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Enzymatic release of phosphate from model substrates and P compounds in soil solution from a peaty podzol

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Abstract Phosphate in solutions of model esters and polyphosphates (glucose phosphate, inositol hexaphosphate, pyrophosphate, ribonucleic acid, tripolyphosphate and trimetaphosphate) was quantitatively released in <6 h by acid phosphatase or phytase at pH 5.0. Interference from insoluble, ion association complexes formed between protein in the enzymes and the phosphomolybdenum blue during the colorimetric determination of the molybdate reactive phosphorus released was removed by adding dimethyl sulphoxide. Filtered (0.45 μm) soil solution from a peaty soil contained 590 μg dm^{-3} total dissolved phosphorus (TDP), of which 13% was molybdate reactive phosphorus (MRP), 26% dissolved organic phosphorus (DOP) and 61% dissolved condensed phosphorus (DCP). When acid phosphatase was added to the soil solution under the conditions used to hydrolyse the model compounds, MRP increased to 54% of the TDP in about 10 h and then remained constant. From a mass balance, at least 25% of the DCP was hydrolysed. Incubation of the soil solution at 35°C without enzyme increased MRP to 44% of the TDP, reflecting native enzyme activity. Soil solution containing a higher concentration of TDP (1.27 mg dm^{-3}) was also obtained. The distribution of MRP, DOP and DCP fractions was similar but acid phosphatase hydrolysed a greater proportion of the P and MRP increased to 64% of the TDP and at least 40% of the DCP was hydrolysed. The results of hydrolysis with phytase were similar to those with acid phosphatase. The protection of part of the DOP or DCP fraction from hydrolysis was likely caused by occlusion within colloids or the existence of P compounds unlike those of the model substrates.

Key words Enzyme · Hydrolysis · Ion association complex · Phosphorus · Soil solution · Podzol

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Introduction

The forms of phosphorus (P) in soil solution determine the mechanism of its transport and potential bioavailability. Because of their complexity, P compounds in soil solutions are classified as molybdate reactive phosphorus (MRP), dissolved organic phosphorus (DOP) and dissolved condensed phosphorus (DCP) (Ron Vaz et al. 1992). Inositol phosphates have been identified in $CaCl_2$ extracts of soil (Wild and Oke 1966) but knowledge of the structure of DCP (polyphosphates) in soil solution is extremely limited.

Organic phosphates are considered as bioavailable P-sources if they can be hydrolysed (Cembella et al. 1984). Alkaline phosphatase has been used to test the bioavailability of phosphate compounds in surface waters and sediment extracts (Shan et al. 1994). In these studies the contribution of DCP to the pool of hydrolysable P is unknown and the P fraction which did not react with molybdate assumed to be DOP although it may have contained polyphosphates. Our aim was to determine the amount of DOP and DCP in soil solution from a peaty soil and the amount of MRP which could be released by hydrolysis with acid phosphatase or phytase. The addition of enzymes introduces proteins which form insoluble complexes during subsequent colorimetric determination of MRP. A procedure for the elimination of the interference is described.

Materials and methods

Soil and soil solution

Soil solution was from a peaty podzol developed on a till derived from quartz-mica-schist at Glensaugh in NE Scotland (National Grid Reference NO663796). Soil was taken from an area which had been improved in 1985 by the addition of lime and fertiliser and reseeded with ryegrass and clover. The site received additions of superphosphate which provided N, P and K at 116, 22 and 46 kg ha^{-1} $year^{-1}$, respectively. In 1987 an area was fenced to exclude herbivores and thereafter herbage was cut and removed. The organic horizon was

