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Temperature selects for static soil enzyme systems to maintain high catalytic efficiency

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ABSTRACT

Knowledge on enzymatic mechanisms of acclimation to temperature is required to predict the effects of warming on decomposition of soil organic matter – the largest C stock in terrestrial ecosystems. Based on Michaelis–Menten kinetics we tested the hypothesis that enzyme affinity to substrate (K_m) is more sensitive to warming at cold than at warm temperatures. We also predicted a gradual increase in K_m values with increasing temperature. The kinetic parameters of six enzymes involved in cycles of C, (cellobiohydrolase, β-glucosidase and xylanase), P (phosphatase), and N (leucine-aminopeptidase, tyrosine-aminopeptidase) were determined after one month of soil incubation at a temperature range 0 –40 °C (with 5° increment).

Contrary to our hypothesis, the increase in K_m with temperature was not gradual for most tested enzymes. Within large range of temperatures from 0 to 15 °C (phosphatase), 0–20 °C (enzymes involved in C cycle) and 0–40 °C (proteases) the hydrolytic activity was governed by enzymes with nearly constant substrate affinity. Temperature, therefore, mainly selected for soil enzyme systems maintaining static K_m. The catalytic efficiency of the enzymes (V_{max}/K_m) increased from low to intermediate temperatures (0–20 °C) as a result of linear increase of V_{max} at constant K_m. Static K_m values were explained either by low flexibility (high structural stability) of a single enzyme type, which catalyzed the reaction over a broad temperature range, or by production of multiple isoenzymes each with different temperature optima but with similar affinity to substrate. Thus, maintaining static K_m with temperature increase ensured high enzyme efficiency within a low and intermediate soil temperature range.

1. Introduction

The temperature sensitivity of soil organic matter (SOM) decomposition is crucial for understanding the consequences of global warming (Davidson and Janssens, 2006; Stone et al., 2012). Since microbial enzymes are responsible for SOM decomposition, knowledge on enzymatic mechanisms of acclimation to temperature is required to predict the effects of warming on the cycling of C and major nutrients (Koch et al., 2007; Conant et al., 2011; Birgander et al., 2013).

Hydrolytic enzyme activity is a saturating function of substrate concentration and is described by the Michaelis–Menten relationship (Michaelis and Menten, 1913). Both parameters of the Michaelis–Menten equation – the maximal catalytic reaction rate at a given temperature (V_{max}) and the half-saturation constant (K_m), which reflects the affinity of the enzyme for the substrate – are temperature sensitive (Davidson and Janssens, 2006; Davidson et al., 2006) and usually increase with temperature (Stone et al., 2012). Temperature sensitivity of potential enzyme activity (V_{max}) is traditionally studied by adding excess substrate. In contrast, little information is available about the temperature sensitivity of K_m in soil enzyme systems; this, however, is especially important for the very low substrate levels common under soil conditions (Hobbie and Hobbie, 2012; Kuzyakov and Blagodatskaya, 2015).

Temperature affects microbial and enzyme activities not only directly (Allison et al., 2010; A'Bear et al., 2012; Wieder et al., 2013), but also indirectly affects enzymatic reactions when a shift in dominating microbial populations changes the temperature sensitivity of the produced isoenzymes (i.e., an enzyme with the

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same function but different structure) (Bárcenas-Moreno et al., 2009; Zimmermann and Bird, 2012; Van Gestel et al., 2013). Temperature, therefore, modulates the rate of biogeochemical processes by controlling microbial metabolism (Leroi et al., 1994; Turner et al., 1996; Cooper et al., 2001).

The parameters of enzyme kinetics – specifically, K_m, which determines the binding affinity of enzyme to substrate – are indicative for detecting enzyme flexibility (the capacity for quick conformation change) (Somero, 1975). Flexible enzymes are characterized by a fast rate of conformation change when converting a substrate to a product at an enzyme active site (Fields, 2001; Bradford, 2013). At low temperatures, flexible enzyme structures ensure a fast rate of catalytic reaction at high binding affinity (i.e. low K_m) (Fields, 2001; Bradford, 2013). With temperature increase, the ability of flexible enzymes to maintain binding conformations decreases, reducing the affinity to the substrate (Fields, 2001). This can be measured as an increase of K_m with temperature (Fields, 2001). Therefore, warmer temperatures are favorable for enzymes with lower substrate binding affinity but with higher structural stability (Zavodszky et al., 1998; Bradford, 2013). However, applicability of the hypotheses established on the basis of single enzyme properties needs to be tested under *in situ* soil conditions considering great functional redundancy of microorganisms. Due to functional redundancy, high catalytic efficiency (determined as V_{max}/K_m) is maintained in soil by numerous enzymes with different temperature optima mediating similar functions (Nannipieri et al., 2012). As enzyme systems are altered by climate warming, a different set of isoenzymes is expected to be expressed at cold and warm temperatures (Somero, 1978; Bradford, 2013). Expression of isoenzymes with higher temperature optima can be produced by the same microbial species adapted to warming (Hochachka and Somero, 2002). Alternatively, isoenzymes can be expressed as a result of changes in microbial community structure caused by warming (Baldwin and Hochachka, 1969; Vanhala et al., 2011). Therefore, K_m determined in soil reflects simultaneous activity of a suite of isoenzymes with different thermal optima rather than single enzyme properties. It remains to be tested, whether functional capacity of suite of soil enzymes is maintained by increase of K_m with temperature (similar to single flexible enzyme) or whether temperature selects for static K_m through the range of temperatures.

