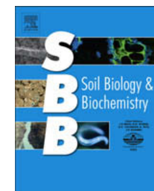
Soil Biology & Biochemistry 41 (2009) 1199–1209

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio



Characterization of humus–protease complexes extracted from soil

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ARTICLE INFO

Article history:
Received 28 November 2008
Received in revised form
24 February 2009
Accepted 26 February 2009
Available online 25 March 2009

Keywords:
Protease activity
Organic matter characterization
Humus–protease complexes
Humus fractionation
Pyrolysis-gas chromatography
Isoelectric focusing

ABSTRACT

Pyrophosphate (140 mM, pH 7.1) extracts of two arable soils and one pasture soil were ultrafiltrated separating the extracted material into three fractions: A_I with nominal molecular weight (nmw) > 100 kD, A_{II} with nmw between 10 kD and 100 kD and R with nmw < 10 kD. Protease activity was determined in the fractions by using three different substrates: N-benzoyl-L-argininamide (BAA), specific for trypsin; N-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL), specific for carboxypeptidases; and casein, essentially a non-specific substrate. The derivative fractions were also analysed for their amino acid N and humic (HA) and fulvic (FA) acid contents. The organic matter of extracts and derivative fractions obtained from the pasture soil was analysed by isoelectric focusing (IEF) and that of fractions analysed by pyrolysis gas chromatography (Py-GC). Activities of the extract were monitored for their thermal stability and those of the extract and derivative fractions for their optimal pH.
Due to the mechanical disintegrating action of sodium pyrophosphate over the humic substances during the fractionation process the amount of total organic C and FA in the fractions was ranked as $R > A_{II} > A_I$. The lowest amino acid N/organic C was found in the R fraction, whereas A_{II} fraction was rich in humic acids, carbohydrates and amino acid N and A_I fraction showed the lowest carbohydrate content. At least 70% of the total BAA- and ZPL-hydrolysing activity was associated to particles with nmw higher than 10 kD and at least 30% of these activities were present in particles with nmw higher 100 kD. Casein-hydrolysing activity was quite evenly distributed among the three fractions (A_I, A_{II} and R). The extracted protease–organic complexes were resistant to thermal denaturation and some of them showed optimal activity at pH values higher than 10 as a result of the polyanionic characteristics of the humic material surrounding enzyme molecules and of the presence of alkaline protease. Comparison of data obtained in Py-GC analyses and in protease activity suggests that BAA-hydrolysing activity was associated to a highly condensed humic matter and ZPL-hydrolysing activity to less resistant humic substances, while at least some of the extracted casein-hydrolysing activity was present as glyco-proteins not associated to humus. BAA-hydrolysing activity was probably inhibited by fresh organic matter of carbohydrate origin whereas lignin derived organic matter probably inhibited ZPL- and casein-hydrolysing activity.
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1. Introduction

All analytical procedures employed to determine the chemical structure of soil show that protein N is the predominant form (Stevenson, 1986; Leinweber et al., 2007). Model studies have shown that enzymes can be adsorbed by pure/dirty (covered by other soil components such as iron oxides) clays, maintaining their activity and being protected against proteolysis (Stotzky, 1986; Nannipieri et al., 1996; Nielsen et al., 2006). In the case of humic molecules, model studies have shown that a better protection of the enzyme against proteolysis is observed when the enzyme is entrapped through oxidative coupling in synthetic humic-like material rather than when it is adsorbed on the preformed polymer (Nannipieri et al., 1996). However, further insights on the state and stability of naturally-occurring enzyme complexes in soil have been obtained after extraction of these complexes from soil and their partial purification (Tabatabai and Fu, 1992; Nannipieri et al., 1996). It has been proposed that enzymes in humus–hydrolases or clay–humus–hydrolases complexes extracted from soil are protected by a network of humic molecules with pores

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0038-0717/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2009.02.032

large enough to permit the passage of substrates and reaction products but not that of large molecules such as proteases (Burns et al., 1972). The resistance of high molecular weight but not low molecular weight humus–urease and humus–phosphatase complexes to pronase and thermal denaturation has been considered as an evidence of the theory proposed by Burns et al. (1972) (Nannipieri et al., 1978, 1988). However, it has been argued that this hypothesis may be only valid in the case of hydrolases active against low molecular weight substrates such as ureases and phosphatases but not for hydrolases active against high molecular weight substrates such as proteases. Indeed if these enzymes are active against, and thus accessible to these substrates, they would be also degraded by proteolytic enzymes (Ladd and Butler, 1975). Present enzyme assays allow evaluating the activity of proteases versus either low or high molecular weight substrates. Among the former there are N-benzoyl-L-argininamide (BAA), specific for trypsin, and N-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL), specific for carboxypeptidases, and among the latter there is casein, which is essentially a non-specific substrate (Ladd and Butler, 1972).

Different solutions have been used to extract naturally-occurring enzyme–organic complexes from soil. Proteases have been extracted from soil by 140 mM sodium pyrophosphate at pH 7.1 (Nannipieri et al., 1980; Ceccanti et al., 1989; Bonmatí et al., 1998), 0.1 M Tris–borate at pH 8.1 (Ladd, 1972), 0.2 M phosphate-0.2 M EDTA buffer at pH 8.0 (Mayaudon et al., 1975; Batistic et al., 1980) and 0.1 M phosphate at pH 7.0 (Hayano et al., 1987; Watanabe and Hayano, 1996; Kamimura and Hayano, 2000). However, pyrophosphate at pH values near to neutrality (7.1–7.5) is more efficient in extracting hydrolases from soil than the above buffer solutions (Nannipieri et al., 1996).

Sodium pyrophosphate extracts of two arable soils and one pasture soil were active against BAA, ZPL and casein (Bonmatí et al., 1998). Yields of both ZPL- and casein-hydrolysing proteases were higher than those of BAA hydrolysing proteases. It was suggested that BAA hydrolysing proteases were mainly associated with condensed humus, ZPL-hydrolysing proteases with less condensed humus and casein-hydrolysing proteases with fresh organic matter. However, protease activities of the derivative fractions obtained after ultrafiltration of pyrophosphate soil extracts have not been determined and their properties are unknown. The aim of the research was to better characterise the BAA-, ZPL- and casein-hydrolysing protease–humic complexes extracted from two arable soil and a pasture soil (Bonmatí et al., 1998) through a better understanding of i) the type and size of organic matrix surrounding the enzymes, and ii) the activity, characteristics and stability of enzymes molecules immobilised by the organic part of the complexes. This information could be important to verify the hypothesis by Ladd and Butler (1975) mentioned above. Therefore we have ultrafiltrated pyrophosphate extracts of two arable soils and a pasture soil (Bonmatí et al., 1998), so as to determine the distribution of three types of proteases among A_I (with nominal molecular weight > 100 kD), A_{II} (with nominal molecular weight between 10 kD and 100 kD) and R (with nominal molecular weight < 10 kD) fractions. This fractionation procedure (Ceccanti et al., 1978) has been successfully used to fractionate phosphatase, urease and protease activity–organo complexes (Ceccanti et al., 1978, 1989; Nannipieri et al., 1988). BAA-, ZPL- and casein-hydrolyzing activity, together with amino acid N and humic and fulvic acid contents, were determined in the derivative fractions. In addition the extract and derivative fractions obtained from pasture soil, that is the soil with the highest yields of the three proteases and organic matter, were characterized by isoelectric focusing (IEF) and the fractions were analysed by pyrolysis gas chromatography (Py-GC). Activities of the extract were monitored for their thermal stability and those of the extract and derivative fractions for their optimal pH to better characterise the properties of immobilized proteases. Finally, comparisons of data obtained by Py-GC analyses and by protease activities were performed through correlation analysis.

