University of South Bohemia in České Budějovice

Faculty of Science

Biochemical inhibition of β-Glucosidase and Leucine-aminopeptidase

Bachelor Thesis

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Annotation

β-Glucosidase and Leucine-aminopeptidase are hydrolytic enzymes found in the soil. This thesis investigated the possible inhibition of these enzymes by their product, i.e., glucose and leucine, respectively.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 09.05.2024

Malak Haddad

Acknowledgments

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1. Introduction

1.1. Soil

1.1.1. Significance of soil for ecosystem functioning

Soil is known to be a living and dynamic ecosystem. It is a physical and chemical complex mixture of minerals, organic matter including any dead animal or plant material returning to the soil, water, air, as well as living organisms (Bot et al., 2005). Numerous vital functions are carried out by microscopic and larger organisms in healthy soil, such as the decomposition of organic materials that involves breaking down and converting these complex molecules into simpler organic and inorganic ones (Juma, 1999). During this biological process, soil organisms get nutrients from the decomposed organic matter and all the surplus nutrients (nitrogen (N), phosphorus (P), and sulfur (S), etc.) are mineralized i.e., released in the soil in inorganic forms available for the plant uptake. Regarding carbon (C), complex structures undergo breakdown and reconstruction, while some carbon is stored within the soil and contributes to the biomass of soil organisms. Additionally, carbon is released in the form of carbon dioxide (CO_2) (Bot et al., 2005). The rate of decomposition is controlled by the soil organisms, the quality of the available organic matter and by the soil's physical properties such as pH, moisture, temperature and texture (Brussaard, 1994).

Most soils are composed of several layers or so-called horizons, each with distinct properties. In general, the amount of organic matter decreases with increasing depth. The humus which constitutes the dark most upper layer absorbs and retains the nutrients. However, factors such as nutrients and oxygen availability affect the microbial activity. Since these conditions become less favourable in deeper layers, the microbial activity is limited there. The distribution of these layers differs depending on the different factors affecting the pedogenesis such as bedrock composition and the environment conditions.

Stevenson and Cole (1999) stated that the biogeochemical cycles happening in the soil by living organisms represent the lifeline of planet Earth. These cycles that are responsible of keeping essential elements available for plants and other organisms, play a vital role in maintaining the balance and sustainability of the earth's ecosystems. Hence, understanding soil is crucial for its utilization as a medium for plant cultivation, the efficient use of both natural and synthetic fertilizers, the proper disposal of waste into the soil, and preventing soil-related pollution of air and water.

Soils possess a vast and, for many of them, a dynamic reservoir of carbon. This soil carbon pool represents more than three times the size of the atmospheric pool and 4.5 times the amount of carbon contained in vegetation (Lal, 2004). Hence, the carbon stored in soil plays a crucial role in the global carbon cycle. Minor changes in the factors affecting the rate of the processes that govern the carbon cycling within the soil own the capacity to release significant quantities of carbon dioxide (CO_2), a greenhouse gas, into the atmosphere, and thereby contribute to the greenhouse effect and its associated environmental consequences (Johnston et al., 2004).

1.2. Microbial roles and challenges in soil ecosystems

Heterotrophic microorganisms in the soil are mainly responsible for the decomposition of organic matter (Swift et al., 1979). During this process, microorganisms employ either secreted or membrane-bound digestive enzymes (referred to as DEs) to break down complex substances such as cellulose and chitin (Burns et al., 2013; Sinsabaugh et al., 1991, 1994). Since plant litter and soil organic matter are composed of macromolecules that are too large to be directly consumed by microbes, such as cellulose, hemicellulose, pectin, chitin, lignin and tannin, depolymerization by extracellular enzymes is needed. The products of these enzymatic degradation processes, such as glucose, amino acids, and phosphate, serve then as essential resources for microbial metabolism and growth (German et al., 2011).

In 2009, a study was conducted by Sinsabaugh and co-authors concerning the functional stoichiometry of heterotrophic microbial communities from different soils and freshwater sediment. Understanding the enzyme activity ratios across diverse habitats aims to enhance knowledge concerning the functioning of microbial communities and nutrient dynamics in ecosystems. The study focused on the activities of four different enzymes (β-1,4-Glucosidase, β-1,4-N-Acetylglucosaminidase, Leucine-aminopeptidase and Acid (alkaline) phosphatase) that are involved in the breakdown of organic matter and acquisition of carbon, nitrogen, and phosphorus nutrients. They found that despite differences in microbial community composition across habitats, there was a consistent relationship between the activities of these four enzymes, with a mean ratio of C:N:P activities near 1:1:1 in all habitats. They concluded the existence of a balance between the composition of microbial biomass elements, the detrital organic matter, and the effectiveness of microbial nutrient assimilation and growth. This suggests that the microbial activity, and thus, the enzymes they secrete, can be reflected by the stoichiometric balance of the measured enzymatic activities. However, the discovered

1:1:1 ratio of C:N:P does not conclusively demonstrate the efficiency of microbial nutrient assimilation. Furthermore, it is important to note that the study lacks a consideration of possible enzyme inhibition, which could be a crucial factor influencing the observed enzymatic activities.

1.3. Soil enzymes and enzyme assays

1.3.1. Enzymes

Enzymes are proteins that are considered to be biological catalysts (also known as biocatalysts) that speed up biochemical reactions in or outside living organisms. They catalyse the reaction by reducing the activation energy and thereby increasing the reaction rate. Enzymes (E) bind to the reactant molecule called substrate (S) to form the enzyme-substrate complex (C_1), which is then catalysed into the enzyme–product complex (C_2) from which the free enzyme and product are released: $S + E \leftrightarrow C_1 \leftrightarrow C_2 \rightarrow P + E$ (Titz et al., 2017).

Microorganisms and larger organisms generate two types of enzymes, categorized by their expression patterns: adaptive i.e., inducible enzymes, and constitutive enzymes. Constitutive enzymes are continually produced, while inducible enzymes are only synthesized in specific conditions or as needed (Bhatia et al., 2023). Enzyme production depends on growth or uptake of organic matter. During the growth phase of microorganisms, there is an escalation in their metabolic activity, leading to a heightened demand for enzymes. As microorganisms grow and multiply, they require enzymes at an increased rate to catalyse essential biochemical reactions. Consequently, the production of enzymes is increased. Concerning the uptake of organic matter i.e., substrates, they can encourage the production of extracellular enzymes. This happens because the presence of these substrates prompt the cells to make the necessary enzymes to break down the substrates. Meaning that specific substrates can trigger the production of specific enzymes. Additionally, natural selection favours enzyme production strategies that minimize the costs of carbon and nutrients while maximizing the associated benefits (Allison et al., 2010).