Importantly, microbial adaptation and acclimation strategies have physiological costs (Schimel et al., 2007) and can reduce enzyme catalytic efficiency (Stone et al., 2012; Tischer et al., 2015). Microbial physiology, however, is evolutionarily selected for most efficient enzyme systems (Hochachka and Somero, 2002; Allison et al., 2010). Therefore, the combined thermal response of both parameters of catalytic efficiency needs to be considered to reveal the mechanisms maintaining effective enzymatic functioning through the wide range of increasing temperatures (Jaenick, 1991; Somero, 1995; Fields, 2001).

This study was designed to test the catalytic properties of enzymes involved in the C, P and N cycles, across the environmental temperatures covering psychrophilic, mesophilic and thermophilic ranges. We hypothesized 1) high enzyme flexibility (and gradual increase in K_m with temperature) within a cold temperature range; 2) in contrast, under warm temperatures we expected more static values and low temperature sensitivity of K_m. To test our hypothesis, we incubated soil for one month over a temperature range of 0–40 °C (with 5 °C steps) and determined the kinetic parameters of six enzymes involved in decomposing soil organics: cellobiohydrolase and β-glucosidase, which are commonly measured as enzymes responsible for consecutive stages of cellulose degradation (German et al., 2011); xylanase, which is responsible for breaking down hemicelluloses (German et al., 2011); acid phosphatase, which mineralizes organic P into phosphate by hydrolyzing phosphoric (mono) ester bonds under acidic conditions (Eivazi and Tabatabai, 1977; Malcolm, 1983). Activities of leucine aminopeptidase and tyrosine aminopeptidase were analyzed to assess the hydrolysis of L-peptide bonds (Koch et al., 2007; Chen et al., 2012).

2. Material and methods

2.1. Soil

Soil samples were taken in the middle of summer from the top 10 cm of the Ap of an arable loamy haplic Luvisol from 4 experimental plots (fallow soil, 5 × 5 m) located on a terrace plain of the Leine River in central Germany (Holtensen, 52°22′40″, 9°41′46″E) (Pausch et al., 2013). The area has a temperate climate with a long-term annual mean precipitation of 645 mm and an air temperature of 8.7 °C (Kramer et al., 2012). The properties of the soil were: pH 6.5; 12.6 g kg^{-1} C, 1.3 g kg^{-1} N, 5.8% sand, 87.2% silt, 5.8% clay; 1.4 g cm^{-3} bulk density, and sampling moisture 60% of WHC (Kramer et al., 2012; Pausch et al., 2013). The samples were kept cold (~4 °C) during transportation to the laboratory. The samples were then frozen at −20 °C until pre-incubation.

2.2. Soil incubation

30 g of soil where incubated in air tight glasses (with rubber seal) with the volume of 125 mL. During the incubation, soil moisture was checked by weighting and was immediately adjusted to 60% of WHC. In order to avoid the anaerobiosis, all the samples have been regularly aerated by opening the cabs for 1 min. After incubation no significant differences were detected in pH of samples. Activity of six enzymes targeting C-, N- and P-containing substrates were determined after incubating the soil at 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C. Nine climate chambers (SBS C120) were used to regulate the temperature (±0.5 °C). The frozen samples were thawed at 4 °C for one day and then pre-incubated at 20 °C for 14 d before the start of 30-day incubation. To minimize the freezing effect on enzymatic activities (Lee et al., 2007; Stone et al., 2012), all samples were frozen similarly, and they were pre-conditioned after thawing. We therefore assume that this pretreatment corresponded to snow thaw in spring and that the freezing effect was strongly reduced after the pre-incubation and was identical for all samples (German et al., 2012).

2.3. Enzyme assays

The kinetics of hydrolytic enzymes involved in C, N and P cycles were measured by fluorimetric microplate assays of 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) (Marx et al., 2005; Dorodnikov et al., 2009). Four types of fluorogenic substrates based on MUF and two types based on AMC were used to assess enzymatic activities; 4-methylumbelliferyl-phosphate (MUF-P) to detect phosphatase activity, 4-methylumbelliferyl-β-D-glucoside (MUF-G) to detect β-glucosidase activity; 4-methylumbelliferyl-β-D-cellobioside (MUF-C) to detect cellobiohydrolase activity; and 4-methylumbelliferyl-β-D-xylopyranoside (MUF-X) to detect xylanase activity. The activities of leucine aminopeptidase (AMC-L) and tyrosine aminopeptidase (AMC-T) were measured using L-Leucine-7-amino-4-methyl coumarin and L-tyrosine-7-amido-4-methyl-coumarin. All substrates and chemicals were purchased from Sigma (Germany).

