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Current standard assays using artificial substrates overestimate phosphodiesterase activity

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ABSTRACT

Phosphodiesterases (PDE) are important enzymes in environments with significant proportion of phosphodiesters. The breakdown of phosphodiesters is a two step process, initiated by PDE hydrolyzing an ester-P bond on the diester molecule, breaking it into an organic moiety and a phosphomonoester. In the second step, the phosphomonoester is further hydrolyzed by phosphomonoesterase (PME) into the second organic moiety and free phosphate, P_i. The methods to assess extracellular PDE activities commonly use the substrate analogues bis-(*p*-nitrophenyl) phosphate (bis-*p*NPP) or bis-(4-methylumbelliferyl) phosphate (bis-MUFP), measure the resulting concentration of *p*-nitrophenol (*p*NP) or 4-methylumbelliferon (MUF), and assume the contribution from phosphomonoester hydrolysis by PME to be insignificant. To verify this, we measured concentrations of MUF and P_i at the end of the 30- and 60-min incubation of wetland plant root segments with a phosphodiester substrate. We found that the hydrolysis was complete (MUF:P_i ratio ~ 2) confirming the importance of step 2. We suggest that a correction for additional hydrolysis by PME should be employed in samples where this is possible, such as plant roots or microbial cultures. Here, the MUF:P_i should be verified for each new sample type tested for its PDE activity, and values obtained from the PDE assay should be lowered accordingly. We do not recommend using the standard artificial substrates for PDE activity measurements in more heterogeneous samples, for example soils, where these assays have low reliability and can produce erroneous results.

1. Introduction

Phosphodiesters, such as nucleic acids and phospholipids, are major sources of phosphorus (P) in fresh organic detritus (Bielski, 1973; Kapoor and Haider, 1982), but their possible utilization, especially by plants, has received very little attention so far (Leake and Miles, 1996). One of the reasons for this discrepancy may be the general belief that, due to high susceptibility of phosphodiesters to hydrolysis, phosphomonesters are the prevailing form of organic P in soils. While this is generally true for most agricultural soils, where the diesters may constitute below 1% of total organic P (Condron et al., 2005), there is increasing evidence that the accumulation of phosphodiesters can be relatively high in soils and sediments from cold and/or wet environments, where they often represent a significant proportion of the organic P pool (Leake and Miles, 1996; Makarov et al., 2002; Turner and Newman, 2005). The mineralization of organic P is a critical link for internal P cycling in these environments and, to a large degree, determines the productivity and water quality of the ecosystems (Robinson et al., 1998). Phosphodiesters are hydrolyzed into phosphomonoesters by extracellular phosphodiesterases (PDE), ubiquitous phosphatases produced by a wide variety of organisms (Duff et al., 1994).

The information on extracellular PDE is still rather limited and the available data, mostly from agricultural soils, deal almost exclusively with acid or alkaline phosphatase, i.e. phosphomonoesterase (PME), activities. In studies, which have included PDE activity measurements, PME generally show a much higher proportion of activity, with PDE activities in some cases an order of magnitude lower (Asmar and Gissel-Nielsen, 1997). In our recently published work (Rejmánková et al., 2011), we indicated the potential ecological importance of PDE, showing that all 21 common wetland plants under study exhibited significant and constitutive root PDE activity, which correlated well with the proportion of diesterase-hydrolyzable dissolved non-reactive P in the sediments. During our preliminary assays, however, we found the PDE activities to be relatively high, with the ratio of PDE:PME >1 and often close to 2. This made us suspicious of potential methodological problems.

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Determinations of phosphatase activities commonly use substrate analogues, i.e., *p*-nitrophenyl phosphate (*p*NPP) for PME and bis-*p*NPP for PDE or 4-methylumbelliferyl phosphate (MUFP) for PME and bis-MUFP for PDE, the hydrolysis product of which can be measured spectrophotometrically or fluorometrically, respectively (Whitton et al., 2005). The enzyme activities are expressed as the amount of *p*NP or MUF released per time.

Based on the reaction stoichiometry (see equations below and Fig. 1), similar activities of PDE and PME are needed to liberate the orthophosphate (P_i) from a phosphodiester to be taken up by organisms, the latter activity being a bottleneck. While PME directly liberates P_i from various phosphomonoesters:

$$\text{MUFP} \xrightarrow{\text{PME}} \textbf{MUF} + P_i \quad (1)$$

PDE hydrolyses only one ester-P bond on the diester molecule, i.e., it is breaking bis-MUFP into MUFP (or bis-*p*NPP into *p*NPP), but does not produce any P_i:

$$\text{bis-MUFP} \xrightarrow{\text{PDE}} \textbf{MUF} + \text{MUFP} \quad (2)$$

P_i can be released only if the second ester-P bond is hydrolyzed by PME, which simultaneously releases more MUF (*p*NP):

$$\text{bis-MUFP} \xrightarrow{\text{PME+PDE}} \textbf{2MUF} + P_i \quad (3)$$

The resulting concentration of MUF (*p*NP), a product measured in the enzyme activity assays, is thus higher in the presence of both enzymes than would correspond to just PDE activity (Eq. (2)). Moreover, one molecule of P_i, the "target" product of the diester hydrolysis must correspond to two molecules of MUF (*p*NP) provided that all the MUFP (*p*NPP) has been hydrolyzed by PME. The top panels in Fig. 1 illustrate the "randomness" of either MUF or P_i release under the simultaneous PDE and PME activity (Eq. (3)); the bottom panels illustrate the MUFP hydrolysis according to the Eq. (1). The potential overestimation of PDE has, to our best knowledge, never been questioned in any papers reporting the activities of PDE, although both enzymes are likely present in most samples analyzed.

