Amino Acids (2008) 35: 411–417
DOI 10.1007/s00726-007-0601-5
Printed in The Netherlands

Amino Acids

Estimation of protease activity in soils at low temperatures by casein amendment and with substitution of buffer by demineralized water

K. Rejsek[1], **P. Formanek**[1], and **M. Pavelka**[2]

[1] Department of Geology and Soil Science, Mendel University of Agriculture and Forestry, Brno, Czech Republic
[2] Laboratory of Ecological Physiology of Forest Trees, Institute of Systems Biology and Ecology, Brno, Czech Republic

Received July 9, 2007
Accepted August 28, 2007
Published online November 2, 2007;

Summary. The aim of this work was to modify the method of Ladd and Buttler (1972), by substituting Tris–HCl buffer (pH 8.52) with demineralized water (DEMI H_2O), in order to assess its suitability for measurement of casein-protease activity at pH levels close to those of real soil in H_2O. Measurements were undertaken over a range of incubation temperatures from 3 to 49 °C. Testing was performed on one organic soil and two different mineral soils. The substitution of Tris–HCl buffer by DEMI H_2O at 49 °C decreased casein-protease activity to 67.25% in mineral soil and to 53.76% in organic soil. With decreasing temperature casein-protease activity decreased the most in organic soil, i.e., 0.07% of original its value at 3 °C. The incubation period was extended to maximally 336 h at 3 °C to totally obtain >10.0% of L-tyrosine equivalents released at optimum or close to optimum temperature and pH conditions. The Q_{10} values of casein-protease activity measured after substituting Tris–HCl buffer with DEMI H_2O were unexpectedly high. Between the temperatures of 3 and 49 °C Q_{10} ranged from 3.46 to 4.25, whereas between 3 and 25 °C Q_{10} ranged from 6.78 to 11.08. Therefore, the modified method of Ladd and Buttler (1972) presented can be used for measurement of soil casein-protease activity under pH conditions close to that of real soil pH and at an averaged soil temperatures measured in the field. This modification makes possible an expression of soil casein-protease activity potential – when being combined with measurements of casein-protease activity under optimum or close to optimum temperature and pH conditions, if high concentration of casein is present.

Keywords: Soil proteases – Enzyme temperature dependence – Q_{10}

Introduction

Proteases (EC 3.4.4) play an important role in mineralization of soil nitrogen. They are involved in cleavage of proteins to polypeptides, and oligopeptides to amino acids. Microorganisms are the main source of proteases in soil and because of the high molecular weight of proteins, the first enzymatic step in protein degradation occurs outside microbial cells. Proteases activity on plant roots or seeds is also of microbial origin (Vágnerová and Macura, 1974; Tjalsma et al., 2004; Aslim et al., 2006; Cazorla et al., 2007).

Soil proteases assays are frequently used. Their activity is commonly measured towards casein where the rate of tyrosine equivalents from casein hydrolysis is measured by the method of Ladd and Buttler (1972) with different modifications (Nannipieri et al., 1979; Gil-Soltres et al., 1992; Dilly and Munch, 1996; Vedder et al., 1996; Trasar-Cepeda et al., 1998; Dilly, 1999; Kandeler et al., 1999; Nunnan et al., 2000; Li et al., 2002; Dinesh et al., 2004; Formánek et al., 2006 etc.).

Temperature has a significant effect on the activity and production of soil proteases. Optimal temperature for production may not be the same as the optimal temperature for activity (Ambrož, 1969). Soil proteases are usually measured in the laboratory at temperature and pH adjusted to express maximum or close to maximum activity. Optimum temperature for soil casein-protease activity is around 55 °C and optimum pH of around 8.0 (Ambrož, 1969; Alef and Nannipieri, 1995). Nunnan et al. (2000) performed experiments whereby casein-protease activity was measured by a modified method of Ladd and Buttler (1972) under optimum or close to optimum conditions (temperature, pH) in combination with laboratory incubations adjusted to averaged daily temperatures in the field. The authors suggested that proteases' activity limited by temperature reflected activity of those enzymes that have recently been secreted from microorganisms and not yet interacted with soil colloids or were only minimally affected by these interactions (Rejsek, 1991). On the

other hand, potential protease activity reflects activity of enzymes bound to soil colloids. Except for adjustment of incubation temperature to averaged temperatures measured in the field, it is also possible to substitute the buffer (pH around 8) applied to soil with substrate with demineralized water in order to measure casein-protease activity under pH values very close to real soil pH.

