European Journal of Soil Biology 70 (2015) 31–37

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Response of amylase activity in buried paleosols and subsoil permafrost to low-molecular-weight compounds

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A R T I C L E I N F O

Article history:
Received 10 February 2015
Received in revised form
30 June 2015
Accepted 5 July 2015
Available online 16 July 2015

Keywords:
Buried and modern soils
Subsoil permafrost
Amylase activity
Alkylresorcinols
Amino acids
Humic acids

A B S T R A C T

Currently, limited information is available on the enzymatic activities in ancient paleosols and permafrost, which are recognized as unique depositories of extant microorganisms. In this work, samples from buried and modern top Kastanozems (Haplic Calcisol), modern tundra (Gleysol) soils, and permafrost were tested for amylase activity based on hydrolysis of starch to low-molecular sugars that are important energy sources for microorganisms. Our study demonstrated the preservation of amylase in buried paleosols (300 and 2000 years old) and permafrost (15,000–60,000 years old). The enzyme activity in buried soils was 5–7 times lower than in top soils and did not significantly ($P > 0.05$) differ from permafrost and tundra soils. Exogenously added alkylresorcinols, valine, and glycine influenced amylolytic activity in soil and permafrost samples in the dose-dependent manner. The amylase response to stimulating concentrations of alkylresorcinols and amino acids was greater in buried paleosols (4.3–11.5-fold) than in top soils (1.9–3.8-fold) over the enzymatic activities in the controls. Positive response of soil amylase even to little changes in concentrations of the low-molecular-weight compounds may be important during recovery of biological activity of microorganisms. Results of this study contribute to a more complete evaluation of hidden biological potential of ancient soils and permafrost and are relevant to paleomicrobiology and soil biology.

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1. Introduction

Buried paleosols from temperate regions and tundra soils and permafrost from cold regions are unique natural depositories of microorganisms that have survived for up to millions of years in the anabiotic state in the absence of available nutrients and water [1,2]. Long-term survival of microorganisms in paleosols and permafrost is ensured by their persistence in the form of dormant cyst-like cells [3] and viable but non-culturable cells that need reactivation to resume the growth [4,5]. Dormant forms comprise a hidden pool of the biological component in such biotops. Along with biota, the biological activity of soils depends upon the functioning of enzymes released from micro- and macroorganisms. Soil enzymes are involved in nutrient cycling and biopolymer transformation, thus contributing to the overall soil productivity and the development of microbial communities [6]. The enzymatic pool is replenished due to metabolic activity of soil biota, including microorganisms (review [6] and references therein). As yet, limited information has been available on the persistence of enzymes in subsoil permafrost [4] and paleosols [7].

One of sensitive indicators of soil activity, ecological changes, fertility, and health are polysaccharide hydrolyzing enzymes, such as amylase [8,9]. Soil amylase is constituted by α-amylase synthesized by plants, animals, and microorganisms and β-amylase produced mainly by plants. Amylase is widely distributed in soils and plays an important role in the breakdown of starch to low molecular weight sugars that are important energy sources for microorganisms [[9] and references therein]. The activity of amylase and invertase was detected in some permafrost sediments even in those samples where

Abbreviations: AR, alkylresorcinol; HR, hexylresorcinol; MR, methylresorcinol; HA, humic acid.

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http://dx.doi.org/10.1016/j.ejsobi.2015.07.001
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culturable microorganisms were absent [4]. As to buried paleosols, no information on amylase activity has been reported as yet. The enzymatic activity in biotops harboring metabolically inactive macro- and microorganisms may be preserved and modulated (often restricted) as result of immobilization and structural modification of enzymes by low-molecular-weight (LMW) compounds.

The interaction of different enzymes (protease, cellulase, amylase of microbial origin and trypsin) with LMW alkylresorcinols (ARs, also designated as alkylhydroxybenzenes) resulted in activation or inhibition of these enzymes with improved stability to denaturing factors [10,11]. Since ARs have phenolic moieties like those in humic acids (HA) [12], it is expected that ARs and HA exert a similar effect on amylase although at different concentrations. However, the enzyme response may differ depending on the chemical nature of humic substances. In particular, HA from buried paleosols are more enriched with aromatic moieties than HA from modern soils [13] and, therefore, it is assumed that humic substances from these ancient objects can exert a prominent effect on amylase. On the other hand, similar (to a certain extent) effects are inherent to other LMW compounds, such as amino acids. Thus, glycine stabilized pure β-galactosidase and lactate dehydrogenase during freezing-thawing and pH changes [14]. As to soil enzymes, little has been known about their response to amino acids: only stimulating or inhibiting effects of glutamic acids and alanine enantiomers were shown for casein protease and acid phosphomonoesterase [15]. Therefore, a response of soil amylase to amino acids cannot be predicted based on this information, as well as similarities or differences with ARs.

The objective of this work was to compare amylase activity in permafrost, paleosols, and modern soils and to study the patterns of enzymatic response to various concentrations of low-molecular-weight AR and amino acids.