2. Material and methods

2.1. Soils, soil extraction, ultrafiltration, and preparation of humic and fulvic acids

The properties, sampling and type of storage of surface (0–20 cm) samples of a Calcaric Fluvisol cropped to maize-rye grass rotation (soil 5; C 2.32%, N 0.27%, pH 7.5) or cropped to tomato (soil 13; C 1.31%; N 0.14%, pH 7.4) and a Dystric Cambisol under a permanent meadow (soil 19; C 4.76%, N 0.56%, pH 5.5) have been already reported (Bonmatí et al., 1998). The same for soil extraction by 140 mM sodium pyrophosphate at pH 7.1 (soil-solution ratio 1:10) and at 37 °C for 24 h in a shaking water bath; N_2 was passed into the Erlenmeyer flasks containing the mixtures before initiating the shaking process, the flasks were hermetically closed immediately afterwards and the mixtures were centrifuged (15,000 g, 30 min) and filtrated (0.22 μm) once the extraction process was finished. Ultrafiltration of soil extracts was carried out at 4 °C against 140 mM sodium pyrophosphate at 50–100 kPa with final concentration to the initial volume as reported by Ceccanti et al. (1978): an Amicon 202 cell with a XM 100A diaflo-membrane, which retained the fraction of nominal molecular weight (nmw) > 100 kD (A_I), was employed; liquid passing through the membrane was further ultrafiltrated using the same cell with a PM 10 diaflo-membrane, which separated the fraction of nmw between 10 kD and 100 kD (A_{II}) from that of nmw < 10 kD (R). Humic acids and fulvic acids were prepared from the three fractions as shown by Bonmatí et al. (1998); 0.5 ml of 2.5 M H_2SO_4 were added to 5 ml of the extract containing the fraction; the mixture was left at 4 °C for 24 h and then centrifuged (4000 g, 10 min); the precipitate was washed with acidulated water and centrifuged.

2.2. Determination of amino acid N, total organic C and Humic acids C of the three fractions (A_I, A_{II} and R) obtained after ultrafiltration of extracts of three soils

Amino acid N content of the derivative fractions of soil extracts was determined as described by Stevenson (1982a). The dialysed samples were previously concentrated by ultrafiltration (cut off at 10 kD nmw) up to a total N content of about 0.1 g l^{-1} as described by Ceccanti et al. (1978). Measurements were replicated three times with each replicate involving the use of a different soil sample.

The dialysed samples were concentrated by ultrafiltration (cut off at 10 kD nmw) to increase the organic C content (about 5 times) and then concentration of total C and humic acids C were determined as described by Bonmatí et al. (1998): an excess of dichromate and 5 M H_2SO_4 was added to the samples an then organic C was oxidised by heating at 150 °C for 15 min; the mixtures were left for 24 h at room temperature and the formed Cr^{3+} determined by measuring the absorbance at 590 nm. Measurements were replicated three times with each replicate involving the use of a different soil sample.

2.3. Protease assays in the three fractions obtained after ultrafiltration of extracts of three soils

Protease activity of the three derivative fractions obtained from the three soil extracts was determined by using three different

substrates (BAA, ZPL and casein) as described by Bonmatí et al. (1998). Prior to the determination of protease activities dialysed samples were concentrated by ultrafiltration (cut off at 10 kD nmw), so as to reach an organic C content of 1 g l^{-1}. Four replicates based on the use of a different soil extract were carried out for BAA- and ZPL-and casein-hydrolysing activities. BAA-hydrolysing activity was expressed as μmol N–NH_4^+ g^{-1} dry soil h^{-1}, ZPL-hydrolysing activity as μmol leucine g^{-1} dry soil h^{-1} and casein-hydrolysing activity as μmol tyrosine g^{-1} dry soil h^{-1}. Ratios between the values of each activity and their relative organic C or amino acid N content were also calculated.

2.4. Isoelectrofocusing of extract of pasture soil and derivative fractions

The pyrophosphate extract of pasture soil (soil 19) and the three derivative fractions were subjected to isoelectric focusing (IEF) analysis because this soil showed the highest yield of organic C (Bonmatí et al., 1998). The analysis was carried out on polyacrylamide gel tubes as described by Ceccanti et al. (1980). Dialysed samples were concentrated by ultrafiltration (cut off at 10 kD nmw) up to an organic C content of 1.5 mg ml^{-1}. Gels with a stable pH gradient between 3.5 and 10 were prepared. The focused organic matter was densitometrically determined at 460 nm with a Nasatron 821 (Milan, Italy) densitometer whereas measurements of gel pH were done by an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, California).

2.5. Organic matter characterization by pyrolysis-gas chromatography–mass spectrometry (Py-GC–MS) of the three fractions obtained after ultrafiltration of the extract of pasture soil

The three derivative fractions of the extract of the pasture soil were subjected to pyrolysis-gas chromatography (Py-GC) analysis. Composite samples of each fraction were prepared by pooling together samples obtained from at least three extracts of the pasture soil. Py-GC–MS was performed as described by Alcañiz et al. (1987) with some modifications (Bonmatí et al., 1998). Previously dialysed composite samples were concentrated by ultrafiltration (cut off at 10 kD nmw) up to an organic C content of 6 g l^{-1} and then dried with a rotatory procedure. Two replicates were analysed for each sample.

We also considered pyrograms of the three soils, the three soils extracts and the three extraction residues (Bonmatí et al., 1998) in the mathematical analyses of pyrograms of A_I, A_{II} and R fractions obtained from of the extract of soil 19.

Pyrograms were compared in the following way as described by Bonmatí et al. (1998):

- Eighteen peaks were selected according to their frequency, their relative area and type of information that they can give for the composition of organic matter.
- Each peak area of the pyrograms was normalised as the percentage of the sum of areas of all peaks; then we calculated the logarithm of each normalized area and this value was used for statistical analysis.
- Differences between pyrograms were evaluated by a Multidimensional Scaling based on the Euclidean Distance.
- Twelve peaks most responsible for the differences between pyrograms were selected through Principal Components followed by Multivariant regression analysis.
- These 12 pyrolysis peaks were grouped in different categories according the organic fraction they represented.

2.6. Stability of protease activity of extract from the pasture soil to thermal denaturation

We studied the thermal stability of the three protease activities of the pyrophosphate extract of soil 19 because this soil showed higher yields of the three enzymes than the other two soils (Bonmatí et al., 1998). The dialysed soil extract (10 ml for each temperature) was concentrated by ultrafiltration (cut off at 10 kD nmw) up to an organic C content of about 1 g l^{-1} and was incubated for 2 h at 22, 30, 50, 65 or 80 °C; then aliquots were taken and assayed for BAA, ZPL and casein-hydrolysing activities as previously described. Measurements were replicated three times.

2.7. Effect of pH on protease activity of the extract of pasture soil and of the three derivative fractions (A_I, A_{II} and R)

BAA, ZPL and casein-hydrolysing activities of the extract of pasture soil and of the three derivative fractions were analysed as previously described using different buffers whose pH ranged from 4.5 to 10.5. Acetate (0.1 M pH 4–5.5), maleate (0.1 M pH 5.5–7), TRIS–HCl (0.1 M pH 7–9) and tetraborate (0.1 M pH 9–11) were used. Buffer was added to the soil extract or the derivative fraction and pH was determined prior the addition of the relative substrate. The casein-hydrolysing activity was not determined between pH 4.5 and 5.5 since casein was precipitated at these pH values. Measurements were replicated three times. Results of enzyme activity were expressed as percentage of the maximal value.