1.3.2. Soil enzymes

Dick and Kandeler (2005) stated that extracellular soil enzymes play a crucial role in ecosystem processes by catalysing reactions in soils that hold significant biogeochemical importance. These enzymes can either reside on the surface membranes of viable cells or be secreted into the soil solution. They proceeded by explaining that extracellular

enzymes break down substances that are too large or insoluble for direct uptake by microbial cells. Hence, they represent an ecological key compound for certain microbial communities.

Four of the most important and most studied extracellular enzymes in the soil are: β-1,4-Glucosidase that is responsible for the cellulose degradation, β-1,4-N-Acetylglucosaminidase that hydrolyses glucosamine from chitobiose, Leucine-aminopeptidase which plays a crucial role for proteolysis, i.e., hydrolyses leucine and other hydrophobic amino acids from the N terminus of polypeptides and finally, Acid (alkaline) phosphatase that hydrolyses phosphate from phosphosacharides and phospholipids (Sinsabaugh et al., 2009).

While the production and activity of soil enzymes have been extensively studied (Dick & Kandeler, 2005), there remains a critical knowledge gap regarding potential biochemical inhibitions that may occur within these enzymatic pathways. The interaction between enzymatic reaction products and their respective enzymes, such as glucose with β-Glucosidase and leucine with Leucine-aminopeptidase, could have significant implications for soil carbon dynamics and ecosystem functioning. This gap in knowledge underscores the need for further investigation into the potential biochemical inhibition of soil enzymes by their reaction products.

1.3.3. Enzyme assays

Enzyme assays serve both qualitative and quantitative purposes. They can be used to detect the presence or absence of a specific enzyme within a sample and measure its activity. Enzymes possess the distinct advantage of being able to be identified through their catalytic reactions (Bisswanger, 2014). Furthermore, Nannipieri et al. (2018) pointed out the fact that, with the conducted enzyme assays, we obtain an approximation of the soil's potential to undergo a particular reaction through catalytic enzymes. These enzymes might have been previously produced by active microorganisms, and subsequently preserved within the soil matrix. And/or they could have been generated from the viable cells present during the sampling period. The assay is typically conducted in slurry of soil sample with the buffer (to keep pH constant) and under laboratory temperature. Slurry is supplemented by a substrate specific to an enzyme class of interest. The enzymatic decay of an enzyme-specific substrate produces a fluorescence dye, which is measured repeatedly over time. The progress curve of reaction product concentration over time is

then analysed and the enzymatic activity is calculated. The method needs to be optimized for each soil.

1.3.3.1. pH and temperature

Enzyme activity is significantly influenced by specific conditions such as temperature, pH and ionic strength. In a study conducted by German et al. (2012), they found out that temperature influences both the maximal rate of reaction velocity (Vmax) and the Michaelis-Menten constant (K_M). Concerning the pH, studies have shown that a deviation of 1.1-1.7 pH units from the optimal pH would reduce the Vmax by 50% (Wang et al., 2012). Despite various studies attempting to replicate the *in-situ* conditions of the soil matrix (German et al., 2011; Peacock et al., 2015), Nannipieri et al. (2018) expressed scepticism about the feasibility of achieving this goal due to the complexity of the soil. They suggested a more accurate way to conduct an enzyme assay which is to run it under optimized conditions that can be different from the *in-situ* ones. The authors recommended measuring the enzyme's activity with both water and optimal pH buffers in order to increase the likelihood of reaching definitive conclusions.

1.3.3.2. Fluorescent substrate and NaOH addition

The usual way to conduct enzyme assays is to use fluorescent-conjugated substrates: 4-methylumbellifery (MUB) and 7-amino-4-methylcoumarin (AMC). It is an established fact that the highest fluorescence of the released fluorescent dye in the assay occurs under alkaline pH conditions (>9; Mead et al., 1955). Since assays are commonly performed at a pH below 9, the addition of sodium hydroxide (NaOH) was frequently employed in order to increase the pH value right before analysing the samples in a fluorometer (e.g., DeForest, 2009; Saiya-Cork et al., 2002). However, there were numerous issues associated with the addition of NaOH. First, not all buffers responded the same way to this addition and second, there was a time-dependent variation in the fluorescence of MUB and AMC after the introduction of NaOH into the assay wells, as observed by DeForest (2009). MUB fluorescence experiences an increase until approximately 20 minutes post NaOH addition, after which it starts to decline. In contrast, AMC demonstrates a decrease in fluorescence over time following the addition of NaOH.

German et al. (2011) investigated the hypothesis that MUB and AMC-based enzyme assays could yield accurate results without the addition of NaOH. They conducted enzyme assays using β-Glucosidase, β-1,4-N-Acetyl-glucosaminidase, Acid phosphatase, and Leucine-aminopeptidase in soils with varying pH values (pH 4.5 and 6.5), measuring

fluorescence with and without NaOH. They concluded that is indeed possible to run enzyme assays using MUB and AMC-linked substrates without adding NaOH to the used buffers.

1.3.3.3. Substrate concentration

When conducting an enzyme assay, the potential activity of the enzyme is estimated by measuring the decay rate of an artificial substrate (German et al., 2011). Most digestive enzymes are considered to be hydrolytic. Thus, they follow a reaction scheme:

$$S + E \leftrightarrow C1 \leftrightarrow C2 \rightarrow P + E$$

From this reaction scheme, the following equation, so called Michaelis-Menten equation, can be derived:

$$\frac{\mathrm{dProduct}}{\mathrm{dt}} = \frac{Vmax \times S}{K_M + S} \qquad \text{Eq. 1}$$

With Vmax being the maximum velocity, S is the substrate concentration, K_M is the Michaelis-Menten affinity constant, Product is the concentration of a reaction product, and t is the time.

From this, two key parameters can be deducted: Vmax and the Michaelis-Menten constant (K_M; the substrate concentration at ½ the maximal velocity), which provides information regarding the enzyme's activity and its affinity for the substrate (German et al., 2011; Malcolm, 1983; Margenot et al., 2018).

