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Temperature-mediated changes of exoenzyme-substrate reaction rates and their consequences for the carbon to nitrogen flow ratio of liberated resources

Christoph A. Lehmeier, Kyungjin Min, Nicole D. Niehues, Ford Ballantyne IV [1], Sharon A. Billings*

Department of Ecology and Evolutionary Biology, Kansas Biological Survey, The University of Kansas, 2101 Constant Ave., Lawrence, KS 66047, USA

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ABSTRACT

Soil microorganisms produce exoenzymes to access resources stored in soil organic matter. Knowledge about the effect of temperature on the rates at which exoenzymes degrade substrates is particularly important for understanding carbon and nitrogen cycling with warming, and possible feedbacks to climate change.

Here, we studied reaction rates of purified enzymes and substrates in controlled laboratory conditions at temperatures ranging from 5 °C to 27 °C. We employed three enzyme-substrate pairings representative of reactions common to soil profiles: β-glucosidase and β-D-cellobioside (BGase/BG), β-N-acetyl glucosaminidase and N-acetyl-β-D-glucosaminide (NAGase/NAG), and peroxidase and 3,4-Dihydroxy-L-phenylalanine (peroxidase/L-Dopa).

Across the entire temperature range studied, BGase showed the highest specific activity ($V_{max\ 27\ °C}$ = 1338 μmol h^{-1} mg$^{-1}_{BGase}$), followed by NAGase ($V_{max\ 25\ °C}$ = 260 μmol h^{-1} mg$^{-1}_{NAGase}$) and peroxidase ($V_{max\ 25\ °C}$ = 36 μmol h^{-1} mg$^{-1}_{peroxidase}$). From 7.5 °C to 25 °C, the specific activities of BGase, NAGase and peroxidase increased by 103%, 111% and 835%, respectively. The activation energy (E_a) required for a reaction to proceed thus was highest for peroxidase/L-Dopa (99.8 kJ mol$^{-1}_{L\text{-}Dopa}$), followed by NAGase/NAG (41.3 kJ mol$^{-1}_{NAG}$) and BGase/BG (31.4 kJ mol$^{-1}_{BG}$). We use a simple model, parameterized with empirical data from these reactions in three different ways, to illustrate how the flow of carbon relative to nitrogen can change with temperature as these resources are liberated from their organic precursors. The results highlight the importance of relative temperature sensitivities among reactions and the substrates' carbon to nitrogen ratio as key determinants of temperature-mediated changes in relative availabilities of carbon and nitrogen to microorganisms.

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1. Introduction

Soil microorganisms produce exoenzymes, which they release into the soil matrix to decompose dead organic material. Through this process, resources such as carbon (C) and nitrogen (N) derived from macromolecular compounds become present in the form of smaller molecules. Such molecules can be taken up by microorganisms, and serve as substrate for their growth and metabolism. Most organic inputs to soils are eventually degraded by microorganisms; their exoenzymes are thus key components in the global cycling of C and N (Lavelle et al., 1993; Sinsabaugh and Moorhead, 1994; Conrad, 1996; Rillig et al., 2007; Nannipieri and Paul, 2009; McGuire and Treseder, 2010; Henry, 2012; Todd-Brown et al., 2012).

Of particular interest for understanding the cycling of C and N in a changing climate is the relationship between temperature and the rates of soil organic matter (SOM) decomposition (Smith et al., 2008; Von Lützow and Kögel-Knabner, 2009; Kleber, 2010; Singh et al., 2010; Conant et al., 2011; Pritchard, 2011; Sierra, 2012). The rate at which a soil exoenzyme degrades a substrate is determined by a multitude of factors, including pH (for all exoenzymes) and O_2 availability (for oxidative exoenzymes), the physical conditions influencing the coupling of exoenzyme and substrates, the substrate concentration, and the temperature (Von Lützow et al., 2006; Kleber, 2010; Kleber and Johnson, 2010). The Van't Hoff–Arrhenius law of reaction kinetics predicts that the probability of an enzymatic reaction occurring increases with rising temperature (see Laidler, 1984; Bosatta and Ågren, 1999; Davidson and Janssens, 2006; Sierra, 2012), and that the response of the reaction rate to

* Corresponding author.
E-mail addresses: Christoph.Lehmeier@gmail.com (C.A. Lehmeier), kjmin.21@gmail.com (K. Min), niehuesn@gmail.com (N.D. Niehues), fb4@ku.edu (F. Ballantyne), sharonb@ku.edu (S.A. Billings).
[1] Present address: Odum School of Ecology, The University of Georgia, 140 E. Green St., Athens, GA 30602, USA.

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temperature increases with the activation energy required to initiate substrate decay:

$$V = A \cdot e^{\frac{-E_a}{R \cdot T}} \quad (1)$$

V is the reaction rate, A the pre-exponential factor, E_a the activation energy, R the gas constant, and T the temperature in K. The degree to which a specific enzyme-substrate reaction, not impeded by limiting enzyme or substrate concentrations or any physical constraints (i.e. when V equals the maximum specific enzyme activity V_{max}), responds to temperature represents the intrinsic temperature sensitivity of the reaction.