2.8. Comparative study between the organic matter composition and the protease activity

Correlation analysis was done between the percentage of each pyrogram peak, calculated as described above, or its logarithm value, and the ratio between BAA-, ZPL- or casein-hydrolysing activities and the organic C content (specific activities). This was done for the three soils, their relative residues and extracts (data obtained from Bonmatí et al., 1998) and the three derivative fractions of the extract from the pasture soil 19.

Table 1
Total organic C, humic acids (HA) and amino acid N contents and amino acid N/organic C ratio of derivative fractions of soil extracts.

Sample[a]	Organic C[b] (g kg^{-1} dry soil)	Amino acid N[b] (mg kg^{-1} dry soil)	Amino acid N/Organic C ratio (mg g^{-1})
5 A_I	0.54 ± 0.03	27 ± 0.8	50
5 A_I HA	0.15 ± 0.01		
5 A_{II}	0.86 ± 0.13	42 ± 0.8	49
5 A_{II} HA	0.40 ± 0.04		
R5	1.50 ± 0.13	37 ± 0.8	25
R5 HA	0.46 ± 0.13		
13 A_I	0.38 ± 0.04	9 ± 0.8	21
13 A_I HA	0.26 ± 0.08		
13 A_{II}	0.46 ± 0.01	15 ± 0.8	30
13 A_{II} HA	0.26 ± 0.01		
R13	0.96 ± 0.23	12 ± 0.8	11
R13 HA	0.23 ± 0.08		
19 A_I	2.50 ± 0.59	61 ± 4.2	24
19 A_I HA	0.97 ± 0.19		
19 A_{II}	3.74 ± 0.43	215 ± 11.9	57
19 A_{II} HA	1.41 ± 0.19		
R19	3.94 ± 0.56	69 ± 12.5	17
R19 HA	0.60 ± 0.19		

[a] A_I, A_{II} and R are fractions with nominal molecular weight >100 kD, between 10 and 100 kD and <10 kD, respectively. The number concerns the used soil (5 and 13 are a Calcaric Fluvisol cropped to maize-ryegrass rotation and tomato, respectively; 19 is a Dystric Cambisol under permanent meadow).
[b] Mean ± SD values ($n = 3$).

Table 2
Protease activities against N-benzoyl-L-argininamide (BAA), N-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL) or casein of derivative fractions of soil 5, 13 and 19. A_I with nominal molecular weight (nmw) > 100 kD; A_{II} with nmw between 10 kD and 100 kD; R with nmw < 10 kD.

Fraction	BAA-hydrolysing activities[a] (μmol $N–NH_4^+$ g^{-1} dry soil h^{-1})	ZPL-hydrolysing activities[a] (μmol leucine g^{-1} dry soil h^{-1})	Casein-hydrolysing activities[a] (μmol tyrosine g^{-1} dry soil h^{-1})
5 A_I	0.235 ± 0.03	0.135 ± 0.03	0.096 ± 0.03
5 A_{II}	0.092 ± 0.01	0.205 ± 0.05	0.165 ± 0.05
R 5	0.124 ± 0.02	0.121 ± 0.01	0.097 ± 0,04
5 Σ[b]	0.451	0.461	0.358
13 A_I	0.058 ± 0.01	0.943 ± 0.15	0.058 ± 0.01
13A_{II}	0.128 ± 0.02	0.030 ± 0.01	0.027 ± 0.01
R 13	0	0.047 ± 0.01	0.051 ± 0.01
13 Σ[b]	0.186	1.020	0.136
19 A_I	0.407 ± 0.08	0.980 ± 0.16	0.220 ± 0.08
19A_{II}	0.406 ± 0.08	1.000 ± 0.25	0.232 ± 0.09
R 19	0.236 ± 0.04	0.120 ± 0.03	0.318 ± 0.13
19 Σ[b]	1.049	2.100	0.770

[a] Mean ± SD values ($n = 4$).
[b] Addition of the activities of the three fractions.

3. Results

3.1. Chemical characterization and proteases activities of the derivative fractions of the three soil extracts

The amount of total organic C in the derivative fractions can be ranked as $R > A_{II} > A_I$ (Table 1), whereas from 50% to 80% of the extracted humic acids and amino acid N had a nominal molecular weight (nmw) higher than 10 kD (Table 1) and the highest percentage (50–70%) of extracted fulvic acids (data not shown) was found in the R fractions (nmw lower than 10 kD). The A_{II} fractions, with nmw ranging from 10 and 100 kDa, had the highest amino acid N content and generally the highest amino acid N/organic C ratio whereas this ratio was the lowest in the R fractions (Table 1).

All three-enzyme activities showed a linear relationship between the amount of formed product and reaction time. From 72 to 100% of the cumulative BAA- and ZPL-hydrolysing activity of the three fractions were present in the A_I and A_{II} fractions (Table 2); the highest percentage were observed for BAA-hydrolysing activities of A_I and A_{II} fractions from soil 13 (100%) and for ZPL-hydrolysing activities of A_I and A_{II} fractions from soil 13 and soil 19 (95%). Casein-hydrolysing activity was quite evenly distributed among the three fractions (from 27% to 41% of the cumulative activity had a nmw lower than 10 kD).

Both BAA- and ZPL-hydrolysing activities of A_I and A_{II} fractions generally showed markedly higher units/organic C ratio values than those of the corresponding R fraction and ratio values of A_I were usually higher than those of A_{II} (Table 3). The casein-hydrolysing activities/organic C ratio values of A_I fractions were generally higher than those of the other fractions but differences between these values were lower than those of BAA- and ZPL-hydrolysing activities (Table 3).

The highest BAA- or ZPL-hydrolysing activity/amino acid N ratio were observed (with the exception of BAA-hydrolysing activity of the A_{II} fraction of soil 13) in the A_I fractions whereas this was not the case of the casein-hydrolysing activity/amino acid N ratio values (Table 3).

3.2. Characterization of the pasture soil extract and derivative fractions by isoelectric focusing (IEF) and pyrolysis-gas chromatography (Py-GC)

The IEF patterns of the three fractions differed because the dominant bands of A_I, A_{II} and R focalised in the pH ranges 6.0–7.0, 5.5–6.5 and 3.5–6.5, respectively (Fig. 1), thus the patterns were differentiated even if each fraction carried impurities from the other two fractions. The number of bands increased by decreasing the nmw of the fraction whereas the sum of peaks area of the three fractions represented the cumulative area of peaks of the soil extract. It can also be observed that the A_{II} fraction represented a large part of the extract from which it was derived.

The normalized areas of the 12 more relevant pyrolysis fragments present in the pyrograms of pyrophophate extract from soil 19 and of the respective derivative fractions are reported in Table 4. The choice of the 12 fragments and the values of the peaks of the soil extract are from Bonmatí et al. (1998). The peaks reported in Table 4 (1, pyridine and *o*-xylene; 2, styrene; 3, cyclopentenone; 5, furfural; 6, pyrrole; 8, benzonitrile; 10, acetamide; 11, guaiacol; 12, p-tolueneitile; 14, p-cresol; 16, ethylphenol; 17, vynil-guaiacol) can be grouped in 5 different organic pools (Table 5) considering their origin during pyrolysis (Bonmatí et al., 1998). The lowest carbohydrate content was observed in the A_I fraction whereas the composition of A_{II} fraction was very similar to that of the extract and the content of humified organic matter was apparently slightly higher in R than in the other two fractions.

