# Characterisation of organic phosphorus compounds in soil by phosphatase hydrolysis

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**Abstract**
Many organic and condensed forms of phosphorus (P) can be found in soils. Phosphate groups have to be hydrolysed from these P compounds to become available to plants and microorganisms. This chemical reaction is catalysed by several hydrolytic enzymes, which can be used experimentally to investigate the nature and hydrolysability of soil organic P. As a prerequisite to this approach, phytases, alkaline phosphatases, acid phosphatases, and an inorganic pyrophosphatase were tested for their specificity against eight P substrates (monoesters, diesters, inorganic and organic phosphoanhydrides, and a phosphonate). The inorganic pyrophosphatase preparation was specific against pyrophosphate.The other enzymes hydrolysed simple monoesters and condensed phosphate completely and differed only in their ability to hydrolyse phytate, DNA and RNA. None of the enzymes hydrolyzed the model phosphonate. The implications for the interpretation of the experiments planned with soils are discussed.

**Key Words**
Enzymes, organic phosphorus, soil, phosphomonoesterase, inorganic pyrophosphatase.

## Introduction

An important part of P in soils is represented by organic and condensed P compounds, which comprise several chemical classes: orthophosphates, pyrophosphates, polyphosphates, orthophosphate monoesters, orthophosphate diesters and phosphonates (Turner *et al.* 2003). These forms of P may function as a source of P for soil organisms and plants after the release of phosphate. The release of phosphate is promoted by enzymatic activity. Several studies using enzyme additions to soils have been published in the recent decades (Jackman and Black 1952; Shand and Smith 1997; Hayes *et al.* 2000; Turner *et al.* 2002; George *et al.* 2007). In these, many different hydrolytic enzymes have been employed. Widely varying conditions during the assay, including choice of buffer and pH value, added enzyme activity, duration and temperature, presence of a microbial inhibitor, end of the reaction and method for determination of product assay conditions make comparison between previous studies difficult or impossible (Bünemann 2008). For this reason, Bünemann proposes a standard protocol for enzyme addition assays. To achieve this, however, it would be necessary to use commercially available preparations that can be easily purchased. A prerequisite to interpret the hydrolysis data correctly is accurate characterisation of the enzymes. Substrate specificity, kinetic properties, and the isoelectric point must be known, and information about the inhibition and inactivation of the enzymes is needed. This fundamental information has been absent in many publications. Furthermore, there is no study that characterizes and compares a comprehensive selection of commercially available phosphatases under the same conditions.

Our ultimate goal is to gain more information about the bioavailability of soil organic P by the use of enzyme addition to soil. Here we compare several commercial enzymes, using model substrates in order to characterize them for their substrate specificity. In subsequent steps, the preparations will be verified for purity, and diesterases will be included in the enzyme list as well. Having characterized the enzyme preparations, tests on soil extracts and subsequently on soil suspensions will be carried out.

## Methods

*Principle of enzyme addition*
Commercially available enzymes are added to model P compounds, and the amount of molybdate-reactive P released is measured using malachite green (Ohno and Zibilske 1991). To ensure completion of the reaction, the release must reach a plateau, and several time points must therefore be sampled.

*Enzymes, buffers and model substrates*

Aqueous solutions of alkaline and acid phosphomonoesterases, phytases and inorganic pyrophosphatase were prepared and added to the appropriate buffers (
Table 1). Four dilutions of each enzyme were pretested on two substrates. The first dilution was chosen based on the indication of enzymatic activity against *p*-nitrophenol given by the supplier and diluted 10, 100 and 1000 times. In order to have the lowest possible consumption of enzyme preparations, and to avoid interferences with the colorimetric analysis, the highest dilution that still achieved maximum hydrolysis was chosen for each enzyme (data not shown).

**Table 1. Hydrolytic enzymes and their respective abbreviation, supplier, source, preparation of the commercial product, activity against *p*-nitrophenol indicated by the supplier, and buffer type and concentration in the well. Freeze dried powder, F; liquid preparation, L; granules, G.**

| Enzyme | Abbr. | Supplier | Source | Prep. | Activity | Buffer |
|---|---|---|---|---|---|---|
| Alkaline phosphatase [a] | AlPS | Sigma | *Escherichia coli* | F | 0.05 – 1.00 µkat/mg protein | 0.2 M Glycine pH 9.0 |
| Alkaline phosphatase [a] | AlPR | Roche | Calf intestine | L | 33.34 µkat/mg | 0.2 M Glycine pH 9.0 |
| Acid phosphatase [b] | AcPS | Sigma | Potato | F | 0.05 – 0.17 µkat/mg solid | 0.2 M Mes pH 5.2 |
| Acid phosphatase [b] | AcPR | Roche | Potato | F | ~0.03 µkat/mg lyophilizate | 0.2 M Mes pH 5.2 |
| Phytase [c] | PhyN | Novozyme | *Peniophora lycii* | L | ≥ 83 µkat/mg | 0.2 M Mes pH 5.2 |
| Phytase [d] | PhyB | BASF | *Aspergillus niger* | G | n.s. | 0.2 M Mes pH 5.2 |
| Pyrophosphatase [e] | PyPS | Sigma | *Saccharomyces cerevisiae* | F | ≥8.34 µkat/mg protein | 0.2 M Hepes pH 7.0 |

