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Effect of fractionation on location of enzyme activities in soil structural units

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Summary. Two different soils were fractionated with three granulometric procedures. Enzyme activity measurements were carried out in four fractions of *real* (undisturbed), *stable* (water-dispersed) and *structural* (water-dispersed and physically disrupted) soil units.

A predominance of catalase (67.3%), dehydrogenase (57.6%) and urease (65.3%) activities was observed in soil structural microunits with diameters of less than 50 μm. Highest protease activity was found in fractions with a diameter of less than 50 μm (66.3%) and in fractions with a diameter ranging from 200 to 2000 μm (18.5%) which contain organic matter of a higher and lower grade of humification, respectively.

All fractionation procedures result in a loss of total enzyme activities, which was more clear in the physically disrupted soil than in the sieved soil (e.g. 38.4% vs. 5.9% loss of urease activity).

Key words: Enzyme location – Fractionation – Organic matter – Structural soil units

Soil organic matter has been considered to consist of three different components that can be evaluated experimentally: A physically recognizable fraction of non-transformed animal, plant and microbial debris, a biochemically recognizable fraction composed of simpler molecules (e.g. carbohydrates, peptides, fatty acids) and a polymeric humic fraction made up of phenolic compounds and other kinds of organic biomolecules (Burns 1983).

Among the physical methods proposed to separate soil fractions containing these types of organic matter, the granulometric procedures cause minimal disturbances (Bruckert 1979a). Soil fractionation by aqueous sieving with and without mechanical disruption has been carried out (Gonzalez Carcedo 1978; Bruckert 1979b) without disruption of the microbial cell integrity as determined by electron microscopy. In the microaggregate fractions, predominantly containing humic compounds, there are niches occupied by microbial cells covered by polysaccharidic substances (Bruckert and Kilbertus 1980).

Extracellular enzymes may be intimately associated with the different kinds of organic matter and/or clay colloids and in such a way acquire a remarkable stability (Burns 1982). Positive correlations between organic matter and/or clay content and enzyme activities have been observed in different soils (e.g. Burns et al. 1972a; Russel and Kobus 1974; Stott and Hagerdorn 1980; Tarafdar et al. 1981). Enzyme-active soil fractions have been obtained in this work after a granulometric procedure which has previously been used to isolate fractions with different types of organic matter and differentiated in size and aggregation state (Gonzalez Carcedo et al. 1983). The tested enzymes are characterized by properties suggesting different locations in soil. Thus, catalase (EC

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1.11.1.6) is an intracellular enzyme that may retain its activity outside microorganisms by becoming associated with colloidal particles (Stotzky 1974). Dehydrogenases (EC 1.1) are unspecific obligately intracellular enzymes of microorganisms whose function is solely determined by metabolically active cells (Skujins 1978). Urease (EC 3.5.1.5) is an extracellular enzyme produced constitutively that may also be stabilized on soil colloids (Burns 1983) while proteinases (EC 3.4) are secreted by living cells and are usually found in the aqueous phase or on soil colloids (Burns 1982).

Materials and methods

Soils

A colluvial Rendzina (pH 8.4; CEC 10.8 meq/100 g; organic C 4.2%; C/N 9.4; sand 26.2%; silt 0.1%; clay 0.21%; $CaCO_3$ 63.2%) and a brown acid soil (pH 4.3; CEC 16.5 meq/100 g; organic C 6.3%; C/N 14.9; sand 17.5%; silt 48.6%; clay 13.3%) were collected from the surface (0–10 cm), air-dried and sieved (< 2 mm) prior to use. Methods of soil analysis have been described elsewhere (Gonzalez Carcedo et al. 1983). Soil enzyme activities at the time of sampling are given in Table 1.

Fractionation procedures

The dried soils were fractionated according to the procedure reported in Fig. 1 (Gonzalez Carcedo et al. 1983). Samples of each soil were sieved at 4°C without pretreatment (A), sieved after dispersion in distilled water (reciprocating agitation for 15 h at 60 rpm) (B) and sieved after mechanical disruption and aqueous dispersion (agitation with 5 agate balls 1 cm diameter for 15 h at 60 rpm) (C). Procedures B and C were performed by adding water on each sieve. Thirty-five grams (dry wt.) soil samples (in 200 ml water) were used for each fractionation to give fractions of 2000–200 μm (F_1), 200–100 μm (F_2), 100–50 μm (F_3) and < 50 μm (F_4).