The aim of this work was to develop a procedure for measurement of exploitation of potential of soil proteases activity against casein in different ecosystems. The assumption for this aim was to modify the method of Ladd and Buttler (1972) for measurement of casein-protease activity by operating at a lower temperatures and substituting Tris–HCl buffer with demineralized water so as to attain a pH close to that of real soil pH. To accomplish the aim, the use of such a modified method should be combined with measurement of potential casein-protease activity at optimum or close to optimum temperature and pH conditions. The ratio between the casein-protease activity assessed by modified method and that obtained at optimum or close to optimum temperature and pH, expressed in per cents, represents the exploitation potential of soil proteases activity against casein when substrate saturation is not taken into account. The description of the dependence of casein-protease activity on the temperature with expression of Q_{10} values based on the application of the modification on samples taken from three different soils is presented as well.

Materials and methods

Soil samples preparation

The design of the experiment was based on samples of very different properties. To have them, the following different soil samples from three different soil units (ISSS-ISRIC-FAO, 1998) have been used in this study:

i. NS sample, Rendzic Leptosol, O horizon, the 40-year-old Norway spruce pure stands;
ii. EBH sample, Haplic Luvisol, A horizon, the 90-year-old mixed European beech and hornbeam stand, and;
iii. G 0–10 sample, Hortic Anthrosol, A horizon, the cultivated vegetable garden.

These samples were taken from only organic soil (O horizon) and topsoil (A horizon) due to their frequent incorporation into ecological studies. The sampling was performed in May 2006.

After transportation to laboratory, the soils were sieved through 5 mm mesh size and stored in a refrigerator for a period of <14 days. The selected properties of the soils are presented in Table 1.

Measurement of casein-protease activity

Casein-protease assesment was performed by the modified method of Ladd and Buttler (1972) (Nannipieri et al., 1979). Wet soil (1 g) was incubated with 0.05 M Tris–HCl buffer (2 ml, pH 8.52) and 2 ml 1% casein (sodium salt, C-8654, Sigma) solution (pH 6.7) at 49 °C for a period of 2 h (Method I). Control samples were prepared by the same way as described above, except that incubations were performed without 1% casein which was applied into reaction mixture at the end of incubation. After stopping the reaction by the addition of 1 ml of 17.5% trichloroacetic acid (TCA) and subsequent centrifugation, 1 ml of supernatant was mixed with 3.7% aqueous Na_2CO_3 solution, and 1 ml 0.06% aqueous $CuSO_4$ solution. After mixing and 30 min incubation at room temperature, 1 ml of Folin-Ciocalteau reagent (diluted 1:3 by demineralized water) was applied. Following a further incubation at 37 °C and 15 min at room temperature, the concentration of aromatic amino acids released by proteolytic cleavage of casein and expressed in L-tyrosine equivalents was determined colorimetrically at 578 nm (Specol 11). The calibration line of dependence of absorbance on L-tyrosine concentration was prepared from stock solution of L-tyrosine in DEMI H_2O and a solution of 0.05 M Tris–HCl / 17.5% TCA (ratio 3:1).

The modification of the above mentioned method consisted of substitution of 0.05 M Tris–HCl bufer (pH 8.52) by DEMI H_2O and incubation of reaction mixtures of 1 g wet soil, 2 ml DEMI H_2O and 2 ml 1% casein solution at temperatures 3, from 6 to 9, 12, 25 and 49 °C and for a different incubation intervals (Method II). These intervals were selected based on preliminary series of experiments to obtain totally >10.0% of L-tyrosine equivalents released from casein under optimum or close to optimum conditions. In the case of incubation in range from 6 to 9 °C temperature fluctuation was noted during the first 8 days incubation at 6 °C and last 2 days was temperature increased to the higher limit of this range. Control samples were in this case prepared by the same way as described above. After stopping the reaction by 1 ml of 17.5% TCA and subsequent centrifugation, exactly the same procedure as mentioned above was used to determine amount of aromatic amino acids released by proteolysis. In this case, the calibration line of dependence of absorbance on L-tyrosine concentration was prepared from stock solution of L-tyrosine in DEMI H_2O and a solution of DEMI H_2O/17.5% TCA (ratio 3:1).