We determined enzyme activities in a range of substrate concentrations from low to high (0, 10, 20, 30, 40, 50, 100, 200 μmol g^{-1} soil). Saturation concentrations of fluorogenic substrates were determined in preliminary experiments. Suspensions of 0.5 g soil

(dry weight equivalent) with 50 mL deionized water were prepared using low-energy sonication (40 J s^{-1} output energy) for 2 min (Stemmer et al., 1998; Koch et al., 2007). 50 μL of soil suspension was added to 100 μL substrate solution and 50 μL of buffer [MES ($C_6H_{13}NO_4SNa_{0.5}$), (pH:6.5) for MUF substrate and TRIZMA ($C_4H_{11}NO_3 \cdot HCl$, $C_4H_{11}NO_3$), (pH:7.2) for AMC substrate] in a 96-well microplate (Koch et al., 2007). Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, slit width of 25 nm, with a Victor3 1420-050 Multi label Counter (Perkin Elmer, USA). All enzymes activities were determined and incubated at exact temperature during 2 h. After each fluorescence measurement (i.e. after 30 min, 1 h and 2 h) the microplates were promptly returned to the climate chambers, so that the measurement time did not exceed 2.5 min. During assay-incubation, microplates in all different temperatures have been covered tight to prevent evaporation of solutions within the microplates.

The assay of each enzyme at each substrate concentration was replicated three times in each plate. Enzyme activities were expressed as MUF or AMC release in nmol per g dry soil per hour (nmol g^{-1} h^{-1}). In addition, for all four incubation replicates, the assay of each enzyme at each substrate concentration was performed in three analytical replicates (12 wells in the microplate). Besides, linear increase of fluorescence over time during the assay was properly checked and data which obtained after 2 h used for further calculation (German et al., 2011). We checked possible temperature effects on the chemical decomposition and thermal hydrolysis of the four MUF-substrates and two AMC-substrates, but no significant effects were detected in the range 0–40 °C (Razavi et al., 2015).

The Michaelis–Menten equation was used to determine parameters of the enzyme activity *(V)*:

$$V = \frac{V_{\max}[S]}{K_m + [S]} \quad (1)$$

where V_{max} is the maximum enzyme activity; K_m represent the half saturation constant, or the substrate concentration at which the reaction rate equals $V_{max}/2$; and S is the substrate amount (Michaelis and Menten, 1913; Segel, 1975; Von Lützow and Kögel-Knabner, 2009). Both V_{max} and K_m parameters were approximated by the Michaelis–Menten equation (1) with the non-linear regression routine of STATISTICA. Analysis of variance (ANOVA) followed by the Tukey HSD at a probability level of $p < 0.05$ was used to define the ranges of temperatures with significantly different K_m ($p < 0.05$). This means that pairwise differences test were applied to distinguish the significant differences for each neighboring pair of independent variables (mean values of K_m at 0, 5, 10, 15, 20, 25, 30, 35, 40 °C) (Melillo et al., 2002; Razavi et al., 2015). Homogeneity of variance and normality of the values was tested by the Leven's test and Shapiro–Wilk's W test. We used the routine Q_{10} function (2) to examine temperature sensitivity and to express temperature responses of each enzyme kinetic parameter (i.e., K_m or V_{max} separately).

$$Q_{10} = \left(\frac{R_{(T+10^{\circ}C)}}{R_{(T)}}\right) \quad (2)$$

where R is the rate of a process or a value of kinetic parameters and T is temperature (Kirschbaum, 1995; Khalili et al., 2011).

3. Results

3.1. Enzyme responses to increasing temperature

The Michaelis–Menten kinetics (enzyme activity as a function of substrate concentration) revealed positive response of six enzymes to increasing temperature (Fig. 1 and Fig. S1). Both V_{max} and K_m increased with temperature, although the increase pattern was not linear and indicated different temperature sensitivity of V_{max} and K_m (Figs. 2 and 3). The temperature response of V_{max} differed across