Table 1. Enzyme activities of the soils selected

Enzymes tested	*Rendzina*	Brown acid soil
Catalase activity (CA)[a]	90.90	103.70
Dehydrogenase activity (DHA)[b]	159.30	101.30
Urease activity (UA)[c]	184.70	255.50
Protease activity (PA)[d]	0.31	0.29

[a] In ml O_2/g soil/h
[b] In μg TPF/g soil/h
[c] In μg NH_4^+/g soil/h
[d] In μmol tyr equiv./g soil/h

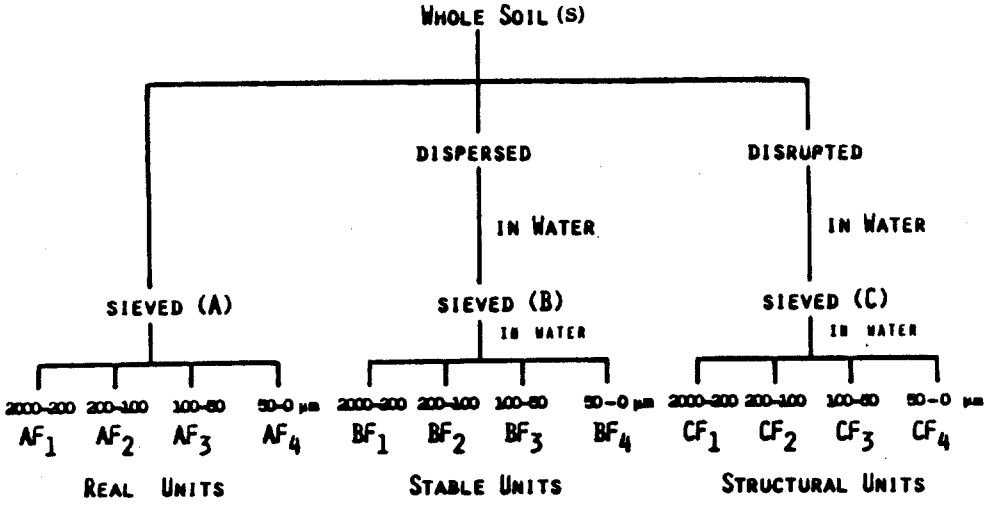

Fig. 1. Fractionation procedures of soil

Enzyme assays

Catalase activity (CA) was determined following a modified gasometric method (Kruglov and Paromenskaya 1966) in which a gasometric apparatus was used to measure the volume of oxygen released by soil decomposition of hydrogen peroxide. Two grams of soil, 0.5 g $CaCO_3$ and 5 ml of a freshly prepared 3% (v/v) solution of H_2O_2 were placed in a vessel and the volume of gas measured every minute during a 7-min incubation period at 20°C. The levels of the manometric columns were equalized for 15 s while stirring before measuring. Three controls with autoclaved soil and NaN_3 (0.1 *M*) were run.

Dehydrogenase activity (DHA) was assayed by measuring the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 24 h as previously reported (Casida et al. 1964). Glucose was added as substrate and $CaCO_3$ as a buffer. The assay was performed anaerobically (under N_2) and in the darkness, thus avoiding the action of other electron acceptors. The red triphenyl formazan (TPF) produced was extracted by shaking the soil 3 times with 5 ml methanol for 30 min. Concentrations of TPF were determined spectrophotometrically (Hiatchi 100–10) at 485 nm using methanol as a blank. A new synthesis of enzymes may occur within 24 h of incubation; thus DHA would reflect the microbial biomass by a glucose-induced activity (Glathe and Thalmann 1970).

Urease activity (UA) was determined by the release of ammonium after incubation of soil (10 g) with 20 ml 0.9 *M* citrate buffer (pH 6.7) and urea solution (10 ml, 10% w/v) at 37°C for 3 h (Hoffmann and Teicher 1961). At the end of incubation, 70 ml distilled water was added, the mixture filtered (Whatman No. 6) and the sediment washed once. An aliquot (1 ml) of the filtrate was mixed with phenol-sodium (OH) reagent and the intensity of the colour was read photometrically after 20 min at 630 nm.