In spite of the great theoretical utility of the Van't Hoff–Arrhenius relationship in studies of SOM decomposition (Davidson and Janssens, 2006; Craine et al., 2010; Conant et al., 2011; Davidson et al., 2012; Sierra, 2012), intrinsic temperature sensitivities of enzyme-substrate reactions relevant for biogeochemical processes remain largely unknown. What is observed in most studies of SOM decay is an apparent temperature sensitivity of reactions, where other variables such as low soil water content, low substrate availability or altered microbial exoenzyme production may constrain or obscure the intrinsic temperature sensitivities of the reactions (Davidson and Janssens, 2006; Subke and Bahn, 2010; Billings and Ballantyne, 2012). Thus, apparent temperature sensitivities of SOM decomposition can deviate substantially from intrinsic temperature sensitivities, as has been concluded in numerous studies (e.g. Giardina and Ryan, 2000; Dioumaeva et al., 2002; Biasi et al., 2005; Fang et al., 2005; Leifeld and Fuhrer, 2005; Wetterstedt et al., 2010; German et al., 2012). Recent studies attempt to determine the extent to which factors such as substrate and exoenzyme availabilities (Davidson et al., 2012; German et al., 2012) and microbial adaptation (Bradford et al., 2008; Hartley et al., 2008) drive any discrepancies between intrinsic and apparent temperature sensitivities of SOM decay. However, because the intrinsic temperature sensitivity of decay for key SOM compounds paired with relevant exoenzymes is not known, quantifying the extent to which microbial adaptation or substrate availability influence apparent temperature sensitivities of decay is difficult. Predictions of how temperature responses of biochemical reaction rates will influence the exoenzyme-mediated release of C and N (and other resources) assimilable by microorganisms are therefore not possible at present.

To this end, we quantified reaction rates of purified (1) β-D-cellobioside and β-glucosidase, (2) N-acetyl-β-D-glucosaminide and β-N-Acetyl glucosaminidase and (3) 3,4-Dihydroxy-L-phenylalanine and peroxidase at multiple temperatures relevant globally to soil profiles. The three pairings are employed as representatives of fundamental reactions occurring during the natural enzymatic degradation of cellulose (1), chitin (2) and lignin (3), respectively, prevalent organic compounds in most soil profiles. We determined the concentrations at which substrate and enzyme availability was not limiting for the reaction rates, and quantified the intrinsic temperature sensitivities of the reaction rates between 5 °C and 25 °C or 7.5 °C and 27 °C, depending on the reaction. We then used the temperature responses of specific β-glucosidase and β-N-Acetyl glucosaminidase activities to assess the influence of temperature on relative C and N liberation rates from their respective substrates. Our work provides a baseline for quantifying differences between intrinsic and apparent temperature sensitivities for these reactions, highlights the importance of distinguishing between relative and absolute temperature sensitivities, and reveals how temperature-induced variations in the rates of these purely biochemical reactions may influence fluxes of liberated C and N in soils.

2. Materials and methods

We measured the fluorescence of fluorescently tagged substrates as they decay to quantify reaction rates of β-D-cellobioside (abbreviated in the following as "BG") with β-Glucosidase (BGase; EC 3.2.1.21; Megazyme, Ireland) and N-acetyl-β-D-glucosaminide (NAG) with β-N-Acetyl glucosaminidase (NAGase; EC 3.2.1.52; New England BioLabs, USA). The substrates (both from Sigma–Aldrich, USA) contained a methylumbelliferyl ("MUB") label, which causes a fluorescence signal when cleaved by the enzyme (Mead et al., 1955). The two reactions are considered to simulate the naturally occurring enzymatic cleavage of single molecules from a chain of glucose molecules (representing cellulose) and from a chain of NAG molecules (representing chitin), respectively (see Lynd et al., 2002; Howard et al., 2003; Baldrian and Valášková, 2008).

The enzyme peroxidase (EC 1.11.1.7) catalyzed the oxidation and structural transformation of the substrate 3,4-Dihydroxy-L-phenylalanine (L-Dopa; both reagents from Sigma–Aldrich, USA) to 2-carboxy-2,3-dihydroindole-5,6-quinone (Mason, 1948) in the presence of hydrogen peroxide (see Nappi and Vass, 2001; Puiu et al., 2010). The oxidation of the chromogenic substrate causes a color change of the reaction volume, which we measured spectrophotometrically to determine the enzyme activity. This reaction is considered to represent an important step in the peroxidase-mediated degradation of lignin in soils (Skujins, 1978).

2.1. Enzyme-substrate assays for fluorescence measurements

Crystalline substrate (BG or NAG) was weighed into deionized water and stirred to dissolution. Aliquots of the enzymes (BGase and NAGase, respectively) were dispensed in 0.2 M sodium acetate buffer solutions at a pH of 6.5. For each assay, 50 μl substrate and 200 μl enzyme solution were pipetted in two columns (i.e. 16 wells) of a black, 96-well plate (Fisher Scientific, USA). One column of the same plate was loaded with substrate controls (50 μl substrate solution and 200 μl buffer), another with enzyme controls (200 μl enzyme solution and 50 μl buffer). The fluorescence of these wells served to correct for any signal detected for the enzyme-substrate wells not caused by the reaction product MUB itself. Ten μM 4-Methylumbelliferone (MUB reference; Sigma–Aldrich, USA) suspensions served to quantify the fluorescence signals of the MUB released in the enzyme-substrate reaction. One plate column was the quench control (50 μl MUB reference solution and 200 μl enzyme solution), and another was the standard control (50 μl MUB reference solution and 200 μl buffer).

We measured the evolution of fluorescence across time with a Spectramax Gemini XS fluorescence plate reader (Molecular Devices, USA) at excitation and emission wavelengths of 365 nm and 460 nm, respectively. Preliminary tests ensured that these wavelengths provided for the highest detection sensitivity to MUB. Values for each control are the average fluorescence values of eight single wells of the respective columns. Fluorescence values of one replicate enzyme-substrate assay refer to the average of fluorescence signals from the 16 assay wells of the plate. Over all measurements, the average coefficient of variation of 16 assay wells was 0.042 for BGase/BG (max. 0.105, min. 0.018) and 0.046 for NAGase/NAG (max. 0.118, min. 0.026). The specific enzyme activities were calculated based on DeForest (2009) and German et al. (2011) as follows:

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