Casein-protease activity of every soil sample and under every case of temperature and pH conditions adjusted in our study was measured in three repetitions with one control. Activity of casein-protease was expressed as follows:

a) in absolute values, i.e., μg L-tyrosine equivalents $\cdot g^{-1}$ dry soil $\cdot$ hours of incubation^{-1} or in μg L-tyrosine equivalents $\cdot g^{-1}$ dry soil $\cdot h^{-1}$;
b) relatively as an exploitation potential of soil to cleave casein by proteases (E_P) which is expressed as a ratio (in %) between casein-

Table 1. Selected physical and chemical properties of three studied soils

Soil	Texture	Clay (%)	Silt (%)	Sand (%)	Total *C* (%)	Total *N* (%)	*C*/*N*	pH H_2O	pH $CaCl_2$	Moisture (%)
NS	N	N	N	N	27.40	1.36	20.15	7.05	7.04	45.9
EBH	clay loam	27.30	40.80	31.90	4.36	0.26	16.77	6.86	6.71	25.3
G 0–10	loam	20.00	33.80	46.20	5.56	0.32	17.38	7.34	7.22	26.5

NS O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden; *N* not assessed. Texture was classified according to USDA-NRCS (1999) and soil units according to ISSS-ISRIC-FAO (1998)

protease activity assessed by incubation of 1 g wet soil with 2 ml DEMI H_2O and 2 ml 1% casein (P_M) at different temperatures, and 1 g wet soil with 2 ml Tris–HCl buffer (pH 8.52) and 2 ml 1% casein at 49 °C (P) [Ep = (P_M/P) *100];

c) relatively when casein-protease activity assessed in 1 g wet soil with 2 ml DEMI H_2O and 2 ml 1% casein at temperatures <49 °C and expressed in μg L-tyrosine equivalents · g^{-1} dry soil · h^{-1} was related to the casein-protease activity measured by the same procedure at 49 °C.

The dependence of casein-protease activity measured in soil after substitution of 0.05 M Tris–HCl buffer by DEMI H_2O on temperature were expressed by exponential equation. In addition, the temperature responses of casein-protease activity was expressed as a Q_{10} function which indicates the change in the casein-protease activity for 10 °C rise in temperature. As reported in the work of Lipson et al. (2001) the Q_{10} was calculated from the exponential coefficient (b) using the formula $Q_{10} = \exp(10b)$, where b was obtained from linear equation $Y = a + bx$ describing dependence of hyperbolic logarithm of casein-protease activity on temperature.

Soil pH (soil:water = 1:2.5, w/v) as well as pH of reaction mixture of 1 g wet soil with 2 ml 0.05 M Tris–HCl buffer (pH 8.52) and 2 ml 1% casein or 1 g wet soil with 2 ml DEMI H_2O and 2 ml 1% casein were measured by combined glass electrode.

Statistical analysis

Statistical comparisons of the differences between casein-protease activity assessed by measurements adjusted differently by buffer and incubation temperature was performed by One-Way ANOVA and multiple comparisons by Fisher LSD test (Statistica 7.0) for every soil sample separately.

Results

The results obtained in this work demonstrate that casein-protease activity measurements can be performed by the modified Ladd and Buttler's method in the temperature range from 3 to 49 °C and with substitution of Tris–HCl buffer by DEMI H_2O. The pH of the soil mixture with Tris–HCl buffer of pH 8.52 and 1% casein solution was 0.41 pH units lower in case of NS sample (Rendzic Leptosol, O horizon, the 40-year-old Norway spruce pure stands) when related to pH of only Tris–HCl buffer (Table 2).