3.3. Thermal denaturation of protease activities of pyrophosphate extract of the pasture soil

The three types of proteases extracted with pyrophosphate from soil 19 were completely denaturated at 80 °C but conserved 25% (ZPL-hydrolysing), 40% (BAA-hydrolysing) or 75% (casein-hydrolysing) of their activities at 65 °C (Table 6). BAA- and casein-hydrolysing activities of samples treated at 30 °C were higher than those of the untreated samples.

Table 3
Protease activities/organic C ratios and protease activities/Amino acid N (AA-N) ratios against N-benzoyl-L-argininamide (BAA), N-benzyloxy-carbonyl-L-phenylalanyl L-leucine (ZPL) or casein of derivative fractions of soil 5, 13 and 19. A_I with nominal molecular weight (nmw) > 100 kD; A_{II} with nmw between 10 kD and 100 kD; R with nmw < 10 kD.

Fraction	BAA-hydrolysing activities		ZPL-hydrolysing activities		Casein-hydrolysing activities	
	(μmol $N–NH_4^+$ g^{-1} organic C h^{-1})	(μmol $N–NH_4^+$ μg^{-1} AA-N h^{-1})	(μmol leucine g^{-1} organic C h^{-1})	(μmol leucine μg^{-1} AA-N h^{-1})	(μmol tyosine g^{-1} organic C h^{-1})	(μmol tyrosine μg^{-1}AA-N h^{-1})
5 A_I	480	8700	276	5000	196	3560
5 A_{II}	116	2190	259	4880	209	3930
R 5	93	3350	90	3270	72	2620
13 A_I	254	7310	4100	117900	252	7250
13A_{II}	457	9140	107	2140	96	1930
R 13	0	0	80	4270	86	4640
19 A_I	217	6780	521	16300	117	3670
19A_{II}	145	1900	356	4700	86	1080
R 19	80	3480	41	1760	107	4680

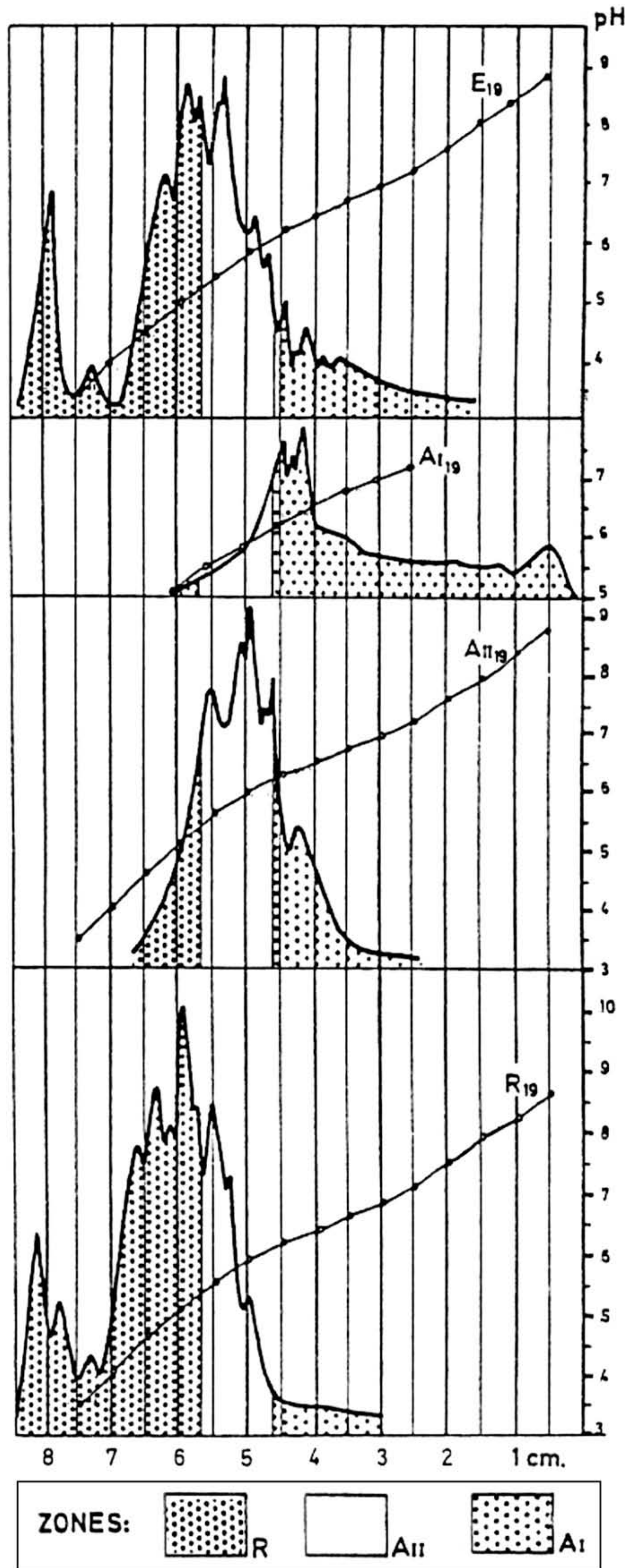

Fig. 1. Isoelectric focusing of pyrophosphate extracts of soil 19 and the derivative fractions; A_I nmw > 100 kDa; A_{II} nmw between 10 and 100 kDa; R nmw < 10 kDa.

3.4. *Effect of pH on BAA-, ZPL- and casein-hydrolysing activities of extract of the pasture soil and the respective derivative fractions*

The highest values (80–100%) of the three proteases activities of the derivative fractions were found at ever higher pH value the lower the nmw of the fraction (Figs. 2–4).

The optimal pH of BAA-hydrolysing activities of soil extract was 6, these activities presented an inflexion at pH 8, showed the lowest value at pH 10 and then increased by increasing the pH value (Fig. 2). By comparing the pH profiles of the soil extract with those of the three derivative fractions we can see that A_I contained the humic–enzyme complex of optimal pH around 8 that was partially masked in the extract. The A_I and A_{II} fractions did not contain the enzyme complexes with optimal pH around 6 and the R fraction did not show that of optimal pH 8.

The optimal pH of ZPL-hydrolysing activities of the soil extract was 6 whereas the lowest value was at pH 8.5 and then these activities continuously increased by increasing the pH value (Fig. 3). The ZPL-hydrolysing activities of A_I fraction showed their highest values between pH 5.5 and pH 7.5 whereas those of the R fraction had the maximum values at pH 10.5; both optimum pH values roughly corresponded to those of the enzyme-complex of the soil extract. On the contrary the pH optimum (8.8) of the A_{II} fraction was not present in the pH profile of the soil extract.

Casein-hydrolysing activity of the soil extract had a broad optimal pH (pH 7–10.5), The R fraction contained the humic–caseinase complexes with pH optima higher than 10, whereas the casein-hydrolysing activities of the A_I fraction showed an optimal pH between 8.5 and 9 and those of the A_{II} fraction showed a pH optimum at 8.5. However casein-hydrolysing activities of both A_I and A_{II} did not increase by increasing pH over 9, as observed for BAA-hydrolysing activities.