To measure the potential enzyme activity and since the reaction follows zero-order kinetic at very high substrate concentrations i.e. the rate of the reaction is not affected by the substrate concentration, it was a widespread practice to add a saturating amount of an artificial substrate to the soil-buffer slurry (German et al., 2011). The recommended amount of the substrate was five times more concentrated than the value of K_M (Malcolm, 1983; Margenot et al., 2018). Then, a linear regression without the intercept was used to estimate the Vmax. Under these conditions and within the initial linear region of product concentration increase over time, the error of the estimated Vmax was close to 20% (Fig.1, Eq.2, Čapek et al., 2021):

$$\frac{\mathrm{dProduct}}{\mathrm{dt}} = Vmax\frac{5 \times K_M}{K_M + (5 \times K_M)} > 0.8 \times Vmax \qquad \text{Eq. 2}$$

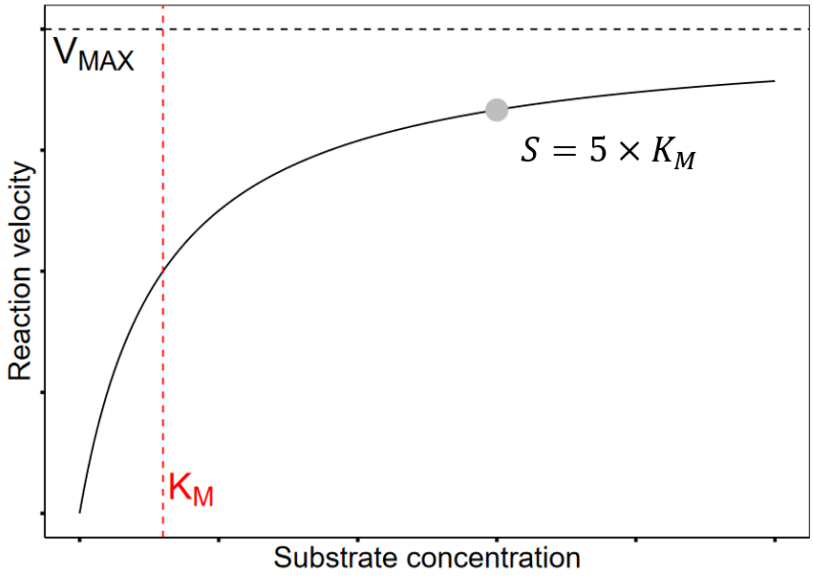

Fig. 1: *Relationship between artificial substrate concentration and the reaction velocity of the enzymatic reaction. The red vertical line represents the Michaelis-Menten constant (K_M; the substrate concentration at ½ the maximal velocity). Vmax is represented by a horizontal cut line. The grey dote shows the substrate concentration which is equal to 5 times K_M.*

However, these enzyme potential estimations as well as both equations 1 and 2 are absent of any inhibition term. It has been reported that in various classes of hydrolytic enzymes typically analysed in soil, biochemical inhibition of the enzymatic reaction by the product of the reaction itself was present. As it is the case for our two enzymes of interest: β-Glucosidase (Gusakov & Sinitsyn, 1992) and Leucine-aminopeptidase (Gilboa et al., 2001).

1.3.4. Biochemical enzymatic inhibition

1.3.4.1. Enzymatic inhibition and its types

Enzyme inhibitors are molecules that interact with enzymes, either temporarily or permanently, causing a decrease in the rate of an enzyme-catalysed reaction or disrupting the normal functioning of enzymes. Regarding the reversible ones, three types exist; Competitive inhibitors closely resemble the substrate, i.e. they compete with it for the enzyme's active site, reducing the formation of enzyme-substrate complexes. Consequently, K_M is increased but Vmax remains unchanged (Eq .3, Ochs, 2000).

$$\frac{\text{dProduct}}{\text{dt}} = \frac{Vmax\ S}{K_M\left(1+\frac{I}{K_i}\right)+S} \qquad \text{Eq .3}$$

K_i denotes the inhibitor constant and I stands for the inhibitor concentration.

Another type is the noncompetitive inhibition in which inhibitors bind to a site other than the active site of the enzyme, so called an allosteric site. Thus, K_M is unaffected but Vmax is reduced (Eq .4, Ochs, 2000).

$$\frac{\text{dProduct}}{\text{dt}} = \frac{Vmax\ S}{K_M\left(1+\frac{I}{K_i}\right)+S\ (1+\frac{I}{K_i})} \qquad \text{Eq .4}$$

In the case of uncompetitive inhibition, the inhibitor is restricted from binding to the unoccupied enzyme; instead, it exclusively binds to the enzyme-substrate complex (C_1). Therefore, the formed ES complex becomes enzymatically inactive. In uncompetitive inhibition both K_M and Vmax are reduced (Eq .5, Ochs, 2000).

$$\frac{\text{dProduct}}{\text{dt}} = \frac{Vmax\ S}{K_M+S\left(1+\frac{I}{K_i}\right)} \qquad \text{Eq .5}$$

Concerning the permanent inhibition, it occurs when the inhibitor irreversibly binds to the enzyme making it permanently inactive. Increasing the concentration of the substrate typically does not overcome this form of inhibition (Kuddus, 2018).

1.3.4.2. Effects of enzymatic inhibition in the soil

As previously mentioned, Soil Organic Carbon (SOC) results from the interactions of several ecosystem processes, including microbial decomposition within the soil (Lal, 2004). Given that enzymes play a vital role in this process, any factor influencing them inevitably extends its impact to the entire carbon cycle (Johnston et al., 2004). Considering that the product of this cycle is carbon dioxide, inhibiting enzymatic reactions does not only affect substrate decay rates but also has broader impact on the carbon dioxide production. Consequently, altered rates of enzyme activity due to inhibition can disrupt the delicate balance of carbon fluxes in soil ecosystems, potentially leading to shifts in carbon storage and greenhouse gas emissions. Thus, understanding the ecological impact of enzymes in the soil requires comprehensive consideration of enzymatic inhibition and its consequences on carbon cycling dynamics and ecosystem functioning.

1.3.4.3. Conducting enzymes assays while considering inhibition

A study conducted by Čapek et al (2021) analysed the type and strength of the product inhibition of the 4-methylumbelliferyl phosphate (MUB-P). They measured the decay rate of MUB-P at different initial MUB-P and inorganic phosphate ($P\text{-}PO_4^{3-}$) concentrations. They, then, analysed the progress curve data using a non-linear analysis. As a conclusion of their study, they found that the product inhibition of MUB-P by $P\text{-}PO_4^{3-}$ might, indeed, bias the estimation of the enzymatic activity (Fig. 2 A, B).