Mineral soils (EBH sample, A horizon, the 90-year-old mixed European beech and hornbeam stand and G 0–10 sample, Hortic Anthrosol, A horizon, the cultivated vegetable garden) mixed with Tris–HCl buffer of pH 8.52 and 1% casein solution gave a pH value 0.10 units lower when related to Tris–HCl buffer. The substitution of Tris–HCl buffer by DEMI H_2O gave in the case of G 0–10 sample conditions almost equal to real soil pH measured in H_2O. Related to soil pH measured only in mixture of soil and H_2O in ratio 1:2.5 w/v, the application of 1% casein solution to NS sample increased pH by 0.30 and EBH sample by 0.23 (Tables 1 and 2). Furthermore, the substitution of Tris–HCl buffer by DEMI H_2O decreased pH of reaction mixture of 1 g mineral soil (EBH and G 0–10 samples) with 2 ml 1% casein by maximally 1.33. In terms of the enzymes activity at temperature of 49 °C, decrease in pH-level caused a reduction in the casein-protease activity to 67.25% (Table 3). For NS sample, the substitution of Tris–HCl buffer by DEMI H_2O led to a decrease in pH of the reaction mixture by 0.76; such decrease at temperature of 49 °C caused the decrease in the casein-protease activity to 53.76% (Table 3). The potential of studied soils to cleave casein (E_P) was at 25 °C exploited into range from 10.32 to 16.34%. Further decrease of incubation temperature decreased Ep to values from 0.76 to 2.15% (12 °C), and to values from 0.09 to 0.31% (at 6 to 9 °C) or to values from 0.07 to 0.19% at 3 °C (Table 3). The absolute values of casein-protease activity assessed under different temperature and pH conditions are listed in Table 4.

The incubation times are reported in Table 5 and differ according to selected temperature. The incubation of soil

Table 2. Real pH of mixture of 1 g wet soil with 2 ml Tris–HCl buffer (pH 8.52) and 2 ml 1% casein (pH 6.7) (I) or modified procedure when 1 g wet soil was incubated with 2 ml DEMI H_2O and 2 ml 1% casein (pH 6.7) (II)

Soil	I	II
NS	8.11	7.35
EBH	8.42	7.09
G 0–10	8.42	7.36

NS O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden

Table 3. Potential of three studied soils to cleave casein by proteases (E_P) at different temperatures (mean ± 1SE; $n = 2–3$)

Soil	49 °C	25 °C	12 °C	6–9 °C	3 °C
NS	53.76 ± 3.87	16.34 ± 1.63	0.76 ± 0.31	0.09 ± 0.01	0.07 ± 0.01
EBH	67.25 ± 5.66	11.87 ± 2.51	2.15 ± 0.83	0.31 ± 0.07	0.19 ± 0.09
G 0–10	67.37 ± 12.63	10.32 ± 1.80	1.40 ± 0.22	0.13 ± 0.03	0.09 ± 0.01

The potential is expressed as a ratio (in %) between casein-protease activity assessed by Method II at different temperatures (P_M), and by Method I assessed at 49 °C (P) [Ep = (P_M/P) · 100 (%)]. *NS* O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden

Table 4. Casein-protease activity in μg L-tyrosine equivalents · g^{-1} dry soil · h^{-1} assessed by Method I (*MI*) at 49 °C, and by Method II (*MII*) at temperatures from 3 to 49 °C (mean value; $n = 2–3$)

Soil	49 °C MI	49 °C MII	25 °C MII	12 °C MII	6–9 °C MII	3 °C MII
NS	449.52^{D}	241.57^{C}	73.40^{B}	3.40^{A}	0.42^{A}	0.33^{A}
EBH	104.87^{C}	68.20^{B}	11.91^{A}	2.04^{A}	0.30^{A}	0.18^{A}
G 0–10	166.82^{C}	108.98^{B}	16.75^{A}	2.29^{A}	0.22^{A}	0.15^{A}

NS O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH sample* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden. Different letters indicate significant ($P < 0.05$) differences

Table 5. The relative amounts of L-tyrosine equivalents (in%) released by casein-protease activity per time of incubation (mean ± 1SE; $n = 2–3$)

Soil	MI	49 °C MII	25 °C MII	12 °C MII	6–9 °C MII	3 °C MII
NS	100	107.52 ± 7.75	65.35 ± 6.51	27.25 ± 10.99	11.30 ± 0.89	12.30 ± 1.22
EBH	100	134.50 ± 11.31	47.47 ± 10.05	77.46 ± 29.98	36.93 ± 8.95	31.66 ± 15.10
G 0–10	100	134.74 ± 25.26	41.29 ± 7.18	50.55 ± 7.74	16.19 ± 3.87	15.49 ± 2.28
Incubation period (hours)	2	4	8	72	240	336

NS O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden. Casein-protease activity in μg L-tyrosine equivalents · g^{-1} dry soil · hours of incubation^{-1} measured by Method I (*MI*) at 49 °C equals 100%, the other measurements were performed by Method II (*MII*)

with DEMI H_2O and casein in range from 3 to 49 °C required a period from 4 to 336 h to get results which, if they are not-recalculated to μg L-tyrosine equivalents released per 1 g dry soil after 1 h and are expressed in μg L-tyrosine equivalents · g^{-1} dry soil · hours of incubation^{-1}, relate to an amount >10.0% and <135.0% of μg L-tyrosine equivalents · g^{-1} dry soil released by casein-protease activity measured using Method I (Table 5).