30 cm deep and was sampled to a depth of 15 cm. Soil sample 1 (soil 1) and soil sample 2 (soil 2) were taken 3 weeks apart in June 1995. Soil sample 3 (soil 3) was taken in October 1995. The soils were screened (6 mm). The soil had a pH of 5.0 in 0.01 *M* $CaCl_2$ and contained 65 mg kg^{-1} acetic acid extractable P expressed on a dry weight basis. Soil moisture was determined by heating at 105°C for 16 h. Field water-holding capacity (matrix potential 10 kPa, Gliński and Lipiec 1990) was 72% w/w. Soil 1 and soil 3 had moisture contents near 73% of field capacity. On the day after sampling, soil 1 and soil 3 were wetted-up to 95% of the field capacity and left for 18 h at 4°C in loosely closed polythene bags. Soil 2 had a moisture content of 95% of field capacity and was not further moistened. Soil solution was obtained by centrifugation (Shand et al. 1994), immediately filtered (0.45 µm) and stored at 4°C.

Model compounds

Dodecasodium inositol hexaphosphoric acid with <1% inorganic phosphate calculated as P, 'AnalaR' KH_2PO_4 and tetrasodium pyrophosphate were from BDH. Dipotassium glucose 1-phosphate (98%), disodium β-glycerophosphate, pentasodium tripolyphosphate (98%) and trisodium trimetaphosphate (98%) were from Sigma. The sodium salt of ribonucleic acid (RNA) containing 7% P was from Fluka.

Enzymes

Acid phosphatase from wheat germ (EC 3.1.3.2) and phytase from wheat (EC 3.1.3.26) were from Sigma and had 0.7 and 0.01 units of activity mg^{-1}, respectively.

Reagents for MRP determination

Reagent A Potassium antimonyl tartrate $1/2H_2O$ (0.2908 g in 100 cm^3 water) and ammonium molybdate $4H_2O$ (12.0 g in 250 cm^3 water) were mixed with 148 cm^3 18 *M* sulphuric acid in 1 dm^3 water and the volume adjusted to 2 dm^3.

Mixed reagent Ascorbic acid (8.8 g) was dissolved in 100 cm^3 reagent A.

P composition of model compounds and soil solutions

The total P contents of disodium β-glycerophosphate and sodium nucleinate were determined accurately by inductively coupled plasma optical emission spectrometry using solutions containing about 10 mg P dm^{-3}. The TDP contents of soil solutions were determined (as MRP) after persulphate oxidation (Williams et al. 1995). The MRP contents of solutions of the model compounds (1.0 mg P dm^{-3}) and soil solutions were determined by mixing 5-cm^3 aliquots with 0.95 cm^3 of the mixed reagent and measuring the absorbance at 882 nm after 30 min (Murphy and Riley 1962). The DOP contents of soil solutions were determined by photo-oxidation (Ron Vaz et al. 1992) and DCP calculated by difference: DCP=TDP – (MRP+DOP).

Enzyme preparations

The acid phosphatase (190 mg) was dissolved in 40 cm^3 pH 5.0, 0.1 *M* sodium acetate buffer and dialysed against buffer using Visking tubing (molecular cut-off 12–14 kDa) to remove MRP. Four changes of solution (800 cm^3 each) were made over 20 h. The phytase (334 mg) was similarly purified but with six changes of the dialysis medium.

A portion of the dialysed acid phosphatase solution (20 cm^3) was centrifuged at 25000 *g* for 1 h. The supernatant was decanted and treated with 3.8 cm^3 1.25 *M* H_2SO_4. After 3 h the mixture was re-centrifuged. The drop of brown liquid adhering to the base of the container was rinsed with water, freeze-dried and the infrared spectrum recorded. A similar procedure was carried out with phytase.

Removal of protein interference

Addition of Triton X-100 to enzyme hydrolysates was made prior to the addition of the mixed reagent to prevent a precipitate reaction between protein and phosphomolybdenum blue during determination of MRP. Alternatively, dimethyl sulphoxide (DMSO) was added after the precipitate had formed. To test the effect of the surfactant and DMSO on the determination of MRP the following experiments were performed.

Triton X-100 A solution of KH_2PO_4 (5 cm^3) containing 0.5 mg P dm^{-3} and 0.25% w/v Triton X-100 was treated with the mixed reagent (0.95 cm^3). The absorption spectra (500–900 nm) were recorded 8, 20 and 60 min later. Continuous absorbance measurements were also made at 797 nm.