[a] EC 3.1.3.1, [b] EC 3.1.3.2, [c] EC 3.1.3.26, [d] EC 3.1.3.8, [e] EC 3.6.1.1

Enzymes were tested against eight model P substrates belonging to different functional classes (
Table **2**). The concentration of total P in the model substrate solutions was determined by autoclaving 0.1 ml of substrate with 1.0 ml of 0.9 M sulfuric acid containing 0.14 M ammonium persulfate. Enzyme solutions, buffers und substrates were prepared with autoclaved Nanopure® water.

**Table 2. The eight P substrates tested in this experiment, their abbreviations, suppliers, functional class and concentrations used (means and standard deviations shown).**

| Substrate | Abbr. | Supplier | Functional class | Conc.(mmol P /L) |
|---|---|---|---|---|
| Myo-inositol hexakiphosphate | Ins6P | Sigma Chemicals | Phosphate monoester | 9.0 ± 0.1 |
| D-Glucose 6-phosphate | G6P | Sigma Chemicals | Phosphate monoester | 9.8 ± 0.1 |
| Glycerol phosphate | GP | Sigma Chemicals | Phosphate monoester | 6.7 ± 0.1 |
| Deoxyribonucleic acid | DNA | Sigma Chemicals | Phosphate diester | 6.5 ± 0.3 |
| Ribonucleic acid | RNA | Sigma Chemicals | Phosphate diester | 5.5 ± 0.1 |
| Pyrophosphate | PP | Riedel de Haën | Phosphoanhydride | 9.7 ± 0.1 |
| Adenosine 5'-triphosphate | ATP | Roche | Organic phosphoanhydride | 9.9 ± 0.01 |
| 2-Aminoethyl phosphonic acid | AEP | Aldrich | Phosphonate | 9.8 ± 0.2 |

*Assay procedure*

Assays were made in flat-bottomed microtiterplates made of polystyrene (Greiner Bio one GmbH, Frickenhausen, Germany). Microtiterplates were put on ice, and the following volumes were added to each well: 60 µl of buffer, 20 µl of different P compounds, 20 µl of enzyme preparations and water to make the total volume up to 300 µl. The assay was performed with 5 analytical replicates. Controls with enzyme only or substrate only were included. An internal orthophosphate standard curve was prepared in duplicate. Plates were incubated at 40 rpm and 30°C (Vortemp™ 56, Labnet international Inc.). Measurements were made just after the addition of enzyme, when the microplate was still on ice, after 30 min., 60 min., 180 min., 24 h and 48 h, in order to verify the completion of the reaction. With each measurement, 20 µl were removed from the incubation plate and diluted 6 times. Finally, 20 µl diluted liquid were transferred to a new plate and made up to 200 µl with water. Molybdate-reactive P was determined using malachite green (Ohno and Zibilske 1991). No additional reagent was used to stop the reaction, as it had been demonstrated in the case of PhyN that the first reagent of the colorimetric measurement stops the enzymatic reaction. Plates were read at 620 nm at room temperature in a computerized microplate spectrometer (Biotek ELx800 Absorbance Microplate Reader, BioTek Instruments, Inc., USA).

## Results

Completion of the reaction was reached for almost all substrates after an incubation period of 24 h. Absorption values of enzyme controls were all below the detection limit; thus, the amount of P released by enzyme hydrolysis was calculated from the difference between the assay with substrate and enzyme and the

control with only substrate.
AEP was not hydrolyzed by any of the enzyme preparations, whereas PP was hydrolyzed almost completely by all enzymes (Table 3). The two alkaline phosphatases gave very similar results: they were active against G6P, GP, and phosphoanhydrides, giving complete recovery, whereas Ins6P, DNA and RNA were hydrolyzed at rates below 12%. The two acid phosphatases hydrolysed monoesters, DNA and phosphoanhydrides approximately at the same rate as the alkaline phosphatases. However, the AcPR showed higher hydrolysis rates for Ins6P and RNA (62.5% and 64.1%, respectively). The two phytases hydrolyzed Ins6P at high levels (93.2% and 77.3%, respectively), and they also hydrolyzed monoesters and phosphoanhydrides. RNA was hydrolyzed to a greater extent by PhyN (86.9%) than by PhyB (11.7%). Finally, PyPS was very specific against PP, which was hydrolyzed at 92.8%, and did not hydrolyze any of the other compounds.