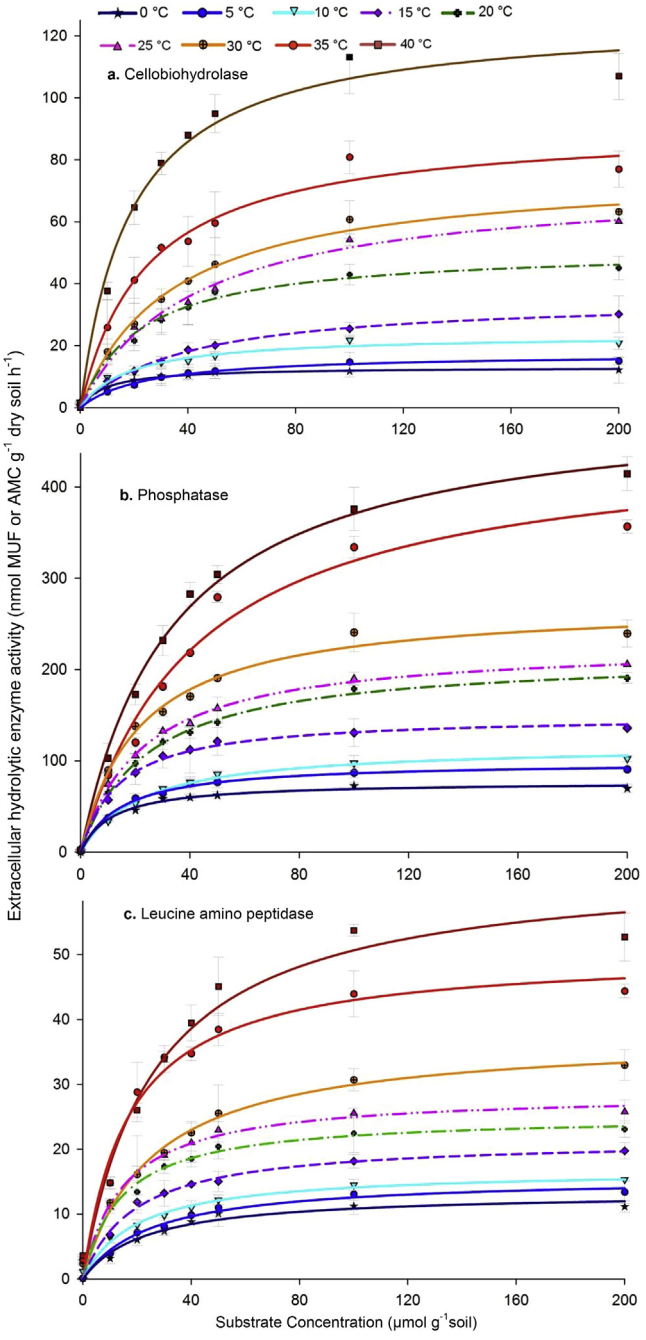

Fig. 1. Examples of Michaelis–Menten kinetics (enzyme activity as a function of substrate concentration) in response to increasing temperature for cellobiohydrolase (a), phosphatase (b), leucine amino peptidase (c) measured at nine temperatures. Each enzyme was assayed at a range of substrate concentrations (8 concentrations) at each of 9 temperatures. Values are means of 4 replications (±SE). (Kinetics' plots of other three enzymes are presented in Fig. S1).

enzymes, corresponding to Q_{10} values of 1.04–2.25. The temperature sensitivity of K_m was less than of V_{max} and varied from 0.90 to 1.89. The changes in V_{max}-Q_{10} and K_m-Q_{10} were not gradual in the whole range of temperatures tested, and were clearly pronounced between 15 and 30 °C (Fig. 3, Table S1). The K_m-Q_{10} demonstrated three enzyme-specific patterns: 1. Nearly constant K_m-Q_{10} at low temperatures with an increase at 20–30 °C and a final decrease; this pattern corresponded to enzymes of the C cycle. 2. The pattern observed for phosphatase K_m-Q_{10} was a gradual increase until 20–30 °C and a decrease thereafter. 3. Nearly constant K_m-Q_{10} for the whole temperature range corresponded to N cycle enzymes (Fig. 3, Table S1). Compared with V_{max} and K_m, the Q_{10} values for catalytic reaction rates varied in the more narrow range of 1.2–1.8.

3.2. Static characters of enzymes in soil

Despite differences between enzymes, the K_m values for each tested enzyme were unexpectedly static at least up to 20–25 °C (Fig. 2). Cellobiohydrolase, β-glucosidase and xylanase demonstrated relatively constant K_m values at low to moderate temperature increase (0–25 °C). The K_m values of these enzymes strongly increased (by around 40%) between 20 and 30 °C (Fig. 2). After such an extreme increase, the K_m values did not change significantly up to 40 °C (Table S1 and Fig. 2). The changes of proteases K_m had a pattern different from the enzymes involved in carbohydrate decomposition. Proteolytic enzymes demonstrated almost constant K_m values within the whole temperatures range (0–40 °C), (Fig. 2 e, f). Phosphatase K_m increased slightly with temperature up to 15 °C and thereafter increased rapidly to 40 °C. However, the K_m values between 5 and 15 °C were relatively constant (Fig. 2d).

Thus, the K_m of all tested enzymes did not change significantly within psychrophilic and mesophilic temperatures, while K_m of proteases remained relatively stable within the whole tested range (0–40 °C).