3.5. *Comparative studies between the protease activities and the organic matter composition*

As already stated in Material and Methods, we define specific protease activities the ratio between each protease activity and the organic C content of the fraction. Specific BAA-hydrolysing activity was negatively correlated ($p < 0.005$) with the logarithm of the furfural peak area and positively correlated ($p < 0.05$) with the logarithm of the styrene peak area; specific ZPL-hydrolysing activity was negatively correlated ($p < 0.01$) with the guaiacol peak area; specific casein-hydrolysing activities were negatively correlated ($p < 0.05$) with the guaiacol peak area and with the benzonitrile peak area.

4. Discussion

4.1. *Chemical characterization and proteases activities of the derivative fractions of the three soil extracts*

The amount of total organic C in the derivative fractions was higher the lower the nmw of the fraction, thus confirming what observed by Ceccanti et al. (1986) for pyrophosphate extracts of two highly organic soils. Molecules bound through electrostatic interactions are probably separated by pyrophosphate during the extraction and ultrafiltration process (Ceccanti et al., 1982). Pyrophosphate can also chelate cations responsible for maintaining organic matter in flocculated conditions, thus leading to solubilization of organic matter (Stevenson, 1982b). During ultrafiltration, the progressive detachment of low molecular weight components from the complex mixtures of extracted humic molecules may make some entrapped cations accessible to pyrophosphate, thus

Table 4
Normalized areas of the 12 more relevant pyrolysis fragments of pyrophosphate extract of soil 19 and the respective derivative fractions.

Peak	1	2	3	5	6	8	10	11	12	14	16	17
E	0.09	4.99	0.61	3.24	16.01	3.20	10.21	1.44	1.07	5.43	1.07	2.84
A_I	0.14	6.60	0.14	0.14	19.13	4.05	0.45	0.21	2.31	5.98	1.87	3.75
A_{II}	0.16	5.15	0.07	1.67	16.20	2.57	9.10	0.36	1.04	6.01	1.34	2.64
R	0.24	6.99	0.24	4.24	20.49	4.51	0.29	0.41	1.35	5.06	0.29	1.41

The values of E (pyrophosphate extract from soil 19) were taken from Bonmatí et al. (1998).
A_I, A_{II} and R are fractions obtained by ultrafiltration with nmw > 100 kD, between 10 and 100 kD and <10 kD, respectively.
Peak 1 is pyridine and *o*-xylene; peak 2 styrene; peak 3 cyclopentenone; peak 5 furfural; peak 6 pyrrole; peak 8 benzonitrile; peak 10 acetamide; peak 11 guaiacol; peak 12 p-tolueneitile; peak 14 p-cresol; peak 16 ethylphenol; peak17 vynil-guaiacol.

accelerating the depolymerization with further accumulation of the organic matter in the low nmw fractions.

The highest content of fulvic acids in the R fractions was in accordance with Ceccanti et al. (1986), who found important similarities between the compositions of the FA and the R fractions in two highly organic soils using the pyrolysis-gas-chromatography and the isoelectric focusing techniques.

The low contents of amino acid N that we found in the R fractions are consistent with the results of Warman and Isnor (1991), who found that from 45% to 85% of the amino acid N extracted from four sandy loam soils by a mild treatment involving a chelation and a successive gel chromatography fractionation procedure, was present in a fraction whose nmw was higher than 5 kDa. The proteins extracted by Murase et al. (2003) from greenhouse soils with 67 mM pH 6.0 phosphate buffer, ranged between very similar values of nmw (35–68 kDa). These results contradict those by Kimber et al. (1990), who observed that 50% of the extracted amino acid N by pyrophosphate from highly organic soils was present in the fraction of nmw <10 kDa. Probably these differences may be due to the presence of proteins, peptides and amino acids associated to non-humified organic matter in the highly organic soils used by Kimber et al. (1990), because the non-humified organic matter is less resistant than the humified organic matter to the disintegrating action of pyrophosphate.

We found that more than 70% of the BAA- and ZPL-hydrolysing activities were present in the humus–protease complexes having nmw higher than 10 kD and that at least 30% of these activities belonged to complexes with nmw higher than 100 kD. About 95% of the cumulative BAA-hydrolysing activity extracted and fractionated by sodium pyrophosphate from a pasture soil had nmw higher than 10 kD (Ceccanti et al., 1989), whereas nmw of ZPL-hydrolysing proteases extracted from an arable soil by 0.1 M PO_4^{3-} at pH 7.0 for two 30 min-shaking period ranged from 35 kD and 75 kD (Kamimura and Hayano, 2000). The milder extraction procedure used by Kamimura and Hayano (2000), compared with our pyrophosphate extraction method, was probably not effective in solubilising high nmw humus–enzyme complexes (Ceccanti et al., 1978; Ruggiero and Radogna, 1984). On the other hand we found that the casein-hydrolysing activity values were not systematically higher in any of the three derivative fractions. These results agree with the findings by Bonmatí et al. (1998), who suggested that BAA- and ZPL-hydrolysing proteases were associated with condensed humic matter, whereas casein-hydrolysing activity was mainly associated to non-humified organic matter (the lower the nmw of the fraction the lesser the condensation degree of the organic matter).

The BAA- and ZPL-hydrolysing activity units/organic C ratio values found in the derivative fractions were generally higher the higher the nmw of the fraction. This trend was also present in the casein-hydrolysing activity/organic C ratio values but differences between the fractions were less marked than in the other two proteases. These results are consistent with those of Ceccanti et al. (1989) who found that the area of BAA-hydrolysing active bands increased by increasing the nmw when A_I, A_{II} and R fractions extracted by sodium pyrophosphate from a pasture soil were submitted to analytical IEF. Nannipieri et al. (1985), using the same extraction and fractionation procedure with a histosol rich in plant debris, observed the same trend for both BAA- and casein-hydrolysing activity units/organic C values of A_I and A_{II} fractions; unfortunately they did not determine protease activity of the R fraction.

Casein-hydrolysing activity/amino acid N ratio values were not systematically higher in any of the three fractions, whereas in the case of BAA- and ZPL-hydrolysing activities these values were generally higher in the A_I fraction. These results (together with those of activity units/organic C ratios) suggest that casein-hydrolysing activity is mainly associated to non-humified organic matter.

4.2. Characterization of the pasture soil extract and derivative fractions by isoelectric focusing (IEF) and pyrolysis-gas chromatography (Py-GC)

The results obtained in IEF show that the acid character of bands and the heterogeneity of the fractions increased by decreasing the nmw of the fraction. These results confirm what already observed by Ceccanti et al. (1982, 1986, 1989) for the IEF of pyrophosphate extracts and fractions obtained from three organic soils and support the hypothesis of the disintegrating action of sodium pyrophosphate during ultrafiltration with progressive unmasking of negative charges of the extracted organic matter. In addition, our results support what suggested by Maggioni and Cacco (1977) that IEF is charge size dependent.

Table 5
Cumulative area of peaks of Table 3 representing pools of soil organic matter.

