Anal 11:835–842

Ognalaga M, Frossard E, Thomas F (1994) Glucose-1-phosphate and *myo*-inositol hexaphosphate adsorption mechanisms on goethite. Soil Sci Soc Am J 58:332–337

Olsen SR, Sommers LE (1982) Phosphorus. In: Page AL, Miller RH, Keeney DR (Eds) Methods of soil analysis, part 2. Chemical and microbiological properties, vol 2. ASA-SSSA, Madison, Wis., pp 403–430

Otani T, Ae N (1999) Extraction of organic phosphorus in Andosols by various methods. Soil Sci Plant Nutr 45:151–161

Pant HK, Edwards AC, Vaughan D (1994) Extraction, molecular fractionation and enzyme degradation of organically associated phosphorus in soil solutions. Biol Fertil Soils 17:196–200

Richardson AE, Hadobas PA, Hayes JE (2000) Phosphomonoesterase and phytase activities of wheat (*Triticum aestivum* L.) roots and utilisation of organic phosphorus substrates by seedlings grown in sterile culture. Plant Cell Environ 23:397–405

Ridley AM, Slattery WJ, Helyar KR, Cowling A (1990) The importance of the carbon cycle to acidification of a grazed annual pasture. Aust J Exp Agric 30:529–537

Schoenau JJ, Huang WZ (1991) Anion-exchange membrane, water, and sodium bicarbonate extractions as soil tests for phosphorus. Commun Soil Sci Plant Anal 22:465–492

Shand CA, Smith S (1997) Enzymatic release of phosphate from model substrates and P compounds in soil solution from a peaty podzol. Biol Fertil Soils 24:183–187

Sharpley AN (1985) Phosphorus cycling in unfertilized and fertilized agricultural soils. Soil Sci Soc Am J 49:905–911

Stace HCT, Hubble GE, Brewer R, Northcote KH, Sleeman JR, Mulcahy JJ, Hallsworth EG (1968) A handbook of Australian soils. Rellim Technical Publications, Glenside, South Australia

Tate KR, Newman RH (1982) Phosphorus fractions of a climosequence of soils in New Zealand tussock grassland. Soil Biol Biochem 14:191–196

Wild A, Oke OL (1966) Organic phosphate compounds in calcium chloride extracts of soils: identification and availability to plants. J Soil Sci 17:356–371

Wyss M, Brugger R, Kronenberger A, Rémy R, Fimbel R, Oesterhelt G, Lehmann M, Loon APGM van (1999a) Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65:367–373

Wyss M, Pasamontes L, Friedlein A, Rémy R, Tessier M, Kronenberger A, Middendorf A, Lehmann M, Schnoebelen L, Röthlisberger U, Kusznir E, Wahl G, Müller F, Lahm H-W, Vogel K, Loon APGM van (1999b) Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. Appl Environ Microbiol 65:359–366