3.3. Catalytic efficiency of enzymes as affected by temperature

The catalytic efficiency of the enzymes (V_{max}/K_m) increased from low to intermediate temperatures (0–20 °C). Further extreme increases in K_m at the 30 °C threshold were always accompanied with

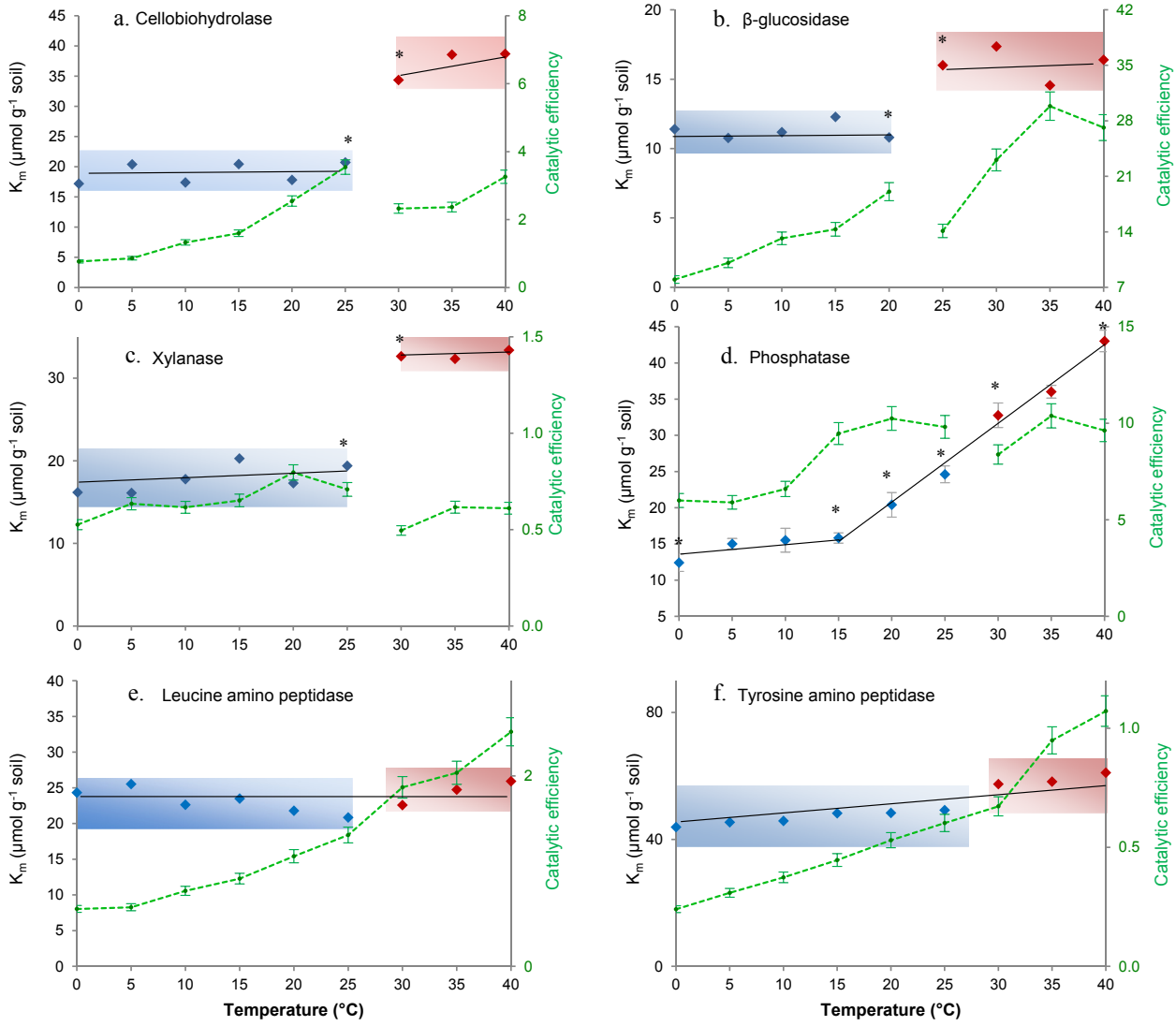

Fig. 2. Catalytic efficiency (V_{max}/K_m) of phosphatase (a), β-glucosidase (b), cellobiohydrolase (c) and xylanase (d) (green dashed line). Changes of K_m at low and intermediate temperatures are indicated by blue markers and at high temperatures indicated by red markers. Shading indicates the range of K_m values ± SE. Asterisk (*) indicates significant differences between K_m values at $p < 0.05$ after Turkey's HSD test. Catalytic efficiency increased at low and intermediate temperatures and dropped at high temperatures (indicated by no dashed line). For lucine aminopeptidase (e) and tyrosine aminopeptidase (f), catalytic efficiency increased gradually. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