Pools of organic matter and peaks of Table 3	Fresh organic matter Peaks 5, 10, 11, 16 and 17	Humified organic matter Peaks 1, 2 and 3	Carbohydrates Peaks 5 and 10	Proteins Peaks 1, 6, 12 and 14	Glycoproteins Peak 8
E	18.8	5.69	13.45	22.6	3.2
A_I	6.42	6.88	0.59	27.56	4.05
A_{II}	15.11	5.38	10.77	23.41	2.57
R	6.64	7.40	4.53	27.14	4.51

The values of E (pyrophosphate extract from soil 19) were taken from Bonmatí et al. (1998).
A_I, A_{II} and R are fractions obtained by ultrafiltration with nmw > 100 kD, between 10 and 100 kD and <10 kD, respectively.
Peak 1 is pyridine and *o*-xylene; peak 2 styrene; peak 3 cyclopentenone; peak 5 furfural; peak 6 pyrrole; peak 8 benzonitrile; peak 10 acetamide; peak 11 guaiacol; peak 12 p-tolueneitile; peak 14 p-cresol; peak 16 ethylphenol; peak17 vynil-guaiacol.

Table 6
Thermal denaturation of protease activities of pyrophosphate extract of soil 19. Values are expressed as percentage of the value measured at 22 °C and values not equal to 100% are significantly different with $p < 0.05$.

Temperature (°C)	BAA-hydrolysing activities	ZPL-hydrolysing activities	Casein-hydrolysing activities
22	100	100	100
30	150	40	135
50	40	60	100
65	40	25	75
80	0	0	0

According to Py-GC results, the content of humified organic matter was quite similar in the three fractions but surprisingly it was slightly higher in R, mostly due to the higher proportions of pyridine + *o*-xylene and cyclopentenone that were present in the pyrogram of this fraction (Tables 4 and 5). On the other hand our results showed that the R fraction had lower ZPL-hydrolysing activities/organic C ratios than the other two fractions (Table 3), thus contradicting what found by Bonmatí et al. (1998) that ZPL-hydrolysing activities of our three soils and their respective extracts were associated to the humified organic matter represented by pyridine + *o*-xylene and cyclopentenone. Probably the mentioned results are only apparently contradictory because the progressive mechanical disintegrating action of sodium pyrophosphate during ultrafiltration may have especially affected the humic–protease complexes represented by the above mentioned peaks. Humic substances represented by the styrene pyrogram peak were probably less affected (and hence more resistant) to pyrophosphate action, because pyrograms of the three fractions contained similar amounts of styrene.

Both IEF and Py-GC results suggest that the A_{II} fraction probably represents the "core" of the soil extract, as already shown by Ceccanti et al. (1986) after the Py-GC characterization of derivative fractions of their two highly organic soils.

4.3. Thermal denaturation of protease activities of pyrophosphate extract of the pasture soil

At least 25% of the activity of the proteases extracted from soil 19 was conserved after incubating the samples at 65 °C for 2 h. Generally microbial proteases are less resistant to thermal denaturation than proteases extracted from soil due to protective action of the associated soil colloids. BAA-hydrolysing protease from *Chlostridium hystolyticum* was totally denaturated after incubation for 5 min at 50 °C (Mitchell and Harrington, 1971); *Aspergillus flavus* caseinase was completely denaturated after its exposure for 10 min at 70 °C (Impoolsup et al., 1981); BAA- and casein-hydrolysing commercial pronase conserved less than 20% of their activity after a 2 h incubation at 60 °C (Nannipieri et al., 1982); three different *Stenotrophomonas maltophilia* azocasein-hydrolases

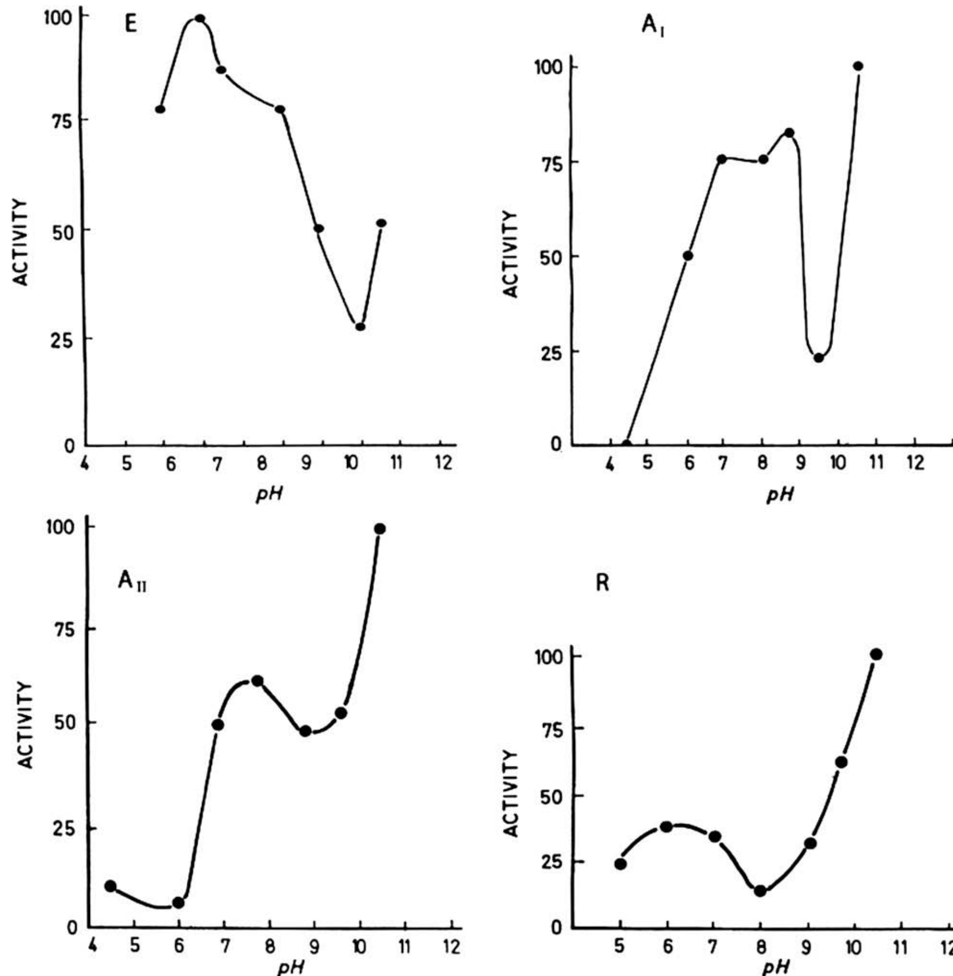

Fig. 2. BAA-hydrolysing activities as affected by the pH of the solution; A_I nmw > 100 kDa; A_{II} nmw between 10 and 100 kDa; R nmw < 10 kDa.