Statistical analysis showed significant ($P < 0.05$) effect of temperature on casein-protease activity, when temperatures from 3 to 49 °C were taken into account. Nonsignificant ($P > 0.05$) effect of temperature was proved when mineral EBH or G 0–10 samples were incubated at temperatures from 3 to 25 °C. The casein-protease activity of organic NS sample was nonsignificantly ($P > 0.05$) different only when soil samples were incubated at temperatures from 3 to 12 °C ($0.775 < P < 0.993$). The incubation at 3 and from 6 to 9 °C gave very similar casein-protease activity in all soil samples (Table 4).

If the effect of temperature on casein-protease activity was expressed in per cents such a way that 100% equaled μg L-tyrosine equivalents · g^{-1} dry soil · h^{-1} released from casein by soil proteases when Method II was used at 49 °C, then the decrease of temperature to 25 °C decreased casein-protease activity in the soils to minimally 15.4 and maximally 30.3%. The decrease of temperature to 3 °C was related to relative decrease of casein-protease activity to values from 0.14 to 0.26% (Fig. 1).

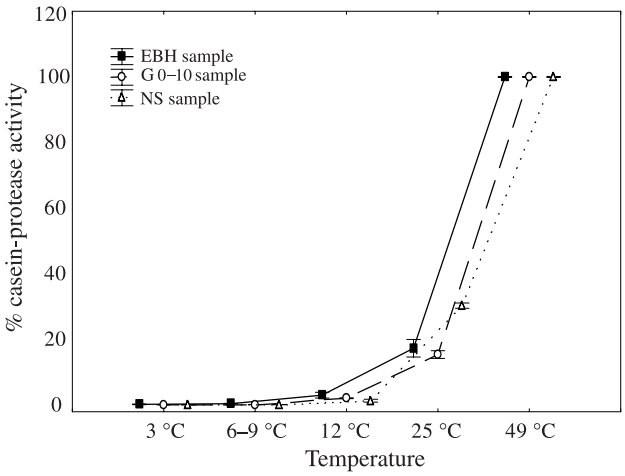

Fig. 1. The dependence of casein-protease activity measured by Method II on temperature expressed in per cents, when 100% equals casein-protease activity measured by this procedure at 49 °C (mean ± 1SE; $n = 2–3$)

The dependence of soil casein-protease activity assessed by Method II on temperature from 3 to 25 °C or from 3 to 49 °C was expressed by exponential equation (Table 6). The values Q_{10} were in case of all three samples lower if casein-protease activity from 3 to 49 °C was taken into account. When casein-protease activity from 3 to 25 °C was only taken into account, the Q_{10} of EBH soil increased from 3.46 to 6.78, respectively and from 3.83 to 8.14 for G 0–10 soil. The Q_{10} of sample from O horizon was the highest of all soils, with the value 4.25

Table 6. The exponential equations describing dependence of casein-protease activity assessed by Method II on temperature in three studied soils, and Q_{10} values

Soil	Exponential equation (3–49 °C)	Exponential equation (3–25 °C)	Q_{10} (3–25 °C)	Q_{10} (3–49 °C)
NS	$y = 0.5379e^{0.1447x}$ $R^2 = 0.8586$	$y = 0.1868e^{0.2405x}$ $R^2 = 0.9861$	11.08	4.25
EBH	$y = 0.2468e^{0.1241x}$ $R^2 = 0.894$	$y = 0.117e^{0.1914x}$ $R^2 = 0.9173$	6.78	3.46
G 0–10	$y = 0.2493e^{0.1344x}$ $R^2 = 0.906$	$y = 0.1085e^{0.2097x}$ $R^2 = 0.9566$	8.14	3.83

NS O horizon, Rendzic Leptosols, the 40-year-old Norway spruce pure stands; *EBH* A horizon, Haplic Luvisol, the 90-year-old mixed European beech and hornbeam stand; *G 0–10* A horizon, Hortic Anthrosol, the cultivated vegetable garden. The exponential equations and Q_{10} values were calculated from casein-protease activity measured at 3, 12 and 25 °C or at 3, 12, 25 and 49 °C

when temperatures from 3 to 49 °C were taken into account, or 11.08 at the temperature range from 3 to 25 °C (Table 6).

