Measuring Phosphatase Activity in Peatland Soils: Recent Methodological Advances

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Abstract

Measurements of phosphatase activity in peatlands are made difficult by the low levels of activity and the characteristically high concentrations of dissolved organic matter within the sediments. These materials may cause high background absorbances in colorimetric assays, and quenching interference in fluorimetric assays. This review describes the development of a new approach which allows such problems to be overcome by using HPLC to separate the interferences from the products of enzymic hydrolysis. This approach is applicable to various environmental samples such as peat, wetland sediment, and sludge which may contain a large amount of interfering organic matters.

Keywords: Methylumbelliferyl phosphate, HPLC, Quench correction, Organic soil

1. Methodological Development of Phosphatase Measurement

Phosphatase is an enzyme that release inorganic phosphate from organic moiety, and is known to play a key role in phosphorus cycle in various ecosystems including soil, lakes, and wetlands.6 Interest in the study of phosphatase in soil dates as far back as 1927, when Parker suggested that transformation of soil organic phosphates was caused by enzymic processes.2 The presence of a soil phosphatase enzyme was later demonstrated in the 1940s by Rogers, by following the release of inorganic phosphate from glycerophosphate.3 Many studies took this same methodological approach in the 1940s. However, it was soon realised that the approach was relatively inefficient, for it was unable to quantitatively account for enzymatically released phosphate which later became adsorbed onto the soil matrix.

A major methodological advance came in the 1950s, when Kroll et al. introduced the use of artificial model substrates.4 Phenyl phosphate was adopted as the substrate, but, instead of measuring inorganic phosphate release, the authors measured the release of the organic part of the molecule, the phenol. The organic moiety could be efficiently extracted and quantified colorimetrically. Since then, measurement of the release of the non-phosphate moiety has become the cornerstone of phosphatase activity measurements, albeit with a range of alternative organic moieties such as -napthylphosphate,5 glycerophosphate,6 and ultimately p-nitrophenyl phosphate.7 The latter was the most sensitive of those early methods, and has become the standard assay for measuring phosphatase in soils.8

Although peatlands are generally classified as soil systems, the conventional p-nitrophenyl phosphate method9 tends to be of limited value in these wetland systems. The major reason is the relative insensitivity of the colorimetric detection procedure used in p-nitrophenyl assays. This is a major limitation in an ecosystem which is characterised by extremely low rates of enzymic hydrolysis.10 This problem appears to be specific to peatland systems, rather than wetlands in general. In highly active wetland ecosystems such as the Florida Everglades, sensitivity is far less of an issue, and p-nitrophenyl assays are more easily applied.11 The sensitivity of the p-nitrophenyl methods is further impaired by the abundance of highly coloured phenolic compounds, which dominate the peatland dissolved organic carbon, and cause high background absorbances.

In other low activity ecosystems, it has been found possible to overcome the methodological limitations of the p-nitrophenyl approach, through the use of the fluorogenic methylumbelliferyl substrates. These compounds have been widely adopted aquatic
ecosystems, where low activity is often found, such as oligotrophic lakes, streams and the oceans, due to the inherently greater sensitivity of the fluorimetric detection procedure. The technique uses the compound methylumbelliferone (MU) which fluoresces at 450 nm emission (330 nm excitation). Chemical attachment of phosphate to the MU causes the loss of the fluorescent property. Upon enzymic hydrolysis of the bond between MU and phosphate, the MU is liberated, and its fluorescence can be readily measured and related to the activity of the hydrolytic enzyme. While this method should in principle be superior to the p-nitrophenyl method for peatlands too, in practice its application has proven more problematic in organic-rich soils and sediments. First, for example, it has been necessary to find ways of minimising the adsorption of MU onto sediments. Secondly, highly coloured phenolic materials dominate the dissolved organic carbon (DOC) pool of organic-rich peat soils. These materials may cause quenching interferences in fluorimetric enzyme assays. In peat homogenates, quench can reduce the intensity of fluorescence (Fig. 1) by absorbing radiation at both the excitation and emission wavelengths used in the fluorimetric detection procedure. Quenching shows tremendous variation on both a temporal and spatial basis. On a spatial basis, for example, quench-induced loss of fluorescence can vary between 13% in an aerobic riparian peat soil with a low DOC concentration (2.3 mg/L) to 93% in an organic rich (DOC 185 mg/L) Malaysian tropical peat swamp (unpublished data). Thus, without appropriate correction procedures, quench can lead to serious, and highly variable, underestimates of fluorescent product release. Unfortunately, quench correction is labour intensive and requires measurements of the detectability of known amounts of MU in every sample matrix and on every sampling occasion. Although, the quenching phenomenon is particularly serious in organic-rich matrices such as peat, it has also been found to restrict the application of the method in some aquatic systems such as organic-rich brown water streams.

2. Development of HPLC-based Method

Recent years have seen the development of an analytical solution to the problem; an HPLC-based chromatographic separation has been developed which allows the detection of the fluorescent MU-product without interferences from quenching agents. In that method, HPLC analyses were carried out using a Dionex 2020i system. Separations were performed on a Spherisorb SSP bonded phase phenyl column, of length 25 cm with 5 μm packing material (Phase Separations Inc, Norwalk CT). An eluent of 50% methanol:water (pH 7.5) was pumped isocratically at a flow rate of 0.5 mL min⁻¹ and the eluted MU was detected fluorimetrically using a Perkin Elmer LS50 detector at 320 nm excitation, 450 nm emission (slit width 2.5). Detection levels as low as 200 nM MU were achievable, with detection linearity extending to more than 200 μM. A 50 μL injection volume was applied throughout. All samples were filtered through a 0.2 μm Anopore filter prior to injection (without detectable losses through adsorption).