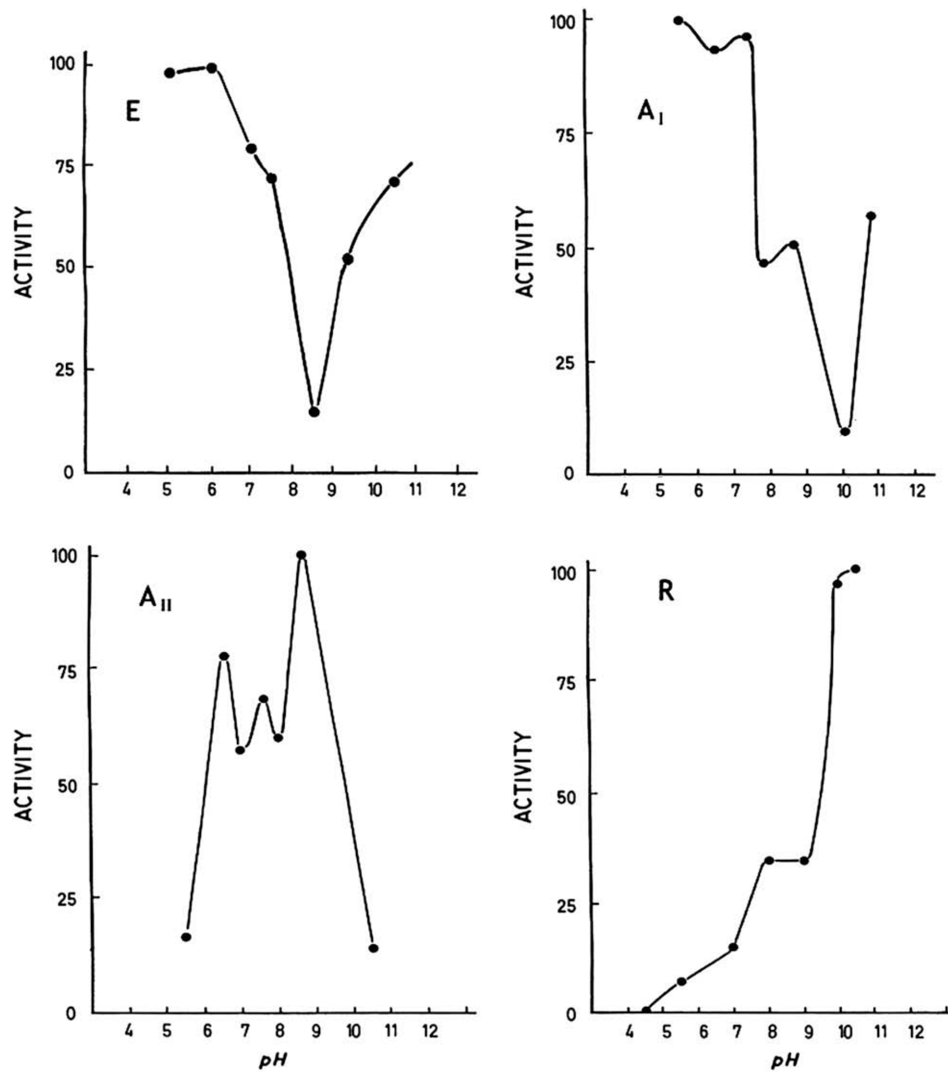

Fig. 3. ZPL-hydrolysing activities as affected by the pH of the solution; A_I nmw > 100 kDa; A_{II} nmw between 10 and 100 kDa; R nmw < 10 kDa.

were completely inactivated after 1 h exposure to 60 °C (Vazquez et al., 2005); *Aspergillus fumigatus* caseinase retained 47% of its activity after exposure for 30 min to 60 °C (Wang et al., 2005).

The higher resistance of casein-hydrolysing activities than BAA- and ZPL-hydrolysing activities to thermal denaturation in our soil extract can be due to the type of organic matter to which the former enzymes are associated. Indeed casein hydrolyzing activity, but not the other two protease activities, was associated to a type of organic matter which was free of mineral fractions (Bonmatí, 1989). The mineral fraction exerts a protective action over the soil organic matter (Chenu, 2001) and probably the humic-BAA-proteases and the humic-ZPL-proteases were separated from mineral component during soil extraction by pyrophosphate, as suggested by Nannipieri et al. (1980), making both proteases more sensitive to thermal denaturation. These results contradict those by Nannipieri et al. (1982), who found a lower resistance of casein-hydrolysing activities to thermal denaturation than BAA-hydrolysing activities of pyrophosphate extracts of a forest and a permanent grassland soil. This may depend on the different soil handling because soil 19 was air-dried and stored at room temperature prior the study whereas Nannipieri et al. (1982) sieved moist soils and stored them at 4 °C. Air-drying of soil can have denaturated thermo-sensitive enzymes but also facilitated adsorption of extracellular enzymes as caseinase by soil colloids making them more resistant to thermal denaturation (Burns, 1982; Mayaudon, 1986; Bonmatí et al., 2003). The higher resistance of BAA-than ZPL-hydrolysing activities of the soil extract to thermal denaturation may depend on the fact that the former enzymes are associated with more humified organic matter than the latter enzymes (Bonmatí et al., 1998). The increase in BAA and casein-hydrolysing activities after incubation at 30 °C may be due to the separation of inhibitors from the enzyme active site caused by the breakdown of the weak bonds linking inhibitors and enzymes, or to the unmasking of some active sites, caused by the increase of temperature as already suggested by Nannipieri et al. (1982).

4.4. Effect of pH on BAA-, ZPL- and casein-hydrolysing activities of extract of the pasture soil and the respective derivative fractions

The values of proteases activities of the derivative fractions were higher at ever higher pH value the lower the nmw of the fraction. Probably this depends on the polyanionic characteristics of the organic matter bound to the proteases molecules; the electric charge of this organic matter increases by decreasing the nmw of the fraction, partially due to the mentioned mechanical disintegration action of sodium pyrophosphate. The polyanionic humic

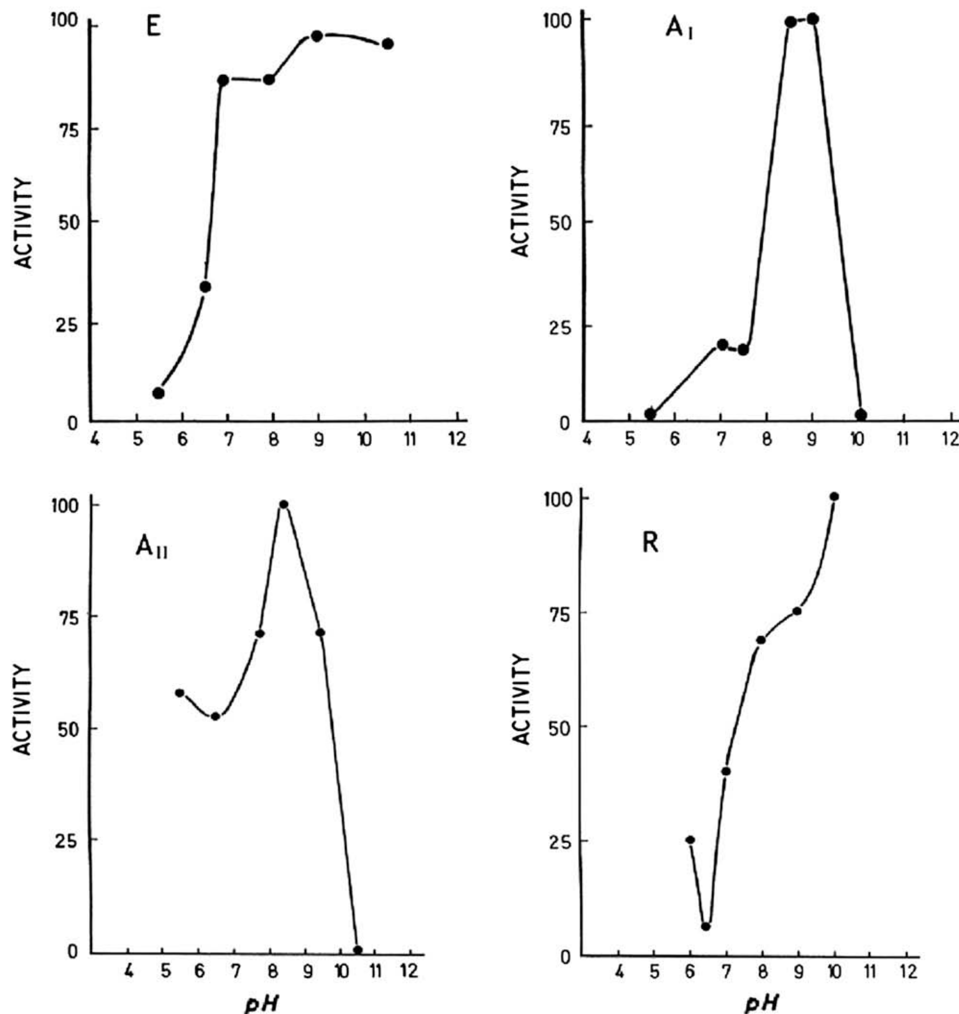

Fig. 4. Casein-hydrolysing activities as affected by the pH of the solution; A_I nmw > 100 kDa; A_{II} nmw between 10 and 100 kDa; R nmw < 10 kDa.