Discussion

The modified method of Ladd and Buttler (1972) presented in this study can be used for measurement of proteases activity in soil samples with DEMI H_2O and 1% casein even at low temperatures. The use of this method can be combined with measurements of casein-protease activity under optimum or close to optimum temperature and pH conditions. The combination both methods offers obtaining data related to percent exploitation potential to cleave casein by proteases, for example in course of vegetation season in soil of different ecosystems. It should be noted that casein-protease activity assessed in the laboratory conditions, where incubation temperature is set for soil temperatures obtained from the field and pH of reaction mixture closed to soil pH measured in H_2O, is still potential. Native soil proteases activity is measured without protein amendment, is by substrate limited achieving significantly lower values (Lipson et al., 1999; Raab et al., 1999). As mentioned by Roberge (1978) proteases activity in water extracts decreased when compared with that in Tris–sodium borate buffer. Instead of DEMI H_2O, Raab et al. (1999) used Na-acetate buffer of pH 5.2 or potassium phosphate buffer of pH 7.2 to match the pH of soils. Presented modification of Ladd and Buttler (1972) method was connected with longer incubation of soil samples upto 336 h at 3 °C. Raab et al. (1999) incubated soil samples with Na-acetate buffer or potasium phosphate buffer and casein at 3 °C for a period from 12 to 18 h. Their incubations were contrary to our study performed with addition of toluene to inhibit microbial proliferation and utilization of protein hydrolytic products, and ninhydrin analysis was used to quantify soluble α-amino N released from casein.

We have not used toluene or any other compounds to inhibit microbial proliferation. It is in agreement with method of Ladd and Buttler (1972) and its modifications presented in many works (Nannipieri et al., 1979; Gil-Soltres et al., 1992; Dilly and Munch, 1996; Vedder et al., 1996; Trasar-Cepeda et al., 1998; Dilly, 1999; Kandeler et al., 1999; Nunnan et al., 2000; Li et al., 2002; Dinesh et al., 2004; Formanek et al., 2006 etc.). These authors incubated soil samples with casein for a short time. Nevertheless, the authors of the paper are aware that the measurement of casein-protease activity at lower temperatures (from 3 to 25 °C) may lead to some difficulties: the incubation intervals at these temperatures ranged from 8 to 336 h and microorganisms were enabled to growth in presence of high concentration of casein what could result to secretion of new proteases and decomposition of products of proteolysis. On the other hand, toluene which has been used often to measure casein-protease activity at shorter or longer incubation intervals upto 16–18 h can serve as a carbon source for fungi and bacteria (Ladd, 1978; Shen et al., 2006). Toluene may have a plasmolytic action and cause the release of intracellular enzymes which can overestimate casein-protease activity.

The effect of toluene depends on soil type, assay conditions and enzyme which is assayed (Ladd, 1978; Rejsek, 1994). According to Skujins (1978) or Nannipieri et al. (1990), in case of the soil samples with a high content of humic substances, an increased extraction of humic substances was regularly recorded after adding toluene to the soil suspension. Informations related to soil proteases and toluene showed that toluene-treated soils hydrolysed gelatine to similar extent as in case of not treated soils. As mentioned in work of Roberge (1978) toluene decreased proteases activity and inhibited soil deaminase. According

to this author an application of toluene had the same impact on proteases activity as sterilization of soil by radiation. When soil was sterilized by radiation, an addition of toluene did not change proteases activity.

The prolongation of period of measurement with decreasing temperatures was accompanied by smell of the casein probably from its autolysis. Extent of casein autolysis should not affect the values measured as Formanek et al. (2005a, b) indicated.