DMSO A solution of KH_2PO_4 (5 cm^3) containing 0.5 mg P dm^{-3} was treated with the mixed reagent (0.95 cm^3) and left for 7 min. DMSO (3.34 cm^3) was added and the absorption spectra (500–900 nm) recorded 8, 20 and 60 min after the addition of the mixed reagent.

Hydrolysis of model phosphates and soil solutions

The MRP content of the solutions of the model compounds (Table 1) used to test the enzymes were first measured using the mixed reagent alone as previously described. Solutions (4.5 cm^3) of each compound were then treated with the enzyme solution (0.5 cm^3) and incubated for 6 h at 35±1°C. The hydrolysate was treated with the mixed reagent (0.95 cm^3) and left for 7 min. DMSO (2 cm^3) was added and the absorbance of the solution at 890 nm recorded 20 min after the addition of the mixed reagent. The MRP content was determined using calibration graphs from standard solutions of KH_2PO_4 carried through the hydrolysis. To assess chemical hydrolysis the procedure was also conducted with buffer alone. Hydrolysates from soil solution (4.5 cm^3) were treated similarly.

Table 1 MRP in hydrolysates of model compounds expressed as a percentage of their initial total P content. The data is derived from solutions containing 1 mg P dm^{-3}

Compound (sodium salt)	Blank (zero time)	Buffer (6 h)	Phosphatase (6 h)	Phytase (6 h)
Glycerophosphate	1.3	0.2	99.8	88.5
Glucose phosphate	2.1	6.3	103	95.3
Inositol	0.4	1.0	96.9	95.7
Pyrophosphate	3.5	3.0	95.6	101.1
Ribonucleic acid	0.7	0.5	104.9	103.2
Tripolyphosphate	2.8	2.4	97.9	102.8
Trimetaphosphate	2.2	1.6	101.7	99.3
Least significant difference ($P \leq 0.05$)			1.04	1.81

Results

Protein interference in the determination of MRP

Storing the mixed reagent at room temperature (25°C) resulted in a reduction in the rate of formation of phosphomolybdenum blue (Fig. 1). The mixed reagent was either used within 1 h of preparation or stored at 4°C and used within 7 h. The absorbance against time profile using mixed reagent stored at 4°C for 7 h was identical to that using reagent stored at room temperature for 1 h. MRP determinations were made at least 5 min after addition of the mixed reagent. In this period, after adding the mixed reagent to solutions of phosphate containing the enzymes, the solutions became blue and cloudy. On standing a blue precipitate formed and the supernatant was virtually colourless.

When the acid phosphatase solution (20 cm^3) alone was acidified with H_2SO_4 to the acidity attained during MRP determination (pH 1), and centrifuged, a small amount (2 mg) of brown liquid collected. The IR spectrum of the material in KBr was dominated by amide absorptions at 3303, 3080, 1652, 1536 and 1407 cm^{-1} typical of protein, with bands at 1223, 1190, 1080, 1051, 886, 590 and 452 cm^{-1} assigned to polyphosphate. A similar material was obtained from the phytase but there was no evidence for polyphosphate.

The addition of Triton X-100 prevented formation of the blue precipitate when added to enzyme hydrolysates but there was a marked shift in the main absorption from 882 to 797 nm. Additionally, the absorption at 797 nm continued to increase even after 1 h. Adding Triton X-100 after precipitation gave a single phase but the visible spectrum showed two maxima of almost equal intensity at 687 and 812 nm. After 1 h there was a shift to longer wavelengths (693 and 816 nm) and the emergence of a third maximum at 877 nm.