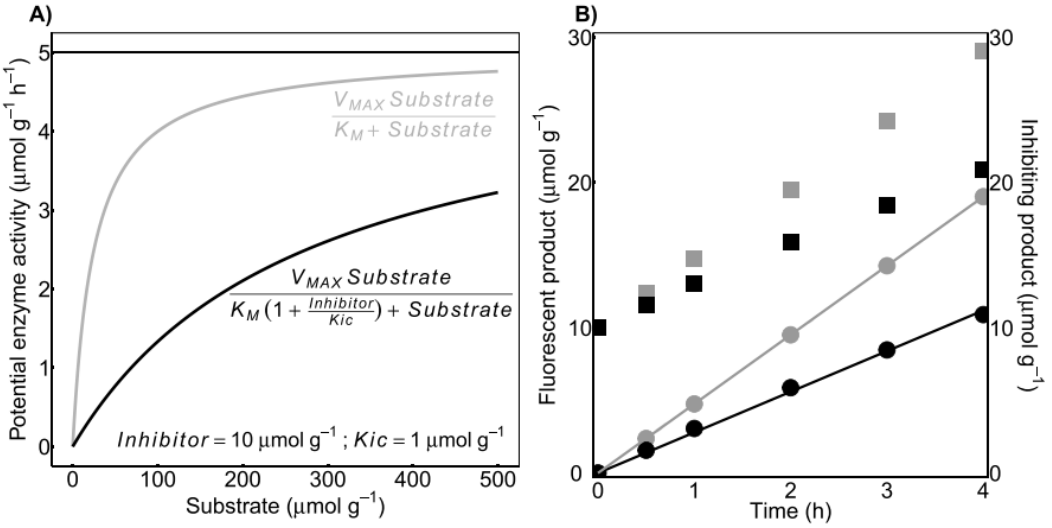

Fig. 2 ***A)*** *Relationship between artificial substrate concentration and uninhibited (grey line) or inhibited enzyme activity (black line) with their respective equations. In both equations,* Vmax*=5 µmol g^{-1} h^{-1} (represented by thin horizontal line) and K_M= 25 µmol g^{-1}. The concentration of inhibitor and value of inhibition constant (*K_i *) is shown in the figure.* **B)** *Increase of fluorescent (i.e., MUB—circles) and inhibiting (squares) product over 4-h incubation time following the uninhibited (grey colour) or inhibited enzyme activity kinetic (black colour) visualized in panel A. Solid lines of different colours represent visualization of the linear regression conducted with the data.*

Previous research regarding the potential inhibitory effects of enzymatic reaction by their products was performed on only one type of extracellular enzyme i.e. inhibition of Acid phosphatase by phosphate (Čapek et al., 2021). However, there remains a gap in our knowledge regarding other extracellular enzymes present in the soil such as β-Glucosidase and Leucine-aminopeptidase. For instance, the interaction between glucose, a product of β-Glucosidase activity, and the enzyme itself was never measured in the soil before. Similarly, the potential inhibitory effects of leucine, a product of Leucine-aminopeptidase activity, on enzyme function warrant further investigation. This understanding is essential for predicting how alterations in enzyme activity, resulting

from biochemical inhibitions, may impact carbon storage, greenhouse gas emissions, and ultimately, ecosystem sustainability. Thus, the present study aims to:

2. Aims of the thesis

1. Identify and measure the inhibition of β-Glucosidase and Leucine-aminopeptidase by the reaction products: glucose and leucine, by estimating the maximum velocity of their enzymatic activity.

2. Compare the inhibition strength between the litter layer and the organic horizon of the soil and between two different types of soils used in previous studies.

3. Identify the effects of benzoic acid, as antimicrobial, on the inhibition strength of the enzymatic reactions.

3. Materials and methods

3.1. Soil samples

For this study, the soil sampling was performed in soils from two different localisations: Plešné (PL) and Čertovo (CT). They are adjacent mountain catchments located in Bohemian Forest (Šumava Mts.) in the south-western part of the Czech Republic. Previous research has shown that both catchments share similarities in terms of vegetation and climatic conditions (Turek et al., 2014). Nonetheless, in a study conducted by Matějka (2015), the bark-beetle gradation and its effect on the regional tree biomass was observed in the Plešné Lake catchment. After comparing both catchments, he reached the conclusion that the bark beetle outbreak had led to an increase in the plant biomass quantity in the ecosystems surrounding the Plešné Lake.

The two horizons used for this study are the litter layer and the organic horizon. The litter layer also called the O horizon is where most of the decomposing organic matter is found. The organic horizon, referred to as the A horizon, which is also known as the top layer or humic horizon, is situated right below the O horizon. It contains mineral particles but also organic matter in lower concentration than the O horizon.

3.2. Sampling and storage

Soil sampling was performed in June 2022. Samples of diagnostic soil horizons denoted here after as a litter layer (O horizon, i.e., top ~ 5 cm containing fragmented but visible plant litter remnants) and organic horizon (A horizon, i.e., between ~ 5 and 15 cm depth containing amorphous organic material) were collected in each catchment. Due to the recent vegetation and soil changes caused by bark beetle outbreak, the criteria of maximum similarity of selected localities were applied. Specifically, localities with mature forest with clear more humic layer were selected. Composite sample of each was created by mixing three subsamples collected from three different soil pits. Composite samples were immediately homogenized by sieving through 5 mm mesh and stored at 4°C until the start of experiments.

3.3. Soil measurements

Prior to conducting the enzyme assays, various soil parameters were assessed. These included measuring the dry weight and pH (both with H_2O and KCl) in a 1:2.5 ratio of soil: solvent. The microbial biomass content was determined using the fumigation-extraction method for C (Vance et al., 1987), N (Brookes et al., 1985), and P (Brookes et al., 1982). The total soil organic carbon (C_T) and total soil organic nitrogen

(N_T) were determined using elemental analyzer NC 2100 (Thermo Quest Italia S.p.A., Rodano, MI). Furthermore, the total soil organic phosphorus (P_T) was determined by HNO_3 and $HClO_4$ digestion according to (Kopáček et al., 2001). In order to quantify the dissolved organic carbon (DOC) and dissolved nitrogen (DN), 4 grams of fresh soil samples were shaken with 40 mL of distilled water for 1 hour at laboratory temperature in the dark. Extracts were centrifuged (3000 g) and filtered through 0.45 μm glass fiber filters (Watrex, Prague, Czech Republic). DOC and DN in water extracts were then measured with a TOC/TN analyzer (LiquicTOC II, Elementar, Germany). Soluble reactive phosphorus (SRP) was measured in water extract by applying the spectrophotometric method described by Murphy and Riley (1962).

3.4. β-Glucosidase and Leucine-aminopeptidase assays

Following the recommendations of German et al. (2011), 4-methylumbelliferone (MUB) and 7-amino-4-methylcoumarin (AMC) were used. These fluorescent dye-conjugated substrates undergo enzymatic conversion, resulting in the formation of the fluorophores and the products, namely MUB and glucose for β-Glucosidase, and AMC and Leucine for Leucine-aminopeptidase. MUB and AMC are measured and produced in a 1:1 ratio to glucose and leucine, respectively.

The soil was diluted and homogenized following the procedure explained by Šantrůčková et al. (2004). One gram of each type of soil was added to one hundred millilitres of buffer solution and was then homogenized an IKA Ultra-Turrax T10 homogenizer (IKA®-Werke GmbH & Co. KG, Germany). All samples were treated the same, with the only exception being the adjustment of the pH of the buffer to match the reported pH values of the corresponding samples in Table 1. The citrate buffer was chosen as the corresponding buffer for the measured pH values.