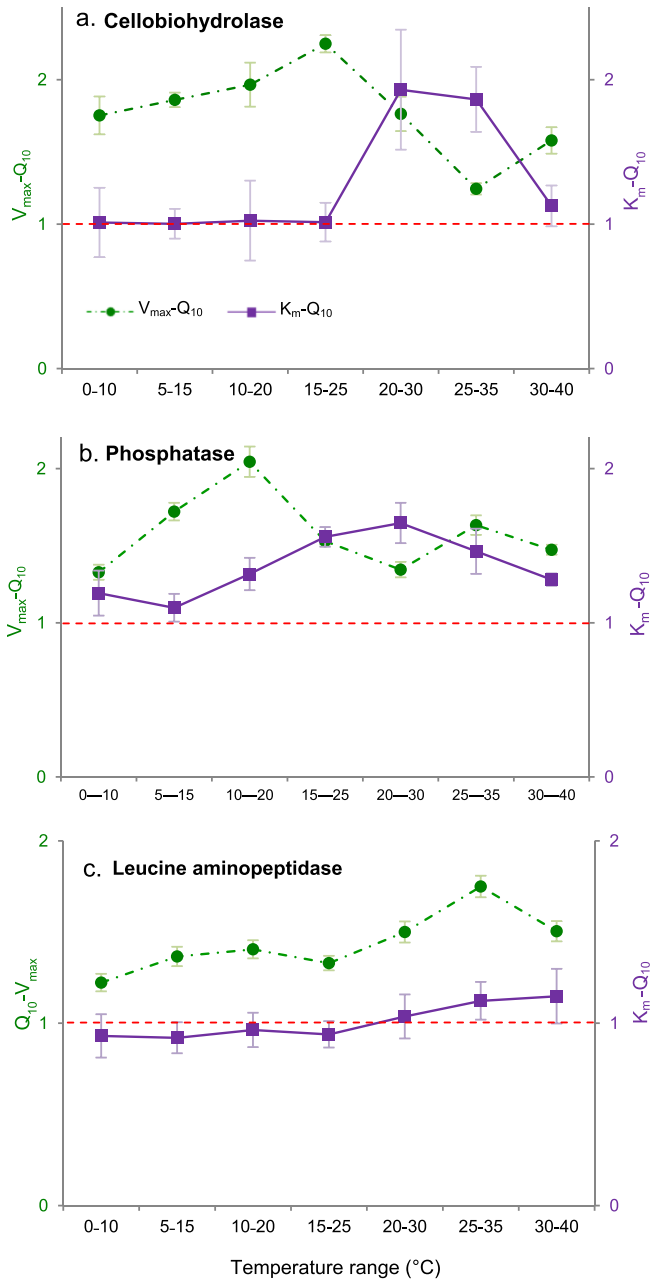

Fig. 3. Temperature sensitivity (V_{max}-Q_{10} and K_m-Q_{10}) of Cellobiohydrolase (a), Phosphatase (b) and Leucine aminopeptidase (c) as a function of temperature with 5° increment. Dashed red line demonstrate where K_m is temperature insensitive (K_m-Q_{10} = 1). Values are means of 4 replications (±SE). V_{max}-Q_{10} and K_m-Q_{10} of all other enzymes are presented in Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

a sharp decrease in the catalytic efficiency of enzymes of C and P cycles (Fig. 2). In contrast, the catalytic efficiency of proteases increased gradually from 0 to 40 °C (Fig. 2e, f).

4. Discussion

4.1. Enzyme temperature sensitivity

The Q_{10} values of reaction rates varied from 1.33 to 1.78 within the low temperature range and decreased to 1.26 at higher temperature. This decrease confirms theoretical predictions (Davidson and Janssens, 2006) and experimental observations on reduced reaction rate Q_{10} values at elevated temperature (Tjoelker et al., 2001; Xu and Qi, 2001; Razavi et al., 2015). The Q_{10} values of reaction rates generally decreased compared to short-term incubation study of Razavi et al. (2015) who found variation from 1.5 to 1.9 within the low temperature range and decreased to 1.4 with increasing temperature. Temperature sensitivity of V_{max} values ranging from 1.11 to 2.25 and K_m-Q_{10} values ranging from 0.90 to 1.93 (Table S1) support previous studies (e.g. German et al., 2012; Stone et al., 2012; Razavi et al., 2015). At least below 20–25 °C, the temperature sensitivity of K_m of all enzymes was lower than that of V_{max} (Fig. 3 and Table S1). Such lower temperature sensitivity of K_m compared to V_{max} obtained in this study was in line with studies of German et al. (2012), Stone et al. (2012) and Razavi et al. (2015). Thus, the effect of temperature on maximal reaction rate is stronger than on enzyme affinity to the substrate (Stone et al., 2012). This indicated potentially strong thermal adaptation of K_m to diurnal and seasonal temperature variations (Bradford et al., 2008; Allison et al., 2010; German et al., 2012) as a possible mechanism to regulate C and nutrient cycling during winter and summer periods (Koch et al., 2007). Moreover, microbial communities and enzymes adaptation to long-term global warming may cause lower temperature sensitivity of enzyme kinetic parameters as reported herein compared to short-term incubation studies (Bradford et al., 2008; Allison et al., 2010; Razavi et al., 2015).