**Table 3 Proportion of P hydrolyzed by enzymatic catalysis after 24h. Means and standard deviations (n=5).**

| Substrate | AlPS | AlPR | AcPS | AcPR | PhyN | PhyB | PyPS |
|---|---|---|---|---|---|---|---|
| | Substrate recovery as hydrolyzed P (%) | | | | | | |
| Ins6P | 8.9 ± 0.3 | 4.1 ± 1.3 | 5.7 ± 0.5 | 62.5 ± 8.0* | 93.2 ± 3.2 | 77.3 ± 2.2 | -0.7 ± 0.3 |
| G6P | 97.5 ± 1.4 | 95.9 ± 3.0 | 92.4 ± 3.9 | 96.7 ± 2.2 | 105.6 ± 3.9 | 94.0 ± 3.1 | -0.4 ± 0.2 |
| GP | 94.9 ± 2.4 | 95.0 ± 3.4 | 90.0 ± 3.0 | 95.7 ± 1.8 | 103.9 ± 3.3 | 76.1 ± 2.0 | -0.4 ± 0.5 |
| DNA | 9.0 ± 0.5 | 11.7 ± 1.1 | 9.4 ± 0.5 | 10.7 ± 0.8 | 11.5 ± 1.3 | 9.3± 0.7 | -1.3 ± 0.1 |
| RNA | 10.7 ± 1.5 | 10.8 ± 1.9 | 13.8 ± 1.3 | 64.1 ± 2.5* | 86.9 ± 4.1 | 11.7 ± 1.0 | -0.3 ± 0.2 |
| PP | 95.9 ± 1.8 | 88.2 ± 1.8 | 90.3 ± 0.9 | 95.9 ± 2.3 | 109.0 ± 4.9 | 93.4± 4.5 | 92.8 ± 4.0 |
| ATP | 96.9 ± 1.9 | 95.1 ± 3.5 | 90.7 ± 4.7 | 98.3 ± 4.2 | 129.4 ± 0.6 | 71.4 ± 1.8 | -0.1 ± 0.3 |
| AEP | 0.3 ± 0.6 | -0.5 ± 0.5 | -0.1 ± 0.7 | 0.3 ± 0.7 | 0.7 ± 0.2 | -0.1 ± 0.6 | -0.9 ± 0.6 |

* Completion of the reaction was not reached

## Discussion

The most specific enzyme tested was PyPS, which released phosphate only from PP. The other enzymes were less specific, but they released Ins6P and RNA at different rates. The poor specificity of AcPR could be due to an impurity of the preparation. The fact that PhyN is able to hydrolyze RNA to an extent of 86.9% is also an indication that impurities are present. The phosphate release from DNA was on average about 10%. This phenomenon could be explained by the fact that monoesterases are able to hydrolyze just the 5' and 3' phosphate residues (Gasmi *et al.* 1991), while the other phosphate groups are not accessible. None of the enzymes triggered P release from the studied phosphonate.

All enzymes tested show a high release of at least one substrate tested. Thus, they are all good candidates for the tests on soil extracts and soil suspensions. The release of PP from solutions can be determined directly with the PyPS. For the enzymes that hydrolyze more than one chemical class, the release of phosphate from soil has to be calculated by measuring the difference in the phosphate release resulting from two or more enzymes. In this way we will be able to calculate the amount of Ins6P available to enzymes by comparing the results from AcPR, PhyN or PhyB with AlPS, AlPR and AcPS. Phosphate released from DNA would be calculated in the same way (Feuillade and Dorioz 1992). Therefore it is important to add a phosphodiesterase to the enzyme set. The further characterization (kinetic properties, inhibition and inactivation) will be made on soil samples. The isoelectric point will be a tool to determine the reason for a low enzymatic activity in soil suspensions, where several factors have to be considered. For example, adsorption on clay surfaces and the presence of proteases play a role in terms of stability and in the ability of an enzyme to hydrolyze organic P compounds in soil (Nannipieri *et al.* 1996).

The use of enzymes with different degrees of substrate specificity and the combination of some of these enzymes enables the characterization of the hydrolysable amount of organic P in the soil. Together with the additional information collected for the single enzymes a better understanding of the role of enzymes in the soil can be obtained.

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