Additionally, each buffer used was made with and without 0.1 % benzoic acid, in order to facilitate a comparative assessment of the two scenarios. It is important to note that benzoic acid possesses antimicrobial properties, capable of inhibiting the growth and survival of microorganisms (Eklund, 1985). Therefore, in the buffers with benzoic acid, the consumption of glucose and leucine was expected to be inhibited, a decrease in Vmax and K_i compared to the samples without benzoic acid was presumed.

While being continuously stirred, 200 μL of the soil slurry was pipetted into a 96-well plate followed by 50 μL of the solution containing either 4-methylumbelliferone-Glucose

(MUB-G) and glucose or 7-amino-4-methylcoumarin –Leucine (AMC-Leu) and leucine at different concentrations.

For each case i.e. one type of enzyme (β-Glucosidase or Leucine-aminopeptidase), one type of soil (Plešné or Čertovo), one type of horizon (litter layer or organic horizon), specificity of benzoic acid (with or without), twenty-five different solutions were prepared. Five concentrations of the substrate MUB-G or AMC-Leu (1, 5, 25, 50, and 100 μmol g^{-1}) were combined with five different concentrations of the product i.e. glucose or leucine (0, 1, 5, 10, 20 μmol g^{-1}). Two hundred microliters of soil-buffer slurry from each soil sample were loaded into the 96 wells plate with 50 μL of one of the MUB-G or AMC-Leu combinations, each combination in a triplicate. To control any spontaneous MUB-G and AMC-Leu decay, the same five concentrations of MUB-G and AMC-Leu without glucose or leucine were added to 200 μL of water. For the calibration, two hundred microliters of soil-buffer slurry from each soil sample were further supplemented with 50 μL of the MUB standards at five different concentrations (5, 25, 50, 125, and 250 μmol MUB L^{-1}) or with 50 μL of the AMC standards at five different concentrations (0.25, 0.5, 1, 2, and 5 μmol AMC L^{-1}). The second set of calibration which represents the blank was 50 μL of the same standard concentrations but mixed with 200 μL of water.

In accordance with Drouillon and Merckx (2005), fluorescence intensity measurements of MUB and AMC were conducted with an excitation wavelength set at 360 nm and emission wavelength at 460 nm. The Spark microplate reader from Tecan Group Ltd., Männedorf, Switzerland, was used for these measurements.

To accurately determine the kinetic parameters, fluorescence readings were taken at short time intervals multiple times within the initial 120 minutes of the reaction. This involved measuring the background fluorescence in all wells without the presence of MUB-G/glucose or AMC-Leu/leucine solutions. The solutions were then added one by one, starting with the lowest concentration, with immediate measurements. The time lapse between adding the respective MUB-G/glucose or AMC-Leu/leucine and the first fluorescence reading was approximately 40 seconds.

Fluorescence measurements were taken every minute for the first 10 minutes and subsequently at 15, 30, 45, 60, 75, 90, and 120 minutes. During the intervals between measurements, the plate was covered to prevent water loss and kept in the dark at laboratory temperature (24 °C).

3.5. Data analysis and statistical evaluation

3.5.1. Polynomial regression

In order to estimate the enzyme activity i.e. the change of the product concentration over time, a third-degree polynomial regression was used (Eq. 6).

$$Product = A_0 + A_1 \times time + A_2 \times time^2 + A_3 \times time^3 \quad \text{Eq .6}$$

Product represents the concentration of a reaction product. A_0 is the product concentration at time 0. A_1, A_2 and A_3 are coefficients of polynomial regression.

The second term of the regression A_1, the regression slope, defines the rate of change of the reaction product concentration over time at time 0 (hereafter referred to as V_0), at which the exact concentration of substrate as well as the inhibitor is known.

Polynomial regression was used to avoid any bias related to the misconception of the accurate relationship between the reaction product concentration and time, in contrast to the limitations of linear regression.

3.5.2. Determining the product inhibition

In order to analyse the possible inhibition and determine its type, a double reciprocal plot i.e. Lineweaver–Burk plot of $1/V_0$ against 1/S was generated (Fig .3). It corresponds to a linear relationship where the y-intercept corresponds to 1/Vmax, and the x-intercept is equal to K_M /Vmax. Vmax and K_M were determined experimentally by measuring V_0 at different substrate concentrations (1, 5, 25, 50, and 100 μmol g^{-1}).

Competitive inhibition can be recognised using Lineweaver-Burk plot if the slope is altered (K_M is increased) and the intercept on the y-axis unchanged (since Vmax remains constant).

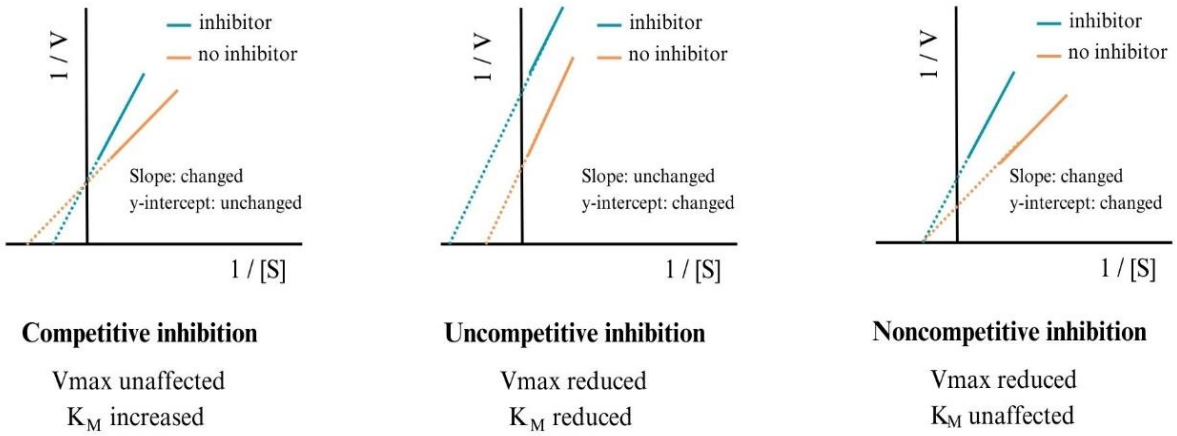

Fig. 3 *Models of Lineweaver-Burk plots for enzyme competitive, uncompetitive and noncompetitive inhibition. It demonstrates the relationship between the inverse substrate concentration and the inverse velocity. Blue lines represent scenarios with inhibitor presence, while the orange line corresponds to the absence of inhibitor.*

The statistical evaluation of the competitive inhibition was performed. First, non-linear regression was used to estimate the kinetic parameters of Michaelis-Menten equation (Vmax, K_M and K_i) with and without inhibition. Afterwards, their goodness of fit was compared employing an F-test, and the corresponding p-values were determined.