4.2. Static character of enzymes in soil

We compared the flexibility of enzyme systems by measuring changes in enzyme affinity (K_m) to substrate (Fields and Somero, 1998; Bradford, 2013) at increasing temperatures (Fig. 4). Contrary to our hypothesis 1, the increase in K_m with temperature was not gradual for most tested enzymes. Within large range of temperatures from 0 to 15 °C (phosphatase), 0–20 °C (enzymes involved in C cycle) and 0–40 °C (proteases) the hydrolytic activity was governed by enzymes with nearly static substrate affinity. This could be explained by an expression of multiple isoenzymes each with different temperature optima (Bradford, 2013) (Fig. 4). Such isoenzymes expression can represent "functional tradeoffs" between enzyme flexibility and functional capacity, given similar flexibilities of isoenzymes under their optimal working conditions (Jaenick, 1991; Somero, 1995; Zavodszky et al., 1998) (Fig. 4a, b and c). Alternatively, static K_m values can be due to low flexibility (high structural stability) of single enzyme type, which catalyzed reaction within broad temperature range. Phosphatase was the only enzyme to demonstrate a gradual decrease in its substrate affinity with increasing temperature. This could be a consequence of enzyme flexibility i.e. the capacity for quick conformation change ensuring a fast rate of catalytic reaction. Due to such a conformation flexibility, however, the affinity of phosphatase to the substrate reduced with temperature increase. We also assume that gradual decrease in its substrate affinity with increasing temperature subjected stepwise expression of isoenzymes degrading organic phosphates (Fig. 4a). Contrary to our hypothesis 2, this flexibility of phosphatase was revealed within moderate (15–25) and elevated (>30 °C) temperatures and was not detected at low temperatures. No significant trends in enzyme affinity were detected within the range 0–25 °C for other enzymes (Fig. 4b). We therefore assume that a similar set of enzymes was expressed below 25 °C by microorganisms adapted to diurnal temperature changes of 10–15 °C under the temperate climate in the studied Luvisol. Furthermore, the proteolytic enzyme systems demonstrated surprising static values of K_m from 0 up to 40 °C (Fig. 4c). This agrees with different temperature responses between amino peptidases and enzymes involved in the C cycle (Hopkins et al., 2006; Koch et al., 2007). Such a static K_m, however,

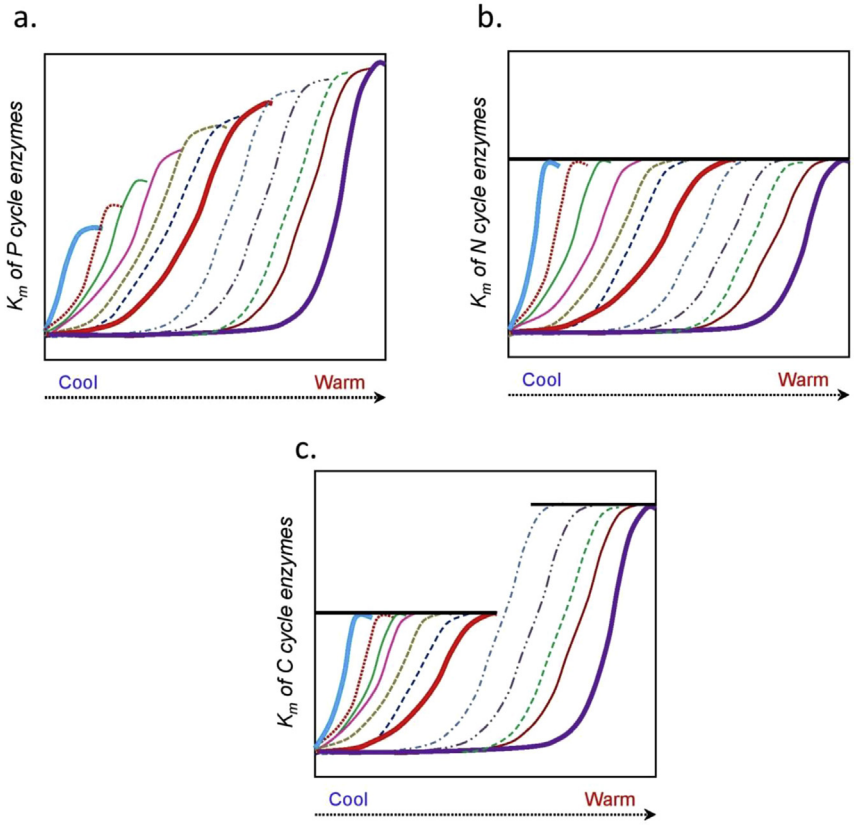

Fig. 4. Generalized thermal responses of K_m to a temperature increase. The scheme explains that functional capacity of soil enzymes is maintained by increase of K_m with temperature (a) or whether temperature selects for static K_m through the range of temperatures (b) as well as a strong-sudden increase of K_m at a temperature increase over 30 °C (c). Thick curves represent single enzymes and thin curves show expression of suite of isoenzymes.

contradicted to our hypothesis that soil enzymes are more flexible at cold temperatures. Thus, in soil, low temperatures selected for enzymes maintaining static K_m values in response to moderate warming.

