Soil Biology & Biochemistry 41 (2009) 1180–1186

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and L-DOPA

Jared L. DeForest*

Environmental and Plant Biology, 315 Porter Hall, Ohio University, Athens, OH 45701, USA

ARTICLE INFO

Article history:
Received 3 September 2008
Received in revised form
23 February 2009
Accepted 23 February 2009
Available online 17 March 2009

Keywords:
Extracellular enzyme activity
Glycosidase
L-DOPA
Ligninolytic
Methods
Methylumbelliferyl (MUB)-linked substrates

ABSTRACT

The purpose of this experiment was to evaluate whether soil storage and processing methods significantly influence measurements of potential *in situ* enzyme activity in acidic forest soils. More specifically, the objectives were to determine if: (1) duration and temperature of soil storage; (2) duration of soil slurry in buffer; and (3) age of model substrates significantly influence the activity of six commonly measured soil extracellular enzymes using methylumbelliferone (MUB)-linked substrates and L-dihydroxyphenylalanine (L-DOPA). Soil collected and analyzed for enzyme activity within 2 h was considered the best measure of potential *in situ* enzyme activity and the benchmark for all statistical comparisons. Sub-samples of the same soil were stored at either 4 °C or −20 °C. In addition to the temperature manipulation, soils experienced two more experimental treatments. First, enzyme activity was analyzed 2, 7, 14, and 21 days after collection. Second, MUB-linked substrate was added immediately (i.e. <20 min) or 2 h after mixing soil with buffer. Enzyme activity of soil stored at 4 °C was not significantly different from soil stored at −20 °C. The duration of soil storage was minimal for β-glucosidase, β-xylosidase, and peroxidase activity. *N*-acetyl-glucosaminidase (NAGase), phosphatase, and phenol oxidase activity appeared to change the most when compared to fresh soils, but the direction of change varied. Likewise, the activities of these enzymes were most sensitive to extended time in buffer. Fluorometric MUB and MUB-linked substrates generally had a 3-day shelf life before they start to significantly suppress reported activities when kept at 4 °C. These findings suggest that the manner in which acidic forest soils are stored and processed are site and enzyme specific and should not initially be trivialized when conducting enzyme assays focusing on NAGase, phosphatase, and phenol oxidase. The activities of β-glucosidase, β-xylosidase, and peroxidase are insensitive to storage and processing methods.

1. Introduction

Soil enzymes are the primary biological mechanism of organic matter decomposition and nutrient cycling. The activities of enzymes can be proxies for microbial activity, decay rates, and the availability of substrates for microbial or plant uptake. Likewise, soil enzymes can be used as metrics of biological diversity, ecosystem functioning, and soil fertility (Kandeler et al., 1996; Marx et al., 2001; Caldwell, 2005). Therefore, measuring extracellular enzyme activity can be a powerful tool for understanding soil biological processes.

Soil enzyme activities are estimated using many different methods (Marx et al., 2001) and those methods often involve harvesting soil days prior to analysis, mixing samples with a buffer and using model substrates and references to estimate activities. The most common methods are colorimetric using *para*-nitrophenyl and fluorometric using 4-methylumbelliferone (MUB)-linked model substrates (Darrah and Harris, 1986; Tabatabai, 1994; Saiya-Cork et al., 2002). Since the publication of Marx et al. (2001) and Saiya-Cork et al. (2002), the use of fluorometric MUB-linked substrates has become more common in soil studies because it is less time consuming and potentially a more sensitive metric of enzyme activities than using *para*-nitrophenyl (Marx et al., 2001; Drouillon and Merckx, 2005). The measurement of ligninolytic enzymes (i.e. phenol oxidase and peroxidase) is typically determined colorimetrically using L-dihydroxyphenylalanine (L-DOPA), but other methods are available (Allison and Jastrow, 2006; Johnsen and Jacobsen, 2008). The measurement of soil enzymes across ecosystems and climates can be used to better understand the relationship among microbial community function, resource availability, ecosystem processes and how ecosystems functionally respond to natural and anthropogenic disturbances.

Nevertheless, direct study comparisons are often difficult because of differences in the handling and processing of soil prior

* Tel.: +1 740 593 0742; fax: +1 740 593 1130.
E-mail address: deforest@ohio.edu

0038-0717/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2009.02.029

to and during enzyme analysis, and because the influence of those differences on enzyme activities independent of potential activity is poorly understood. Because both the temperature at which soils are stored (e.g. refrigeration vs. freezing) and the time between soil collection and enzyme assay can vary greatly among studies, it is possible that disparate results can be explained, in part, by this variation. Given that the methodology sections of most published works lack specific procedural details, result comparisons are even more difficult (Marx et al., 2001; Saiya-Cork et al., 2002). For example, is the delay between mixing soil with buffer and dispensing the MUB-linked substrate an important factor in reported values? It is also possible that diluting enzymes from soil could alter enzyme activity. Likewise, it is unclear if the substrates or reference standard solutions have a viability shelf life that alters the sensitivity of the substrate which would alter reported enzyme activity. Because these details are not reported, it is difficult to determine the severity, if any, of this problem.