The Q_{10} of proteolysis assessed by modification of method of Ladd and Buttler (1972) (Nannipieri et al., 1979) when Tris–HCl buffer was substituted by DEMI H_2O was unexpectedly higher than the Q_{10} values reported for soil enzymes which are normally <2 (Tabatabai, 1994). Lipson et al. (2001) measured casein-protease activity in samples from A horizon of a skeleton-loamy pergelic Cryumbrept under dry meadow, at temperatures from 2 to 22 °C. The Q_{10} of proteolysis reported by the authors was 1.98. Acosta-Martínez and Tabatabai (2000) found in case of soil amino acid arylamidase the Q_{10} to be from 1.32 to 1.71. The Q_{10} of soil L-glutaminase ranged from 1.19 to 1.85 (Frankerberger and Tabatabai, 1991) and soil urease at temperatures from 5 to 45 °C was approx. 2 (Moyo et al., 1989). Soil L-phenylalanine deaminating activity had the Q_{10} maximally 2, soil rhodanase Q_{10} at temperatures from 10 to 60 °C ranged from 1.25 to 1.45 (Tabatabai and Singh, 1979). The evident difference between the Q_{10} values obtained by the proposed methodology and the Q_{10} values obtained by methods using buffers, with or without toluen and shorter incubations seem to be crucially influenced by the time of incubation: the length of incubation proposed was derived from the reason to gain measurable concentrations of L-tyrosine equivalents determined colorimetrically at 578 nm.

Conclusions

Modification of method of Ladd and Buttler (1972) offers possibility to measure proteases' activity in soil of different ecosystems under conditions of pH close to real soil pH, and under temperatures measured in the field when high concentration of substrate (casein) is present. The use of this method in combination with measurement of proteases' activity under optimum or close to optimum conditions offers posibility to find out the effect of temperature and pH of soil on proteolysis in different ecosystems or differently treated ecosystem, and, for example, in different periods of vegetation season.

Acknowledgements

The development of the modification was supported by the Ministry of Environment of the Czech Republic (the project Czech-Carbo No. VaV/640/18/03), and by Grant VZ MSMT 6215648902 Forest and Wood: the support of functionally integrated forest management and use of wood as a renewable raw material, Part 4/2/2 – "The soil as a component of site parameters and forest management strategy in nature conservation areas".

The authors would like to acknowledge the efforts of Dr Avtar Matharu, the University of York (U.K.), for constructive comments on the manuscript.

References

Acosta-Martínez V, Tabatabai MA (2000) Arylamidase activity of soils. Soil Biol Biochem 64: 215–221

Alef K, Nannipieri P (1995) Methods in applied soil microbiology and biochemistry. Harcourt Brace & Company, London

Ambrož Z (1969) Temperature as an ecological factor regulating production of exoproteases and their occurrence in soils (in Czech). Rost Výr 15: 443–448

Aslim B, Sağlam N, Beyatli Y (2006) Determination of some properties of *Bacillus* isolated from soil. Turk J Biol 26: 41–48

Cazorla FM, Romero D, Pérez-García A, Lugtenberg BJJ, De Vincente A, Bloemberg G (2007) Isolation and characterization of antagonistic *Bacillus* subtilis strains from the avocado rhizoplane displaying biocontrol activity. J Appl Microbiol (in press)

Dilly O (1999) Nitrogen and phosphorus requirement of the microbiota in soils of the Bornhöved lake district. Plant Soil 212: 175–183

Dilly O, Munch JC (1996) Microbial biomass content, basal respiration and enzyme activities during the course of decomposition of leaf litter in a black alder (*Alnus glutinosa* (L.) Geartn.) forest. Soil Biol Biochem 28: 1073–1081

Dinesh R, Ghoshal Chaudhuri S, Sheeja TE (2004) Soil biochemical and microbial indices in wet tropical forests: effect of deforestation and cultivation. J Plant Nutr Soil Sci 167: 24–32

Formanek P, Klejdus B, Vranova V (2005a) Bio-available amino acids extraction from soil by demineralized water and 0.5 M ammonium acetate. Amino Acids 28: 427–429

Formanek P, Vranova V, Rejsek K, Klejdus B (2005b) Bio-available amino acids in soil under differently managed meadow ecosystems. 9th International Congress on amino acids and proteins – abstracts. Amino Acids 29: 31

Formanek P, Rejsek K, Janous D, Vranova V, Houska J (2006) Casein-protease, urease and acid phosphomonoesterase activities in moderately mown and abandoned mountain meadow soils. The Beskids Bulletin 19: 53–58

Frankenberger WT, Tabatabai MA (1991) L-glutaminase activity of soils. Soil Biol Biochem 23: 869–874

Gil-Soltres F, Trasar-Cepeda MC, Ciardi C, Ceccani B, Leirós MC (1992) Biochemical characterization of biological activity in very young mine soils. Biol Fertil Soil 13: 25–30

ISSS-ISRIC-FAO (1998) World reference basis for soil resources. World Soil Resources Reports, Rome, FAO