Compared with animal digestive enzymes or organisms inhabiting aquatic systems (Hofer et al., 1975; Somero, 1978; Johns and Somero, 2004), such a temperature response pattern of exoenzymes in soil seems to be unique or at least more relevant for the terrestrial environment. Less temperature response or even a constant K_m value has been observed for soil enzymes (Koch et al., 2007; Stone et al., 2012). Likewise, German et al. (2012) found no variation in K_m for four of five tested enzymes in a cold-climate soil. Similarly, the K_m of intracellular enzymes of invertebrates are unresponsive to temperature (Somero, 1978; Hochachka and Somero, 2002; Huestis et al., 2009). Thus, maintaining the equal binding affinity to substrate (constant K_m) ensured efficient enzyme conformation within a broad temperature range. Demonstrating that expression of static enzymes with higher efficiency is a preferred microbial strategy in the studied soil (Stone et al., 2012).

Overall, for β-glucosidase, cellobiohydrolase and xylanase we found a strong increase in K_m by 30–40% at high temperatures (30 versus 25 °C), reflecting a two-fold reduction of the enzyme-substrate affinities. Following the strong increase at 30 °C, the K_m remained nearly constant from 30 to 40 °C. Such a static K_m is in line with the finding of Somero and Field and the theoretical prediction of Bradford (2013) regarding the stability of enzyme systems at high temperatures. In fact, such a pronounced increase in K_m (comparable to or stronger than the increase in V_{max}) was responsible for the reduced temperature sensitivity of catalytic efficiency. The general trend of catalytic efficiency demonstrated a gradual increase with temperature both at cold and at warm temperatures. The only remarkable exception occurred at 30 °C, where a strong increase in K_m was accompanied by a significant decrease in catalytic efficiency.

The revealed static properties of extracellular hydrolytic enzymes calls for the studies on meta-proteomics and microbial community structure at increasing temperatures in soils from various climate zones as well as for extended range of hydrolytic and oxidative enzymes, such as peroxidases and phenoloxidases.

The static K_m values of hydrolytic enzymes within mesophilic temperature range were revealed in assays of bulk soil enzyme kinetics, which is complementary to classic approaches in enzymology based on enzyme extraction either from pure microbial cultures (Marx et al., 2005) or from soil (Giagnoni et al., 2012). Use of isolated enzymes enables direct study on thermal stability and conformational flexibility to characterize selected enzymes (Zavodszky et al., 1998). Thus, classic enzymology demonstrates a pattern of temperature response for single isolated enzyme, whereas our results rather reflect a behavior of suite of soil isoenzymes occurring *in situ* (Nannipieri et al., 2012). Combination of both approaches is important, therefore, for predictions and for understanding the mechanisms of temperature sensitivity of enzymatic reactions.

Note that the K_m measured in this study could be considered as "apparent" K_m (German et al., 2011; Stone et al., 2012) because of the confounding effects of many temperature-sensitive processes in soil. Desorption and adsorption reactions are temperature sensitive and could affect V_{max} and K_m values of exoenzymes (Nannipieri and Gianfreda, 1998; Davidson and Janssens, 2006). With increasing enzyme adsorption and stabilization, K_m will

increase and consequently the catalytic efficiency will decrease. Adsorption by clays increases enzyme stability against thermal denaturation and proteolysis (Stotzky, 1986; Nannipieri et al., 1996). Furthermore, thermal denaturation – usually occurring at temperatures much higher than 40 °C (dos Santos et al., 2004; Goyal et al., 2014) – affects the kinetic constants of enzymes and also increases K_m (Dick and Tabatabai, 1987). These indirect mechanisms of K_m increase with temperature due to interactions of exoenzymes with soil particles underline the importance of our findings for soil systems. The changes in enzyme kinetics in response to elevated soil temperature revealed here indicate altered enzyme systems and a possible shift in community composition (Buyer and Drinkwater, 1997; Lipson et al., 2002). To our knowledge, no previous studies have investigated variability of catalytic properties for the set of enzymes in soil and at a temperature resolution comparable to our study (9 temperature levels). The further evidence we provide of sharp changes in K_m could be an indicator of isoenzyme expression at warm temperatures (Baldwin and Hochachka, 1969). Furthermore, isoenzyme synthesis could occur because of a major shift in species dominance above 30 °C (Khalili et al., 2011; Bradford, 2013): these temperatures are uncommon in the original community under the temperate climate with an annual soil temperature of 8–9 °C (Bárcenas-Moreno et al., 2009).

Finally, we found three types of K_m changes: 1) gradual increase up to 30 °C, 2) abrupt rise at 30 °C and 3) relatively static K_m. Our results demonstrated an ability of soil enzymes to maintain static systems with high substrate affinity within psychrophilic and mesophilic temperature ranges. Such a static character of soil enzyme systems at increasing temperature ensured efficient enzymatic functioning under warming climate. This pattern seems to be unique for soil microorganisms and for the heterogeneous nature of soil and may differ with soil type and climate. We conclude that predicting and modeling the consequences of warming for C, N and P cycles cannot assume linearity of enzyme-based processes. Rather, they should consider possible temperature thresholds triggering strong changes in catalytic efficiency and, thus, in the process rates.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.02.018.

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