Protease activity (PA) was determined using 2.5 ml casein (10 mg/ml) as substrate dissolved in 0.1 *M* Tris-borate buffer (pH 8.1) after being shaken with 0.5 g soil for 60 min at 50°C (Ladd and Butler 1972). The reaction was stopped by adding 1 ml 17.5% trichloroacetic acid, by separating the sediment by

Table 2. Properties of the various soil fractions (Gonzalez Carcedo et al. 1983)

Fraction type[a]		*Rendzina*				Brown acid soil			
		F_1	F_2	F_3	F_4	F_1	F_2	F_3	F_4
A	W_i[b]	86.6	8.5	2.9	2.0	82.9	7.0	4.0	6.0
	C[c]	3.5	0.4	0.2	0.1	5.2	0.5	0.3	0.3
	C/N	9.3	10.6	15.0	9.0	15.3	13.8	17.0	13.4
	pH[d]	7.8	7.7	7.7	7.7	4.1	3.9	3.9	4.3
	CEC[e]	7.9	8.4	7.0	5.6	9.7	7.1	6.2	7.2
	$CaCO_3$[f]	64.5	54.6	50.9	48.4	ND[g]	ND	ND	ND
B	W_i%	32.2	7.6	8.1	52.1	32.8	3.2	6.7	57.3
	C	1.3	0.2	0.1	1.9	1.9	0.6	0.5	2.8
	C/N	13.1	11.5	16.8	10.2	23.7	16.9	17.7	13.4
	pH	7.9	8.0	8.1	7.6	4.6	4.1	4.2	4.3
	CEC	5.0	3.5	2.9	11.0	3.9	3.1	2.6	7.3
	$CaCO_3$	72.6	66.3	60.0	53.2	ND	ND	ND	ND
C	W_i%	16.4	7.6	10.6	65.4	8.1	1.7	13.1	77.1
	C	0.4	0.4	0.3	2.5	0.2	0.2	0.5	3.4
	C/N	19.5	15.3	16.5	9.7	22.5	18.7	18.6	13.7
	pH	8.1	7.9	8.3	8.1	4.9	4.2	4.5	4.2
	CEC	2.3	3.3	3.5	6.8	3.9	4.2	3.7	5.0
	$CaCO_3$	94.3	57.4	52.7	56.3	ND	ND	ND	ND

[a] A sieved, B sieved after dispersion, C sieved after disruption
[b] weight of each fraction i (in %)
[c] in g/100 g total soil
[d] in water (1 : 5)
[e] in meq/100 g
[f] in g/100 g each fraction (%)
[g] not determined

centrifugation and by mixing 2.0 ml supernatants with diluted Folin reagent (1 ml) and Na_2CO_3 (3.0 ml, 2.8 *N*). The extinction of the colour was read at 700 nm and related to tyrosine standards. All determinations were made in triplicate.

Calculations. The absolute results (in units g^{-1} h^{-1}: ml O_2 for catalase; μg TPF for DHA; μg NH_4^+ for UA and μmol Tyr equiv. for PA) of each of the 12 fractions of the two soils were converted to relative values by taking into account the contribution of each fraction, W_i, to the total weight of soil. Thus the relative activity (R_i) of each fraction (AF_i, BF_i or CF_i) is expressed as a percentage and it is calculated in the following way:

$$R_i\,\% = \frac{FAb_i \times W_i(\%)}{S\,Ab}$$

where FAb_i is the absolute enzyme activity of each fraction and *S Ab* is the measured activity of the original unfractionated soil. These W_i values and other chemical properties of soil fractions are reported in Table 2.

Statistical analysis. All results were tested by standard deviation and variation coefficient, not exceeding (except five cases) 5% of this coefficient. With these samples a variance analysis of a rank classification (Kruskal-Wallis 1952) was performed. For a significance level, $\alpha = 0.05$, experimental constants $<$ 5.66 were obtained for this statistical test.

Results and discussion

The distribution of the enzyme activities in the twelve fractions of both soils is shown in Table 3. When no pretreatment is carried out during the fractionation procedure, more than 66% of CA of both original unfractionated soils is found in the largest aggregates (AF_1). Nevertheless, when these aggregates are dispersed or disrupted into units of smaller size, the activity is predominantly located in fractions with diameters of less than 50 μm (BF_4, CF_4). DHA and UA show similar patterns in rendzina and brown acid soils.

Soil organic matter has a characteristic distribution in the structural units (Gonzalez Carcedo et al. 1983) with the humic compounds concentrated in the CF_4 fraction, as is demonstrated by the lowest C/N ratio (Table 1). Bruckert and Kilbertus (1980) have shown by means of electron microscopic techniques that there are bacteria on and within these microaggregates (CF_4 units). Besides, due to the fractionation procedure all clays are accumulated in CF_3 and CF_4 (Bruckert et al. 1978). Therefore the soil components, such as clays and humic molecules, traditionally related to enzyme activity (Burns 1978) occur in fraction CF_4. Intracellular enzymes such as catalase might be associated with microbial cells and/or associated with humic colloids and anionic clays. Urease may also be immobilized on clay or humic colloids isolated in CF_4. The CF_4 fraction is the only one that retains significant levels of DHA (57.6%), thus indicating that CF_4 is a specific locus of microbial colonization.