moiety increases the concentration of hydrogen ions in the microenvironment of the active site on the bound enzyme (Goldstein and Katchalski, 1968; Rowell et al., 1973; Nannipieri et al., 1982). Consequently caution is required in interpreting optimal pH values of derivative fractions because these values may be only apparently higher than those of the same humic–enzyme complexes of the initial extract.

BAA-hydrolysing activities extracted with pyrophosphate from a forest and a permanent grassland soil (Nannipieri et al., 1982) showed an optimal pH value (7.0) that was similar to that of our soil extract (6.0) but these enzyme activities were not assayed at pH values higher than 9.0. Though BAA is a typical trypsin-like enzyme with optimal pH about 8 (Keil, 1971), other microbial and plant (cistein-, with optimal pH between 5 and 8, or acid-like, optimal pH between 2 and 5) proteases are able to hydrolyze this substrate (Glazer and Smith, 1971; Matsubara and Feder, 1971; Mitchell and Harrington, 1971).

ZPL is a specific substrate for carboxypeptidases, which have generally a pH optimum between 5.5 and 8.5, acid proteases, and alkaline proteases (optimal pH between 8 and 11). Some of these proteases have microbial or plant origin (Satoh and Fujii, 1985; Kase et al., 1990). ZPL-hydrolysing activity extracted from soil by phosphate at pH 7 had two pH optima, at about pH 5 and 9 (Kamimura and Hayano, 2000), whereas the same enzyme activities extracted by 0.1 M Tris–borate at pH 8.1 from an arable soil showed optimal pH values between 7.3 and 8.9 (Ladd, 1972). The activities of both extracted enzyme activities did not increase by increasing pH values over than 9 as it occurred in our work, probably because phosphate and Tris–borate were less effective than pyrophosphate in extracting stable and active enzymes bound to humic materials (Masciandaro et al., 2008). The higher humic content of our extracted enzymes would cause the displacement towards higher optimal pH values due to the effect of the polyanionic humic moiety, as already mentioned. The pH optimum (8.8) of our A_{II} fraction was not present in the pH profile of the soil extract probably because it was masked, but it was similar to that (nmw 37 kDa, optimal pH 9) of the phosphate soil extract of Kamimura and Hayano (2000).

The broad optimal pH range present in the profile of casein-hydrolysing activity of the soil extract was probably due to the presence of various proteases and various protease organo-complexes (Nannipieri et al., 1982). Casein-hydrolysing activity of pyrophosphate extracts of two soils investigated by Nannipieri et al. (1982) had also a broad pH optimum (pH 6.5–8.2). The casein-hydrolysing activities extracted from various soils with a mild extraction procedure followed by humic matter separation showed a pH optimum of 8.5 (Mayaudon et al., 1975). The shift towards higher optimal pH activity values of the casein-hydrolysing of our soil extract may be due to the pyrophosphate extracted polyanionic organic matter surrounding the enzyme molecules as mentioned

above. The fact that casein-hydrolysing activities of A_I and A_{II} did not increase by increasing pH over 9, as observed for BAA-hydrolysing activities, may be a further evidence of scarce association of casein-hydrolysing activities with humified organic matter.

4.5. Comparative studies between the protease activities and the organic matter composition

Specific BAA-hydrolysing activity was negatively correlated with the logarithm of the furfural peak area and positively correlated with the logarithm of the styrene peak area. Probably the carbohydrate content of the recently incorporated organic matter represented by furfural can inhibit this enzyme activity. These results also confirmed the hypothesis by Bonmatí (1989) and Bonmatí et al. (1998) that BAA-hydrolysing activity is associated with the highly condensed humic matter represented by the styrene pyrogram peak, which was the less affected by the disintegrating action of pyrophosphate. Both specific ZPL-hydrolysing activities and casein-hydrolysing activities were negatively correlated with the guaiacol peak area; probably this was due to the inhibition of these enzyme activities by some lignin or lignin-like products since guaiacol represents lignin-derived fresh organic matter (Bonmatí et al., 1998). Indeed lignin decomposition products or lignin-like substances inhibited enzyme activity (Saña and Soliva, 1987; Ichimura et al., 1998). Specific casein-hydrolysing activity was also negatively correlated with the benzonitrile peak area. Since benzonitrile represents glycoproteins (Bonmatí et al., 1998) it may be possible that at least some of the extracted caseinases were present as glycoproteins and this can explain the resistance of this enzyme activity to thermal denaturation. Mayaudon (1986) suggested the existence in soil of stable enzymes as glycoenzyme complexes surrounded by lipo polysaccharides; these latter protect the enzymes against microbial attack but also render casein difficult to access to the active centre of the enzyme (Rowell et al., 1973).

5. Conclusions

The humus–protease complexes, obtained after extraction of soils with pyrophosphate and ultrafiltration, were resistant to thermal denaturation. Some of these complexes showed an optimal activity at pH values higher than 10 probably as the result of the influence of the humic material surrounding the enzyme molecules and of the presence of alkaline proteases. Complexes of nmw greater than 100 kD had a low carbohydrate content and had the highest BAA- and ZPL-hydrolysing activity. Complexes of nmw between 10 and 100 kD, representing the core of the soil extract, had intermediate BAA- and ZPL-hydrolysing activities and were rich in humic acids, carbohydrates and amino acid nitrogen. Complexes of nmw lower than 10 kD had the lowest BAA- and ZPL-hydrolysing activity and amino acid nitrogen content and were rich in fulvic acids. Differences in casein-hydrolysing activities among the three fractions were less marked than those present in BAA- and ZPL-hydrolysing activities. Comparison of data obtained in Py-GC analyses and in protease activity allowed us to hypothesise that BAA-hydrolysing and ZPL-hydrolysing activities were probably associated to highly condensed humic matter and to less resistant humic substances, respectively; the caseinases were present, at least partially, as glyco-proteins not associated to humus. BAA-hydrolysing activity was probably inhibited by fresh organic matter of carbohydrate origin whereas lignin derived organic matter probably inhibited ZPL- and casein-hydrolysing activities. Future research should involve other analytical approaches such as those based in ^{15}N-NMR and/or X-rays spectroscopy (Nitrogen K-edge XANES) used to better characterize protein-N in these complexes.

Acknowledgements

We wish to express our gratitude to Professor J.M. Alcañiz (CREAF Universitat Autònoma, Barcelona), and to Professor L. Comellas and his staff (CETS, Institut Química de Sarrià, Universitat Ramon Llull, Barcelona) for their assistance in the performing and interpretation of pyrograms. The work has been partially supported by the CIRIT of the Autonomous Government (Generalitat) of Catalonia.

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