In this paper, we investigate the hypothesis that phosphomonoesters resulting from the hydrolysis of artificial phosphodiesters by PDE are further hydrolyzed by PME in natural samples. In order to test this hypothesis, we employed the following approaches: We measured the concentration of P_i at the end of the incubation of root segments of a wide range of wetland macrophytes with bis-MUFP substrate. To further test if the presence of PME increases the yield of MUF (or *p*NP) we used the commercially available enzymes, individually, and in combination, and incubated them with the corresponding substrates.

2. Methods

2.1. Root material

Measurements of P_i and MUF released by the enzymatic hydrolysis of bis-MUFP substrate by root PDE and PME were conducted at the Wetland Lab, UC Davis. We did not measure P_i released by the hydrolysis of bis-*p*NPP, because potassium phosphate in the terminator (see below) would interfere with the P_i measurements. Note: at the time of conducting the tests we were not aware of the existence of alternative terminator solutions (e.g., $CaCl_2$ and Tris-NaOH, see Browman and Tabatabai, 1978). We used root material from *Eleocharis cellulosa* from a P-depleted greenhouse culture, as well as roots of *Juncus mexicanus* and *Carex* sp. from serpentine seep wetlands located in the California Coastal Ranges (Freestone and Inouye, 2006), and *Eleocharis macrostachya*, *Schoenoplectus acutus*, *Schoenoplectus californicus*, *Paspalum distichum*, *Alisma plantago-aquatica*, and *Typha latifolia* from marshes of the Cosumnes River Preserve in the Central Valley of California (Hammersmark et al., 2005).

2.2. Enzyme activities

The enzyme activities were determined using the most frequently used artificial substrates for phosphatase assays: *p*NPP or MUFP, and bis-*p*NPP or bis-MUFP as substrates for PME and PDE, respectively (Table 1). Individual plants with at least a part of their root system intact and soil attached were collected and kept at

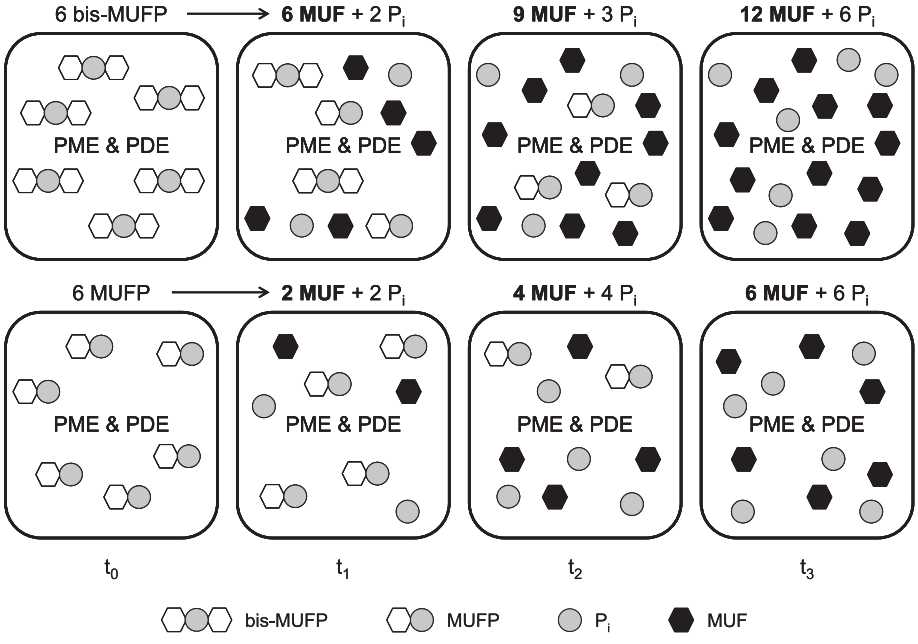

Fig. 1. Schematic hydrolysis of artificial substrates by a mixture of PME and PDE in a sample. Equal amounts of either substrate (left), bis-MUFP (top = Eq. (3)) or MUFP (bottom = Eq. (1)), results in equal amounts of released P (grey circles), but distinct amounts of MUF (black hexagons) fluorescence (right panels) at different times (t_0–t_3). The comparison shows very variable release of MUF fluorescence from bis-MUFP, dependent on sample properties and the length of the assay.