The statistical evaluation was performed for both β-Glucosidase and Leucine-aminopeptidase enzymes, in the presence and absence of benzoic acid and for both the litter layer and the organic horizon separately. All data analyses were done in the statistical program R version 4.3.0 (R Core Team 2023).

4. Results

4.1. Soil characteristics

All soil samples from both catchments were highly acidic (Table 1). The pH ranged from 3.72 to 4.80 and it appeared that the Plešné soil was more acidic. Generally, the concentrations of all measured chemical and microbial biomass characteristics were higher in the litter layer compared to the organic horizon.

Table 1: Basic chemical characteristics (pH, C_T, total soil organic C content; N_T, total soil N content; P_T, total soil phosphorus content; DOC, water extractable organic C; DON, water extractable organic N; SRP, water extractable soluble reactive P) and microbial characteristics (MBC, microbial biomass C; MBN, microbial biomass N; MBP, microbial biomass P) of litter layer and organic horizon sampled in mountain spruce forest catchments of lakes Plešné and Čertovo. The non-italic numbers indicate the mean and the italic font shows the standard deviation of the mean.

Catchment	Horizon	pH	C_T	N_T	P_T	DOC	DON	SRP	MBC	MBN	MBP
			mmol g^{-1}		µmol g^{-1}						
Plešné	Litter Layer	3.72	39.8	1.33	32.8	87.5	30.8	1.6	477	58.1	25.5
			0.65	*0.05*	*4.49*	*1.43*	*0.58*	*0.16*	*26.31*	*3.18*	*0.80*
	Organic Horizon	3.75	37.6	1.33	26.8	49.2	28.1	0.5	313	35.9	20.7
			2.39	*0.21*	*7.05*	*0.92*	*0.66*	*0.06*	*13.76*	*2.29*	*0.28*
Čertovo	Litter Layer	4.80	38.8	1.50	35.3	60.1	13.8	0.6	378	46.1	13.4
			0.21	*0.00*	*2.62*	*0.84*	*0.14*	*0.04*	*5.88*	*0.91*	*1.09*
	Organic Horizon	4.67	28.7	1.13	28.3	41.1	8.80	0.30	260	29.7	14.4
			5.44	*0.17*	*3.46*	*0.52*	*0.19*	*0.07*	*13.17*	*0.97*	*0.44*

4.2. Biochemical product inhibition determination

4.2.1. β-Glucosidase enzyme

The Lineweaver Burk plots for the β-Glucosidase enzyme showed a similar pattern in soils from both catchments, regardless of the presence of benzoic acid (Fig. 4). The decay rate of MUB-G substrate was measured first without any addition of the inhibitor. Then, higher glucose concentrations were added. The graph (Fig. 4) which represents Čertovo catchment, shows that the more glucose was added, the increased the slope is, and thus, the decreased reaction velocity. In other words, we observed a clear correlation between increasing glucose concentrations and a decrease in velocity, indicating enzyme inhibition by its product. Additionally, it was noted that all lines intersected at a single point on the y-axis, indicating an increase in K_M while Vmax remained unaffected. This characteristic pattern suggests the presence of competitive inhibition.

Concerning the litter layer, p-values shows clear statistical significance of competitive product inhibition in soil from both catchments, whether with or without benzoic acid. This significance is particularly pronounced in the Čertovo catchment for both scenarios. In Plešné catchment, statistical significance of competitive inhibition was observed only in the absence of benzoic acid. In the organic horizon, all cases exhibited statistically significant inhibition, with p-values slightly above 0.05 considered marginally significant.

The addition of benzoic acid displayed varying effects: in Plešné catchment, it decreased Vmax and K_M, while in Čertovo catchment, increased Vmax and K_M and lower K_i was recorded in its presence.

In general, the Vmax recorded in competitive inhibition was higher in the litter layer compared to the organic horizon (Table 2). Similarly, K_i values indicated stronger inhibition whereas K_M values suggested that β-Glucosidase enzymes have a lower substrate affinity for their substrates, MUB-G, in the litter layer (Table 2).