Adding DMSO after formation of the precipitate also gave a single phase. About 10% v/v DMSO was effective but 40% v/v was required for rapid dissolution of precipitates from solutions containing >1 mg P dm^{-3}. The absorption spectrum of the phosphomolybdenum blue showed a small increase in the wavelength of the main maxima from 882 to 890 nm (Fig. 2). The reduction in absorbance sensitivity was the result of dilution. With 40% v/v DMSO, absorbance measurements with a 4-cm cell were directly proportional to phosphate concentration up to 1.5 mg dm^{-3}. After 1 h there was little change in the absorption intensity at 890 nm but a new absorption band emerged at 821 nm. The addition of DMSO to hydrolysate at 40% of the sample volume, after the addition of mixed reagent, was used to determine MRP in enzyme hydrolysates.

Hydrolysis of model phosphate compounds

The model substrates contained ≤3.5% MRP and there was little hydrolysis with buffer alone, except for glucose phosphate (Table 1). Acid phosphatase or phytase quantitatively (>95%) hydrolysed the compounds within 6 h except glycerophosphate, which was 88% hydrolysed by phytase.

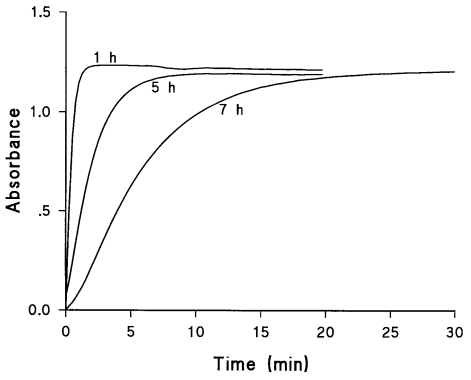

Fig. 1 Effect of storing the mixed reagent at room temperature (25°C) on the rate of formation of the phosphomolybdenum blue complex for a solution of KH_2PO_4 containing 0.5 mg P dm^{-3}. The reaction was monitored at 882 nm using a 4-cm path cell

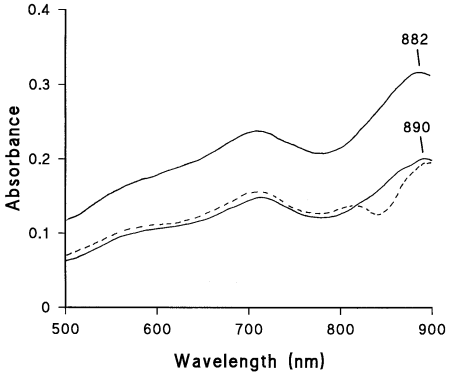

Fig. 2 Absorption spectra of the phosphomolybdenum blue complex from orthophosphate solution containing 0.5 mg P dm^{-3} in a 1-cm cell. *Top* without DMSO, *lower solid* with DMSO (40% v/v of standard solution) 20 min after adding the mixed reagent, *dashed* with DMSO (40% v/v of standard solution) 60 min after adding the mixed reagent

Hydrolysis of phosphate compounds in soil solutions

Soil solution from soil 1 contained 590 μg dm^{-3} TDP. The addition of enzyme caused rapid hydrolysis and the MRP concentrations reached a plateau (Fig. 3). The data was fitted with a logistic sigmoid function which accounted for >99% of the variation. At the end of the hydrolysis the MRP concentrations were about 320 μg dm^{-3} for acid phosphatase and phytase hydrolysis, representing 54% of the TDP (Table 2). Incubation of the soil solution at 35°C with buffer alone resulted in a substantial hydrolysis with a 31% increase in MRP from 13% to 44% of the TDP. Thus only an extra 11% was hydrolysable by the added enzymes.