2. Material and methods

2.1. Soils and permafrost layers

We studied four samples of buried and top Kastanozems (Haplic Calcisol according to IUSS Working Group WRB. 2014) from the Volga-Don interfluve in the arid steppe zone of the Volgograd region (Russia). Samples of buried soils were taken from sites under kurgans (49°3.291′ N, 44°7.247′ E and 47°54.072′ N, 43°52.445′ E) from 2 to 3 profiles with different heights. Modern background soils were sampled from the top horizons (Table 1). All the samples were representatives of specimens taken aseptically from 10 microsites from each horizon. Soils were placed in sterile bags and stored at room temperature [1]. The age of buried soils was estimated by archeological dating and radiocarbon method [1].

The other four samples of subsoil permafrost layers and tundra soils (Table 1), kindly provided by Prof. Gilichinsky (Institute of Physicochemical and Biological Problems of Soil Science, Pushchino) in 2007, were from the upper reaches of the Bolshoy Khomus-Yuryakh river (70.8210 N, 153.2829 E) in the Kolyma lowland in the north-eastern Siberia (Russia). Permafrost specimens were taken aseptically using the drilling technique, placed into airtight containers and stored at −7 to −12 °C [2]. The age of permafrost was determined by radiocarbon, paleomagnetic, palinological, and paleontological analyses [2, 4 and references therein]. The number of cultivable bacteria in the studied samples was determined upon plating on nutrient agar in the previous studies [1,5].

2.2. Assay of amylase activity

All eight samples (Table 1) were tested for amylase activity. Samples of dry paleosols and top soils were ground in a mechanical mortar and pestle and passed through a 2-mm sieve. Frozen samples were prepared without a long exposure to room temperature. Pre-weighted (5 g) samples were suspended in 5 ml of phosphate buffer (pH 5.5) plus 5 ml of 1% starch as a substrate and 0.2 ml of toluene. Suspensions were incubated at 30 °C for 24 h according to the protocol used in soil amylase assays [8]. The amylolytic activity in soil and permafrost solutions was assessed from the amount of maltose released from soluble starch according to the dinitrosalicylic acid procedure [16] with absorbance measurements at $\lambda = 508$ nm (UV-VIS Specord, Carl Zeiss, Germany). The activity was expressed in milligrams of maltose produced in 24 h at 30 °C at pH 5.5 [8] with corrections for its background content in samples.

The activity of commercial β-amylase (EC 3.2.1.1, Type II-B, Sigma) in phosphate buffer was assayed using the above method with the difference that the pH of the reaction mixture was of 4.8 as prescribed in Refs. [16]; one activity unit corresponded to the amount of enzyme required to liberate 1 mg maltose in 3 min at 30 °C. (Tests showed no differences in β-amylase activity at pH 4.8 and 5.5).

Suspensions of soil and permafrost samples were also preincubated at room temperature for 30 min before amylase assays with hexylresorcinol (HR, 0.02–1.8 mg g^{-1}), methylresorcinol (MR, 0.5–2 mg g^{-1}) both from Sigma, glycine, D-valine, or L-valine (Ajinomoto Inc., Japan) at concentrations 0.002–4 mg g^{-1}. Solutions of commercial β-amylase (40 μg ml^{-1}) were treated with MR (0.01–0.2 mg ml^{-1}), HR (0.01–0.16 mg ml^{-1}), or D -valine (0.02–0.2 mg ml^{-1}). MR, amino acids, and were added in the form of aqueous solutions. HR was dissolved at various concentrations in ethanol and added to samples at the proportion 1:20 (v/v).

2.3. Isolation of humic acids

The sample 3 of buried paleosol (2000 years) and the sample 2 of modern top soil (Table 1) were sources for humic acid fractions (HA1 and HA2, respectively) to compare their effects on pure β-amylase, depending on the differences in the content of aromatic structures [13]. A sample of leached Chernozem (the Voronezh region, Russia) with high content of organic matter was a source of HA3. HA4 was commercially available humic acid (Aldrich). The fractions HA3 and HA4 served as controls in β-amylase assays.

Fractions of humic acids (HA 1- HA3) were extracted from 5-g soil samples with 0.1 M NaOH after decalcification with 1 M HCl. Mineral admixtures were removed from the extracts by centrifugation at 3000 *g* for 15 min. Humic acids were separated from fulvic acids by precipitation at pH 1–2 after adding 6 M HCl; the residue was washed several times with distilled water and dissolved in 50 ml of 0.1 M NaOH. The total carbon content was of 0.012 mg ml^{-1}, 0.015 mg ml^{-1}, 0.188 mg ml^{-1}, and 0.148 mg ml^{-1} for the HA 1–4 fractions, respectively. Aqueous solutions of fractions HA1 − HA4 were added to commercial β-amylase solutions at final concentrations 0.2 μg ml^{-1}–15 μg ml^{-1} and tested for the enzymatic activity as described above.

2.4. Assay of alkylresorcinol content

Amylase-tested samples 1 and 2 of buried and top soils and samples 5 and 6 of permafrost (Table 1) were taken to prove the presence of native alkylresorcinols that are analogs of MR and HR used in our study of soil amylase response. Additional seven samples (Table A.1) were used to evaluate the ARs content in soils and permafrost of different age.

Alkyresorcinol fractions were extracted from 2-g samples with two portions (4 ml) of *n*-propanol. The AR amount in extracts was determined using the colorimetric AR reaction with o-dianisidine

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