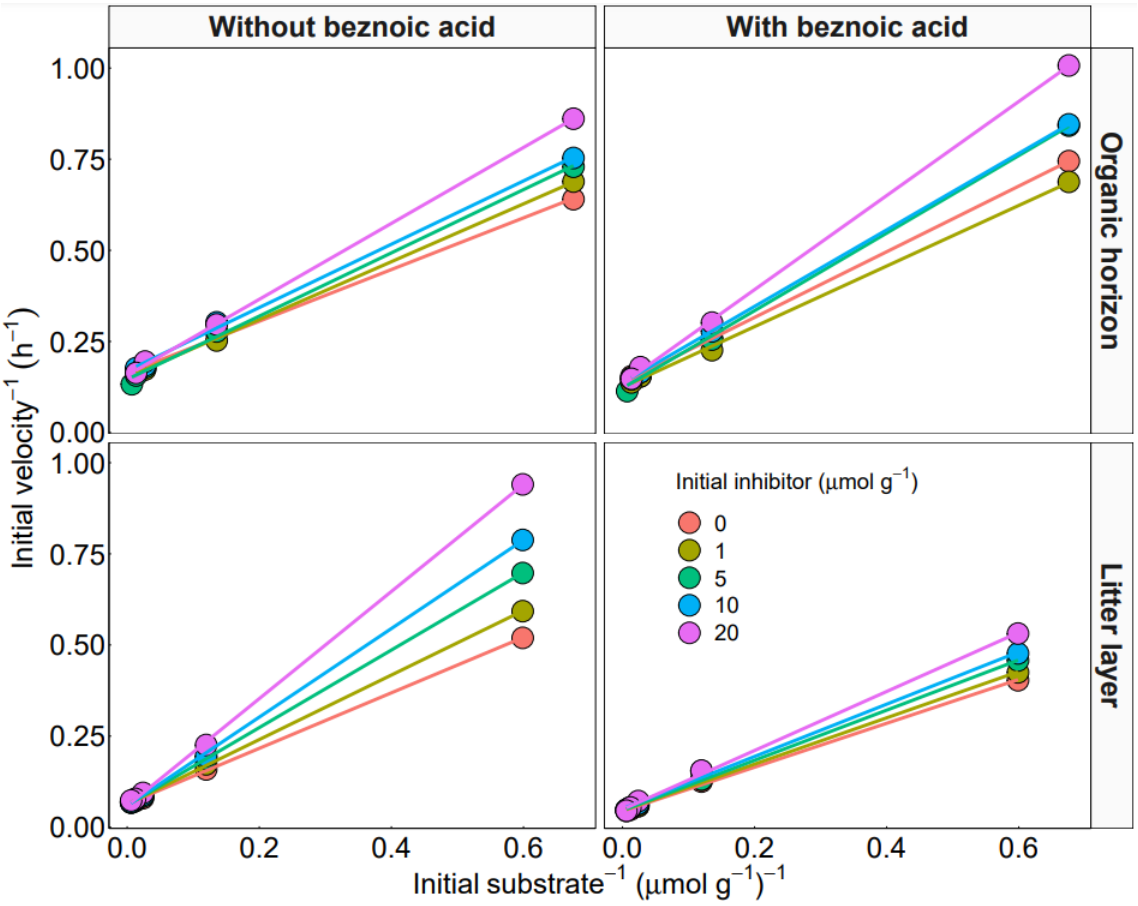

Fig. 4 *Relationship between the inverse initial substrate concentration (µmol g^{-1})$^{-1}$ and the inverse initial velocity (h^{-1}) of β-Glucosidase in Čertovo catchment. The figure is divided into 4 plots representing different treatments. The treatments' identity is defined in the upper and right facets. The colour code distinguishes the different concentrations of the added inhibitor*.

Table 2: Enzyme kinetic parameters (Vmax, K_M, and K_i) and statistical significance of competitive inhibition (F and p-values) for β-Glucosidase activity in soil samples from Plešné and Čertovo catchments in both litter layer and organic horizon, with (true) and without (false) the addition of benzoic acid. The values indicate the mean estimates of non-linear regression. Confidence intervals (95%) are shown in parentheses.

Catchment	Horizon	Benzoic Acid	Vmax	K_M	K_i	F	P
Plešné	Litter Layer	False	15.29 (14.70-15.98)	10.80 (8.87-13.10)	43.62 (23.05-149.5)	6.817	0.016
		True	8.72 (8.29-9.25)	8.257 (6.23-10.53)	NA	0.015	0.903
Čertovo		False	15.98 (15.77-16.17)	13.30 (12.4-14.15)	30.22 (24.62-38.28)	111.0	< 0.001
		True	23.75 (23.16-24.41)	16.59 (14.5-18.57)	35.25 (24.38-59.79)	26.73	< 0.001
Plešné	Organic Horizon	False	7.711 (7.51-7.95)	5.836 (4.80-6.78)	25.37 (12.90-88.92)	6.545	0.020
		True	3.648 (3.47-3.82)	2.380 (1.71-3.26)	25.99 (10.10-251.34)	3.479	0.076
Čertovo		False	6.665 (6.44-6.98)	5.571 (4.17-6.79)	41.01 (18.19-237.53)	3.795	0.067
		True	7.766 (7.40-8.21)	6.714 (4.83-8.31)	28.05 (13.53-120.74)	6.253	0.022

4.2.2. Leucine-aminopeptidase enzyme

Concerning the possible inhibition of Leucine-aminopeptidase by its product i.e. leucine, the conducted experiment showed no conclusive results, in both the litter layer and organic horizon. An example of the obtained data is shown in the graph below (Fig. 5) that represents Leucine-aminopeptidase in Plešné catchment without any addition of benzoic acid. It does not show a linear relationship between the substrate concentration and the rate of the enzymatic reaction at any inhibitor concentration. Therefore, it does not conform to any equation reported in the introduction (section 1.3.3.1.). Consequently, it was not possible to determine any inhibition type.

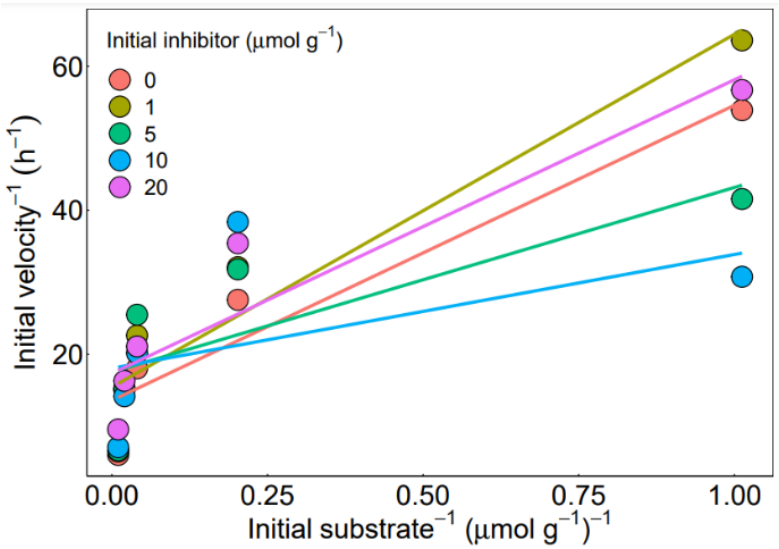

Fig. 5 *Relationship between the inverse initial substrate concentration (µmol g^{-1})$^{-1}$ and the inverse initial velocity (h^{-1}) of Leucine-aminopeptidase in the litter layer of Plešné catchment without the addition of benzoic acid. The colour code shows the different concentrations of the added inhibitor.*

5. Discussion

5.1. Biochemical product inhibition

The main aim of this thesis was to determine the biochemical inhibition of β-Glucosidase and Leucine-aminopeptidase by the reaction products i.e. glucose and leucine, respectively. According to our hypothesis, the decay rate of MUB-G substrate, specific to β-Glucosidase and AMC-Leucine, specific to Leucine-aminopeptidase, would decrease when the product concentration is increased.

Concerning the β-Glucosidase enzyme, the non-linear regression analysis showed the presence of competitive product inhibition (Fig. 4, Table 2). This specific enzyme being inhibited by its product has not been subject to previous research. However, if compared to other studies concerning other extracellular hydrolytic enzymes in the soil, namely the Acid phosphatase, it appears that our findings are consistent with other studies implying the presence of competitive inhibition by the reaction product for this type of enzymes (Bezerra & Dias, 2007; Čapek et al., 2021).

In the case of Leucine-aminopeptidase, the findings did not support the initial hypothesis which suggested the biochemical product inhibition by leucine. The data (Fig. 5) revealed that there was no specific correlation between the decay rate of AMC-Leu and the concentration of either the substrate or the product, leucine. One hypothesis that could explain this behaviour is that the Leucine-aminopeptidase does not follow the

Michaelis-Menten mechanisms. However, this appears improbable as it contradicts numerous research findings (German et al., 2011; Sinsabaugh et al., 2013). Another explanation would be that the concentration of this enzyme, present in our soil samples from both Plešné and Čertovo catchments was too low to be able to reliably measure its activity. The last explanation and maybe the most probable one is the high abiotic degradation of the substrate. This would explain the inconsistent and not conclusive results because the measured decay rate can be resulting from the spontaneous degradation of the substrate or by the actual enzymatic reaction. This would mean that the proportion of the spontaneous degradation is much greater than the enzymatic reaction. Therefore, even if enzymatic reactions are occurring, their effects might not be clearly observed due to the dominance of the spontaneous degradation.