Kandeler E, Luxhøi J, Tscherko D, Magíd J (1999) Xylanase, invertase and protease at the soil-litter interface of a loamy sand. Soil Biol Biochem 31: 1171–1179

Ladd JN (1978) Origin and range of enzymes in soil. In: Burns RG (ed) Soil enzymes. Academic Press Inc., London, pp 51–96

Ladd JN, Buttler HA (1972) Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivates as substrates. Soil Biol Biochem 4: 19–30

Li HC, Ma BL, Zhang TQ (2002) Soil bulk density effects on soil microbial populations and enzyme activities during the growth of maize (*Zea mays* L.) planted in large pots under field exposure. Can J Soil Sci 82: 147–154

Lipson D, Raab TK, Schmidt SK, Monson RK (2001) An empirical model of amino acid transformations in an alpine soil. Soil Biol Biochem 33: 189–198

Lipson D, Schmidt SK, Monson RK (1999) Links between microbial population dynamics and plant N availability in an alpine ecosystem. Ecology 80: 1623–1631

Moyo CC, Kissel DE, Cabrera ML (1989) Temperature effects on soil urease activity. Soil Biol Biochem 21: 935–938

Nannipieri P, Pedrazzini F, Arcara PG, Piovanelli C (1979) Changes in amino acids, enzyme activities, and biomasses during soil microbial growth. Soil Sci 127: 26–34

Nannipieri P, Ceccanti B, Grego S (1990) Ecological significance of the biological activity in soil. In: Bollag JM, Stotzky G (eds) Soil biochemistry. Marcel Dekker, New York, pp 293–356

Nunnan N, Morgan MA, Scott J, Herlihy M (2000) Temporal changes in nitrogen mineralization, microbial biomass, respiration and protease activity in a clay loam soil under ambient temperature. Biology and Environment: Proceedings of the Royal Irish Academy, vol. 100B, pp 107–114

Raab TK, Lipson DA, Monson RK (1999) Soil amino acid utilization among species of the cyperaceae: plant and soil processes. Ecology 80: 2408–2419

Rejsek K (1991) Acid phosphomonoesterase activity of ectomycorrhizal roots in Norway spruce pure stands exposed to pollution. Soil Biol Biochem 23: 667–671

Rejsek K (1994) Experimental approaches to the determination of acid phosphomonoesterase activity in soil. Folia Microbiol 39: 245–248

Roberge MR (1978) Methodology of soil enzyme measurement and extraction. In: Burns RG (ed) Soil enzymes. Academic Press Inc., London, pp 341–370

Shen RF, Cai H, Gong WH (2006) Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. Plant Soil 285: 149–159

Skujins J (1978) History of abiontic soil enzyme research. In: Burns RG (ed) (1978) Soil enzymes. Academic Press Inc., London, pp 1–50

Tabatabai MA (1994) Soil enzymes. In: Weaver RW et al. (eds) Methods of soil analysis: microbiological and biochemical properties, part 2. Madison, WI, SSSA Book Ser, vol. 5, pp 775–833

Tabatabai MA, Singh BB (1979) Kinetic parameters of the rhodanese reaction in soils. Soil Biol Biochem 11: 9–12

Tjalsma H, Antelmann H, Jongbloed JDH, Braun PG, Darmon E, Dorenbos R, Dubois JYF, Westers H, Zanen G, Quax WJ, Kuipers OP, Bron S, Hecker M, Van Dijl JM (2004) Proteomics of protein secretion by *Bacillus* subtilis: separating the ''secrets'' of the secretome. Microbiol Mol Biol R 68: 207–233

Trasar-Cepeda C, Leirós C, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26: 100–106

USDA-NRCS (1999) Soil taxonomy. Agricultural Handbook No. 436, 2nd ed. USDA, Washington

Vágnerová K, Macura J (1974) The effect of microorganisms and sorption on the protease activity of plant roots. Folia Microbiol 19: 329–335

Vedder B, Kampichler C, Bachmann G, Bruckner A, Kandeler E (1996) Impact of faunal complexity on microbial and N turnover in field mesocosms from a spruce forest soil. Biol Fertil Soils 22: 22–30

Authors' address: Klement Rejsek, Department of Geology and Soil Science, Mendel University of Agriculture and Forestry, Zemedelska 3, 613 00 Brno, Czech Republic,
Fax: +420-5-45211422, E-mail: kr@mendelu.cz