Protease activity presents a different behaviour in comparison with the other three enzymes (Table 3). It is predominantly located in fractions with the largest size both after sieving (AF_1 and AF_2) and after aqueous dispersion and wet sieving (BF_1, BF_2). In the structural units of the largest size (CF_1), PA is retained at higher levels than are the other enzymes. In the rendzina soil the activity is entirely associated with CF_1 and CF_4 fractions, while in the brown soil all fractions show the activity with a predominance in CF_3 and CF_4 fractions. Partially humified organic matter (C/N = 15.3–22.5) is found in structural units with diameters larger than 50 μm (CF_1, CF_2, CF_3), and the presence of plant debris has been observed with a low-power microscope. Proteases decomposing high-molecular-weight substrates are necessarily extracellular enzymes, generally released from bacteria, fungi and plant roots (Payne 1980) and that may be involved in the early hydrolytic stages of organic matter degradation.

The location of enzyme activities in the fraction of structural units CF_4 (Fig. 2) probably agrees with the model proposed by Burns et al. (1972b). This model suggests that stable enzymes are physically and chemically immobilized and protected within organomineral complexes which survive disruption during the fractionation procedure used in this work. Thus CF_4 fractions would contain organomineral complexes with immobilized enzymes. Nevertheless, according to this model, binding of proteases to soil colloids might protect the enzyme proteins but might also render them inaccessible and thus inactive towards substrates of high molecular weight.

Effect of fractionation

Table 3 shows that the sum of the enzyme activities in the soil fractions does not coincide with activity of the

Table 3. Enzyme activities in the soil fractions

Soil		Real units (A)		Stable units (B)		Structural units (C)	
		R%[a]	EF%[b]	R%	EF%	R%	EF%
Catalase activity (CA)							
Rendzina	F_1	81.31		14.39		5.10	
	F_2	9.49		4.01		4.75	
	F_3	2.41	− 5.21	4.27	−11.16	4.51	−18.31
	F_4	1.58		66.17		67.33	
Brown acid soil	F_1	66.56		18.34		1.92	
	F_2	12.58		4.20		3.90	
	F_3	5.73	− 9.16	9.15	−15.76	10.66	−17.07
	F_4	5.96		52.55		66.45	
Dehydrogenase activity (DHA)							
Rendzina	F_1	87.57		19.05		1.07	
	F_2	10.40		4.36		3.84	
	F_3	0.45	− 1.58	3.16	−34.96	2.47	−35.06
	F_4	0.00		38.47		57.56	
Brown acid soil	F_1	69.06		8.83		0.60	
	F_2	7.74		1.22		0.41	
	F_3	4.36	−12.26	3.21	−48.48	3.56	−29.27
	F_4	6.58		38.26		66.16	
Urease activity (UA)							
Rendzina	F_1	71.32		22.64		5.35	
	F_2	11.85		4.28		3.93	
	F_3	2.18	−14.03	4.30	−16.50	5.52	−19.85
	F_4	0.62		52.28		65.35	
Brown acid soil	F_1	79.55		33.68		2.61	
	F_2	6.70		7.66		2.28	
	F_3	4.37	− 5.92	5.59	−28.31	6.15	−38.41
	F_4	3.46		24.76		50.55	
Protease activity (PA)							
Rendzina	F_1	95.79		28.29		18.49	
	F_2	13.94		14.05		0.00	
	F_3	8.46	+23.38	7.36	−50.30	0.00	−15.22
	F_4	5.19		0.00		66.29	
Brown acid soil	F_1	136.82		30.20		5.26	
	F_2	8.76		7.21		5.64	
	F_3	2.64	+56.63	8.94	−42.51	18.31	−39.52
	F_4	8.41		11.14		31.27	

a Enzyme activities calculated as reported in "Materials and Methods"
b Effect of the fractionation procedure

original soil (100%). Fractionation processes usually alter soil enzyme activities, causing either losses or even increases (Perez Mateos 1983). The effect of fractionation (EF) on enzyme activity has been indicated in Table 3 as a percentage difference between the sum of relative activities in the four fractions and the activity of the original soil.