Previous studies investigating storage on enzyme activity indicate refrigeration for the shortest duration is best (Ross, 1965; Kandeler and Gerber, 1988; Lee et al., 2007). However, all of these studies used colorimetric methods for measuring enzymes and cold storage duration was measured infrequently at short intervals. The primary goal of this study was to determine if the duration and temperature of soil storage significantly influences the activity of commonly measured extracellular enzymes β-glucosidase, β-xylosidase, acid phosphatase, *N*-acetyl-glucosaminidase (NAGase), peroxidase, and phenol oxidase in three different soils. Specific objectives were to: (1) provide detailed protocols for performing soil extracellular enzyme assays using microplates with MUB-linked substrates and L-DOPA; (2) quantify how enzyme activities change as a function of time since soil collection; (3) determine the best method for preserving soil samples (4 °C vs. −20 °C) prior to analysis; (4) determine if time in buffer solution has a significant effect on enzyme activities; and (5) assess the viability of MUB-linked substrate to improve consistency among studies and repeated measures.

2. Materials and methods

2.1. Study sites

Three different acidic forest soils were used in this experiment. (1) A fine-loamy, mixed, active mesic Ultic Hapludalfs (HAP) collected under a oak–maple forest at the Ridges Land Laboratory within the unglaciated Allegheny Plateau region of southeast Ohio, USA (39° 19′ 25″ N, 82° 07′07″ W). The HAP soil moisture content was 16%, pH was 5.0 and carbon content was 31.1 g C kg^{-1}. (2) A coarse-loamy, mixed, active, mesic Typic Dystrudepts (DYS) collected under a oak–beech forest at the Waterloo Wildlife Area in southeast Ohio, USA (39° 21′ 11.5″ N, 82° 16′ 40″ W). The DYS soil moisture content was 33%, pH was 4.1, and carbon content was 44.2 g C kg^{-1}. (3) A fine-silty, mixed, active, mesic Aeric Fragiaqualfs (FRA) collected under a mixed-mesophytic forest located at the Holden Arboretum (41° 36′ 39.6″ N, 81° 17′29.0″ W) within the glaciated Lake Erie Plains. The FRA soil moisture content was 20%, pH was 4.0, and carbon content was 86.4 g C kg^{-1}. All soil types are common over a wide geographical area and were specifically selected due to proximity to a laboratory for rapid analysis.

2.2. Soil collection

Soils were harvested to a depth of 5 cm using a 6.4 cm diameter soil core in July 2007 for the HAP, November 2007 for the DYS, and December 2007 for the FRA to provide a broad temporal scale. Three cores of mineral soil were composited within a 1.0-m^2 plot. Soil samples were extracted from three plots within three different stands of the forest for a total of nine replicate samples. The plots were approximately 50 m apart and the stands were between 100 and 250 m apart. Exact time was recorded and soil samples were put into plastic bags and immediately placed in a cooler on ice and rushed to the laboratory for immediate processing.

2.3. Experimental design

The potential activity of six commonly reported extracellular soil enzymes was measured: β-glucosidase, β-xylosidase, *N*-acetyl glucosaminide, acid phosphatases, peroxidase, and phenol oxidase (Marx et al., 2001; Saiya-Cork et al., 2002; DeForest et al., 2004; Sinsabaugh et al., 2005). Extracellular enzyme activity was measured on field fresh samples (i.e. <2 h from harvest) and then 2, 7, 14, and 21 days from harvest to determine the influence of time since harvest on enzyme activity. A subset of the samples was stored above (4 °C) or below (−20 °C) freezing conditions to determine the influence of soil storage temperature. The field fresh samples were assumed to be the best measure of potential *in situ* enzyme activity and used as a benchmark for all statistical comparisons. For the first 7 days (three trials in total), a buffer treatment was included to determine the influence of 2 h in buffer prior to further processing on enzyme activities. For example, soil stored for 2 days at 4 °C and −20 °C were analyzed for enzyme activity with less than 20 min in buffer and 2 h in buffer. Soil was homogenized in acetate buffer (see below) and substrate was added to the resulting slurry as soon as possible (<20 min) or at 2 h. The buffer treatment was not continued after the second day for glucosidase enzymes and the seventh day for the remaining enzymes. Each enzyme was measured nine times for each soil and had eight analytic replicates.

2.4. Buffer and MUB-linked substrates

Buffer and substrate recipes followed Saiya-Cork et al. (2002), but with several additional methodological details. A 50-mM acetate buffer solution was made by mixing sodium acetate trihydrate (certified ACS grade, crystalline, Fisher Scientific) with deionized (DI) water. The pH was adjusted using 12 M HCl (technical grade, Fisher Scientific) and stored in a 20^{-1} HDPE carboy. Because enzyme activity is pH sensitive, the pH of the buffer was adjusted to the mean soil pH of the study site within 0.5 units. The large volume of buffer was to ensure consistency among samples. Acetate buffer was kept at 4 °C between analyses, used within 8 days, and new buffer was made for days 14 and 21.

Activities of the non-ligninolytic enzymes, β-glucosidase, β-xylosidase, acid phosphatase, and NAGase were measured fluorometrically using MUB-linked model substrates (Fernley and Walker, 1965; Marx et al., 2001; Saiya-Cork et al., 2002) (Table 1). Each MUB-linked substrate (Sigma–Aldrich, St. Louis, MO) was a 200-μM solution well mixed in DI water and stored in opaque

Table 1
Extracellular enzymes with corresponding substrate and the corresponding Sigma–Aldrich product number.

Enzyme	MUB-linked substrate	Sigma no.
Acid phosphase	4-MUB-phosphate	M8883
N-Acetyl-glucosaminidase	4-MUB-*N*-acetyl-β-D-glucosaminide	M2133
β-Glucosidase	4-MUB-β-D-glucopyranoside	M3633
β-Xylosidase	4-MUB-β-D-xylopyranoside	M7008
Peroxidase	L-DOPA	D9628
Phenol oxidase	L-DOPA	D9628

4-MUB, 4-methylumbelliferyl; DOPA, 3,4-dihydroxyphenylalanine.