Table 2 Initial composition of soil and hydrolysis characteristics of their soil solutions

Soil sample number	Soil moisture (%[a])	Initial soil solution composition				MRP (%) in soil solution after hydrolysis			
		TDP (mg dm^{-3})	MRP (%)	DOP (%)	DCP (%)	Hydrolysis time (h)	Phosphatase	Phytase	No added enzyme
1	73	0.590	13	26	61	32	54	55	44
2	95	1.27	14	26	60	26	64	67	49

[a] Expressed on the basis of field capacity

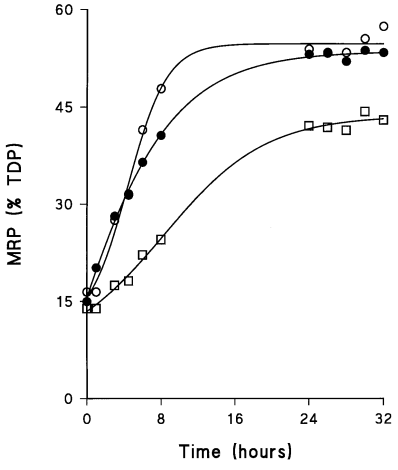

Fig. 3 MRP released from soil solution: ○ phytase, ● acid phosphatase, □ no added enzyme (pH 5.0, 35°C). Standard errors of means (n=4) were <1% of the TDP

The soil solution from soil 2 contained twice the concentration of TDP compared with soil 1 but it had a similar distribution of P-forms (Table 2). Hydrolysis of soil solution from soil 2 with acid phosphatase or phytase yielded a higher percentage of MRP than soil solution from soil 1 (Table 2).

The phosphatase activity of solution from soil 3 was 1.11 nmoles min^{-1} cm^{-3} measured with p-nitrophenyl phosphate (Vaughan et al. 1978), equivalent to 0.3% of the activity of the acid phosphatase added to the soil solutions in the hydrolysis experiments.

Discussion

To study the hydrolysis of phosphate compounds in soil solution it was necessary to overcome the precipitation of ion association complexes (Kirkbright et al. 1971) formed between protein in the enzymes and the phosphomolybdenum blue. Acidification of the enzyme solution alone also caused precipitation of protein. Pant et al. (1994) did not report the formation of precipitates using the same enzymes with water extracts of soils. Kallner (1975) found that addition of polyvinyl alcohol prevented the precipitation of complexes between malachite-green and protein during the determination of phosphate in serum and urine but we were unable to obtain the same polymer. The addition of Triton X-100 prevented precipitation but gave a complex with a different absorption spectra. The addition of Triton X-100 after formation of the precipitate gave a single phase but the spectral characteristics changed on standing. We found that the addition of DMSO to the hydrolysate (40% v/v) after formation of the precipitate gave the best results. The visible absorption spectrum was similar to the phosphomolybdenum blue described by Murphy and Riley (1962) and was stable for at least 20 min. Shida et al. (1994) used DMSO as a solvent for a similar ion association complex formed between phosphomolybdenum blue and a quaternary ammonium cation.

The acid phosphatase and phytase hydrolysed bonds in a range of substrates including monoesters, a complex diester (RNA), pyrophosphate, a linear polyphosphate and a cyclic polyphosphate, demonstrating a lack of specificity and no requirement for other cofactors.

Only partial hydrolysis of the combined DOP-DCP fraction was observed in soil solutions. Incomplete hydrolysis of soil solution phosphates may be explained by the occlusion of phosphate within colloidal material (Hannapel et al. 1964) providing protection from enzyme attack and could explain the non-availability of part of the soil solution P to plants found by Rogers et al. (1940) as discussed by Martin (1970). Alternatively, the soil solutions may not have contained simple phosphate linkages of the kind in the model compounds. From our data it can be deduced that up to 40% of the DCP fraction in soil solution from soil 2 was hydrolysed by phytase, indicating at least part of this largely uncharacterized form of P may be bioavailable.

Hydrolysis occurred in soil solutions without added enzyme, indicating chemical lability or enzyme activity in the 0.45-µm-filtered soil solutions. The phosphatase activity measured using p-nitrophenyl phosphate was about 1 nmole min^{-1} cm^{-3} equivalent to 0.3% of the activity of the acid phosphatase added, implying a high efficiency towards hydrolysis of phosphate compounds in soil solution. Plant phosphatase can have molecular sizes smaller than the 0.45-µm exclusion limit of our filters (Ozawa et al. 1995).

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