Enzymes, such as urease or catalase (Fig. 3), which may be immobilized by soil clays and/or humic molecules, are probably denaturated during fractionation procedures. Aqueous dispersion and even more mechanical disruption increase these losses since they can disrupt and dissolve some immobilized enzymes (Table 3). The largest losses (more than 35%) occur with DHA, which is the most sensitive to fractionation procedures with aqueous dispersion and disruption. The washing process probably removes viable cells (location of intracellular dehydrogenases) that

are not strongly attached to soil particles. The microorganisms within the CF_4 fractions seem to be more resistant to washing and disruption. For the rendzina soil, the inactivation coefficients (EF) for DHA do not present great differences between BF and CF units. However, in the brown soil, the disruption process shows an additional DHA in the CF_4 fraction which did not appear in BF_4 and that probably does not contribute to the total activity of the original soil. This would explain that the activity loss for structural units is lower than the loss observed when isolating stable units (Table 3). Thus the fractionation procedure may actually have made accessible enzymes to substrates and cofactors.

Protease activity behaved differently with respect to the other enzymes. The fractionation in real units (AF) causes increases of activity in comparison with the original soil. Thus the isolation of real units facilitates the access of the macromolecular substrates to the enzymes (especially in AF_1, where activity reaches 136.8%). The proteolytic action, which could be associated with the untransformed organic matter (fractions with the biggest C/N ratios), develops within the real units. Nevertheless, when the water dispersion is applied, a large loss of activity is observed and extracellular proteases seem to be partially lightly bound to aggregates. On the other hand, the CF_4 fraction shows a significant increase in activity over that measured in BF fractions.

To know the distribution of enzymes among different soil fractions would be very useful when studing extraction and purification of soil enzymes.

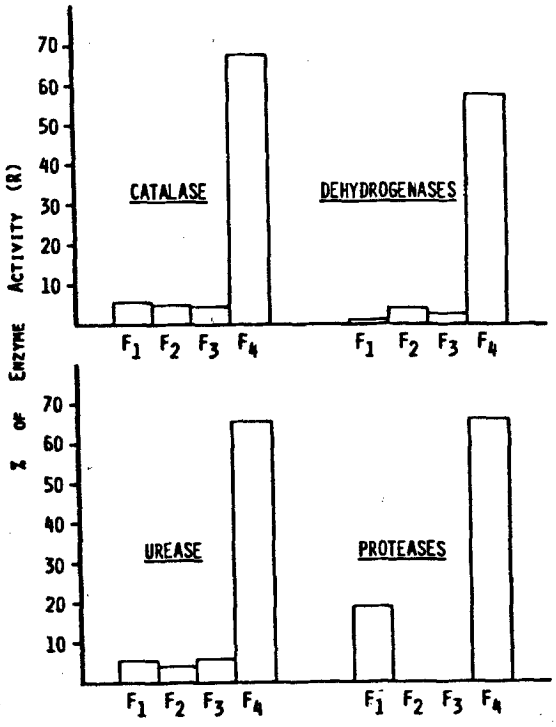

Fig. 2. Distribution of the enzyme activity in the soil structural fractions (CF) of rendzina soil

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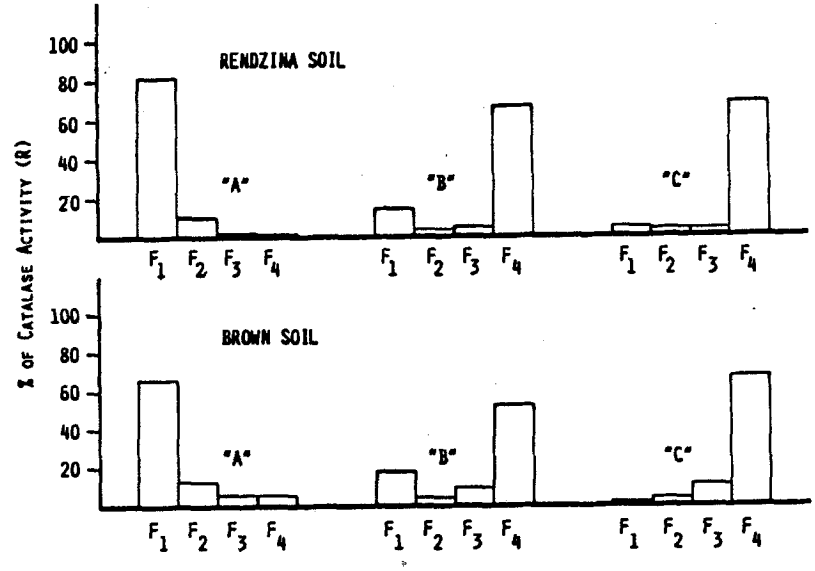

Fig. 3. Distribution of the catalase activity in the soil fractions. *A*, sieved; *B*, sieved after dispersion; *C*, sieved after dispersion and disruption

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