Separation of quenching phenolics from methylumbelliferone was evaluated by adding a Dionex VDM II variable wavelength detector to the system (in series with the fluorimetric detector) and monitoring absorbance at 450 nm (MU emission wavelength). Water samples were extracted by centrifugation of peat from an acid bog, calcareous fen and flush channel peatland in north Wales, and a Malaysian tropical peatswamp. A sample was also collected from the brown-water river Conwy. Each sample was spiked with MU to give a concentration of 100 μM and diluted to 50% with methanol, prior to injection onto the HPLC system. Quenching materials tended to elute from the column in an unresolved form (Fig. 2(b)-(c)), rather than as the distinct peak produced by MU (Fig. 2(a)). This is likely to be due to the highly heterogeneous nature of the phenolic materials of the peat soils. Elution of quenching material was complete after between 3.3 (acid bog) and 4.9 min (fen), while MU began to elute from the column after 5.8 min. Thus, the HPLC approach appeared to efficiently separate MU from quenching materials in diverse samples.

A confirmation of the ability of the HPLC method to overcome the problem of quench was achieved by adding increasing amounts of quenching materials to a fixed concentration of MU (100 μM). The effects of the additional quenching agents on conven-
Fig. 3. Detection of MU using fluorimetry (shaded) and HPLC-fluorimetry (unshaded) following addition of increasing amounts of quenching phenolics. Error bars indicate s.e. values (n = 3) (From 22).

tional-fluorimetric and HPLC-fluorimetric detection were compared. The phenolics used in the additions were extracted from a sample of fen peat using centrifugation. A marked decline in fluorescence was detected with conventional fluorimetry with each increase in the abundance of quenching agents. However, additional quench did not significantly impair the detection efficiency of the HPLC approach (p > 0.05; Fig. 3). Thus, it would appear that the HPLC technique, is a sensitive assay with none of the problems of quench that are associated with earlier techniques.

3. HPLC-UV Detector Method

While monitoring the absorbance of the eluent at the excitation and emission wavelengths, it was noted that the UV detector was able to detect the elution of the free methylumbelliférole (Fig. 4). This suggested that it might be feasible to eliminate the fluorometric detector, and thus allow the measurement of phosphatase activity with standard laboratory equipment; namely a low cost isocratic HPLC with standard UV detector. To test this, we prepared an homogenate for phosphatase assay containing 10 mL peat and 22.5 mL methylumbelliferyl phosphate (MUP). The mixture was blended by stomacher for 30 sec. After the reaction at 10°C, 0.7 mL of the soil slurry was added to 2.1 mL of 100% methanol (HPLC grade) and mixed with a vortex mixer for 20 sec. This step was included to stop the reaction and to increase the recovery of free-MU released from the soil slurry. The sample was filtered with a 0.2 μm ion chromatography syringe filter (Gelman) and loaded to the HPLC with a Spherisorb S5P column and a detector set at 250 nm and eluent of 50% methanol.

The method was applied to three sediments which were collected from a bog (Migneint, UK grid reference SH 805 458), a fen (Gors Goch, SH 497 826), and a swamp (Cwm-y-Glo, SH 554 626). The results were compared with the activities determined by conventional method to test the sensitivity. The peaks for MUP and MU appeared at 4.9 and 8.3 min respectively (Fig. 4). Methanol was efficient as a terminator of the reaction as well as an extraction reagent. The recovery rate of MU free acid was 99% (n=3).

The activities measured by the conventional method were lower compared to the HPLC method (Fig. 5). When the method was applied to three representative wetland sediments in north Wales, for all sites, HPLC detected 17%, 39% and 15% higher activities than fluorimeter method for the bog, the fen and the swamp sediments, respectively (Fig. 5). The results imply that the conventional method might underestimate phosphatase activity, when samples with high phenolics are assayed.

Although internal control (i.e., measurement of recovery rate for MU free acid) for each measurement and correction of calibration curve using water from the field could be used as an alternative to the HPLC method, it is time-consuming and tedious. Furthermore, the highly heterogeneous spatial and temporal distribution of phenolics would impede a proper setting of internal controls unless carried out with every sample analysed. The HPLC-UV method presented here provides a simple and precise method for the assay of phosphatase activity in samples with high phenolics content, such as wetland soils, sediments and dark-coloured water.
4. Development of Multiple-enzyme Assay

Our current work has taken the evolution of the methodology one step further. In Fig. 4, it will be noted that in addition to the MU product peak, it is also possible to detect a second peak (methylumbelliferyl phosphate substrate). We have been studying the feasibility of taking a radically different approach to the MU assay which involves measuring the rate of substrate depletion, instead of product formation. The main benefit of this new approach, is that it becomes possible to monitor the hydrolysis of a suite of substrates in a single assay. Thus for the same number of man-hours as is required for a single phosphatase assay, it becomes feasible to study the interactions between a range of enzymes associated with the cycling of phosphorus. This approach has successfully been applied to wetland samples.

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References