5.2. Horizons comparison in Plešné and Čertovo soils

Overall, competitive inhibition by the product, glucose, was detected for β-Glucosidase enzyme in samples collected from both Plešné and Čertovo soils in both, litter layers and organic horizons. Consistently, the Vmax values were notably higher in the litter layer compared to the organic horizon for both soil types, reflecting a greater enzymatic activity in the upper layer where microbial biomass concentration is typically elevated (as demonstrated by MBC values in Table 1). This observation is consistent with our hypothesis that the higher enzymatic activity in the litter layer can be attributed to the increased microbial biomass and metabolic activity in this upper layer of the soil (Bárta et al., 2014; Šantrůčková et al., 2004). These microorganisms play a crucial role in enzyme production, thus resulting in elevated Vmax values (Allison, 2012). Furthermore, the K_M values which are higher in the litter layer suggest a lower substrate affinity of the enzyme. This can be explained by the trade-off between enzyme specific activity and substrate specificity. Microbes can either produce enzymes which work fast (have high Vmax) but have lower affinity to substrate (high K_M) or vice versa. The enzymes in the litter layer and organic horizons then represent the opposite ends of the spectra – i.e. fast unspecific enzymes in the litter layer and specific enzymes with lower Vmax in organic horizons. The obtained K_i values indicate a significant similarity between the litter layer and the organic horizon. However, there remains a lack of information regarding whether this variability correlates with substrate concentration, microbial biomass, or other enzyme characteristics such as Vmax and K_M. None of these effects can be clearly identified from our dataset.

5.3. Benzoic acid effect

The addition of benzoic acid, known for its antimicrobial properties, was anticipated to inhibit microbial consumption of glucose, thus potentially impacting enzymatic reactions. Our hypothesis suggested a decrease in both Vmax and K_i in the presence of benzoic acid. However, the results presented in Table 4 indicate that, overall, the addition of benzoic acid did not significantly alter the enzymatic reactions.

Specifically, in the case of Plešné, no inhibition was observed in the litter layer with the addition of benzoic acid. Conversely, in the organic horizon, Vmax decreased while K_i remained relatively constant. Analysis of samples from Čertovo revealed increases in both Vmax and K_M in both layers, with K_i increased in the litter layer but decreased in the organic horizon.

From these results, it is evident that benzoic acid does not exhibit a consistent pattern of effects on enzymatic reactions. Further research testing the addition of benzoic acid in different concentrations are required in order to elucidate the specific mechanisms and effects of benzoic acid on these enzymatic reactions.

5.4. Enzymatic inhibition and carbon dynamics in soil ecosystems

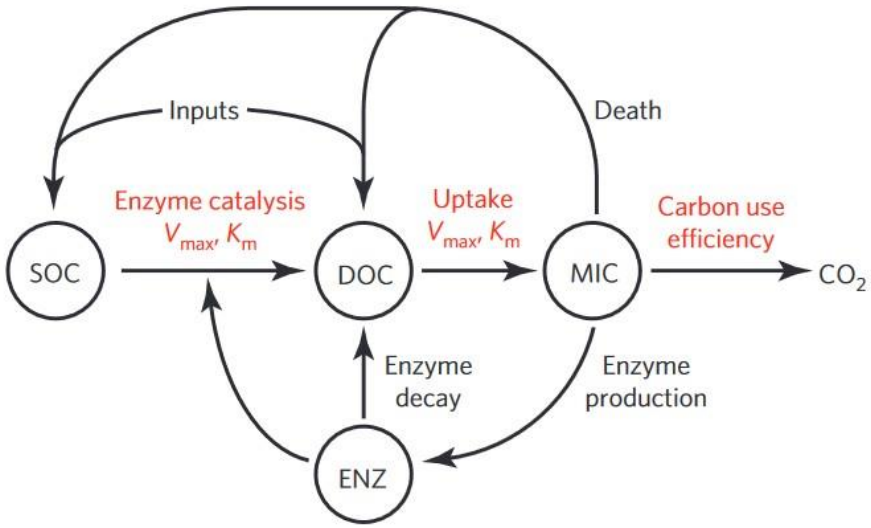

Fig. 6 *Scheme representing the relationship between the soil organic carbon (SOC), dissolved organic carbon (DOC), microorganisms (MIC) responsible of releasing enzymes (ENZ) that catalyse all the transformations occurring within these processes. The final byproduct is the release of carbon dioxide (CO_2).*

In order to understand the impact of the enzymatic inhibitions on the carbon dynamics in the soil, it is important to identify the carbon's origin and its subsequent transformations (Fig. 6). Soil organic carbon, representing carbon stored in organic matter, undergoes enzymatic transformation to convert into dissolved organic carbon.

Microorganisms present in the soil use the dissolved organic carbon to produce energy which is necessary for their growth and metabolism. As a result of this process, microbial respiration releases carbon dioxide as a byproduct.

Moreover, several studies have demonstrated that these microorganisms secrete extracellular enzymes responsible for the decomposition of both SOC and DOC (Burns et al., 2013; Sinsabaugh et al., 1991, 1994; Swift et al., 1979). Intriguingly, the interaction between these enzymes and their respective substrates introduces a complex interplay that can be influenced by various factors, including enzymatic inhibition by end products.

The inhibition of enzymes in the soil by their own products as in the case of β-Glucosidase by glucose has, therefore, significant consequences affecting more than just the decay rate of organic matter. This inhibition can reduce the glucose excess for microbial biomass at certain times e.g. shortly after litter is shed or in patches with high organic carbon content. Therefore, the enzymatic inhibition can decrease the loss of organic matter from soil via respiration due to reducing the excess of substrate, which could be otherwise respired, and thus, can influence the release of the greenhouse gas CO_2, impacting the balance of carbon in soil ecosystems.

6. Conclusion

This study revealed that β-Glucosidase enzyme undergoes competitive inhibition by its product, glucose, while no inhibition was observed for Leucine-aminopeptidase. We suggest conducting the same analysis with other soils in order to understand the variability of K_i and its correlation with substrate concentration, microbial biomass, or other enzyme characteristics. These findings emphasise the importance of considering enzymatic inhibitions. We highly advocate further research to take into account the possible inhibition in order to ensure an unbiased measurement of enzymatic activity.

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