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Enzymatic hydrolysis of ester sulphate in soil organic matter extracts

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Summary. A method of assessing the enzymatic hydrolysis of ester sulphate in soil organic matter was developed. Soil organic matter extracted using a mild, chelating resin extraction procedure was incubated with a sulphatase from *Helix pomatia* in 0.05 *M* sodium acetate buffer (pH 4–8) at 37 °C for 2 h and the sulphate released was determined by a high performance liquid chromatography-conductivity detector system. The effect of some soil factors on the enzymatic hydrolysis of ester sulphate was examined. The study showed that part of the ester sulphate in soil organic matter was biochemically reactive. In the three Podzols studied, the ester sulphate hydrolysed accounted for 2%–12% of the hydriodic acid-reducible organic sulphate extracted. The largest amount of hydrolysable ester sulphate was found in the soil with a low pH, high inorganic sulphate and high hydriodic acid-reducible organic sulphate.

Key words: Enzyme – Organic sulphur – Podzol – Soil organic matter – Sulphatase – Sulphate – Sulphur mineralization

Organic sulphates constitute 30%–88% of the organic S in soils (Speir and Ross 1978; Freney 1986). The importance of the hydrolysis of organic sulphates in the overall mineralization of soil organic S has been emphasized previously (Fitzgerald 1976; Speir and Ross 1978). Although arylsulphatases are involved in the hydrolysis of soil organic sulphates (Tabatabai and Bremner 1970; Cooper 1972; Speir and Ross 1978), attempts to correlate their activity with inorganic sulphate or plant-available S levels in soil have often been unsuccessful (Tabatabai and Bremner 1972; Kowalenko and Lowe 1975; David et al. 1983; Hoque et al. 1987; Appiah and Ahenkorah 1989). The unsuccessful correlation may be partly attributed to the difference in biochemical stability between soil organic sulphates and the synthetic substrate (*p*-nitrophenyl sulphate) used in the assay of soil arylsulphatase activity. The validity of this enzyme assay that uses *p*-nitrophenyl sulphate as a substrate has been questioned (Speir and Ross 1978; Fitzgerald et al. 1985). Therefore, information on the nature, concentration and stability of soil organic sulphates and their impact on S mineralization is highly desirable.

Studies on the chemical nature and biochemical stability of soil organic sulphates have been hampered by the lack of suitable methods. Houghton and Rose (1976) attempted to use enzyme preparations to determine the stability of soil organic sulphates. They could not detect any release of sulphate from a purified "humic acid" and therefore concluded that the ester sulphate groups in the humic fraction extracted with NaOH were resistant to hydrolysis. However, data on the nature and stability of soil ester sulphate may have been obscured by the modifications to soil organic matter that occur during extraction with NaOH.

The objectives of the present study were (1) to develop a suitable method to assess enzymatic hydrolysis of ester sulphate in soil organic matter extracted by a mild chelating resin extraction procedure, and (2) to examine the effect of some soil factors on the formation and hydrolysis of soil ester sulphate.

Materials and methods

Extraction of soil organic matter

Three Pugwash sandy loam soils (Orthic Humo-Ferric Podzols), with different pH, inorganic sulphate, and hydriodic acid-reducible S levels were used in this study. Soil samples were taken from the Ap horizons (0–20 cm) and stored at 2 °C in sealed polyethylene bags until analysed. Some characteristics of the soils are listed in Table 1. Soil organic matter was extracted by the chelating resing extraction procedure according to Warman and Bishop (1985), except that the soil samples were not treated before the extraction. Chelating resin has advantages over NaOH because the latter has been shown to alter organic S compounds (Freney et al. 1969).

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Table 1. Some characteristics of the soils and soil organic matter extracts

Soil no.	Soils			Soil organic matter extracts	
	pH (0.01 M $CaCl_2$)	Organic C (%)	SO_4^{2-} (μg S g^{-1} soil)	Organic C (mg C ml^{-1} SOM)	HI-S (μg S ml^{-1} SOM)
I	5.95 (±0.01)	2.16 (±0.02)	16 (±1)	0.29 (±0.01)	0.8 (±0.0)
II	5.53 (±0.01)	1.92 (±0.01)	100 (±5)	0.26 (±0.02)	2.3 (±0.6)
III	4.28 (±0.01)	2.85 (±0.01)	504 (±27)	0.34 (±0.01)	40.4 (±0.5)

The field-moist soils (10 g oven-dry weight) were shaken with 30 g Na-form Chelex 100 resin (50–100 mesh, Bio-Rad Laboratories, Richmond, California, USA) and 100 ml deionised distilled water for 16 h. The mixture was then centrifuged at 10000 rpm for 20 min and filtered through Whatman no. 41 filter paper. The filtrate was again centrifuged at 12000 rpm for 30 min to remove clay particles and then stored in a refrigerator at 2 °C until analysed.

Sample analysis

The organic C contents of the soils and the soil organic matter extracts were determined by a modified Mebius procedure (Nelson and Sommers 1982). Inorganic sulphate was extracted with $Ca(H_2PO_4)_2$ solution (500 ppm P) and determined using a high performance liquid chromatography-conductivity detector system according to Warman and Sampson (1992). This system consisted of a Beckman model 421 controller, a Beckman model 110A pump, a Waters IC-Pak anion-exchange column fitted with an Anion Guard Pac guard column, and a Waters model 430 conductivity detector. The mobile phase was a gluconate-borate buffer solution prepared as recommended by Waters (Division of Millipore Co.). The flow rate was set at 1.2 ml min^{-1}. The chromatography data were collected and processed using Chromatochart-AP software (Interactive Microware, Inc.). The hydriodic acid-reducible organic sulphate in the soil organic matter extracts was determined as described by Tabatabai (1982).

Enzyme assay of ester sulphate in soil organic matter extracts

The lyophilised powder of sulphatase enzyme (EC 3.1.6.1) from *Helix pomatia* was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). It had a sulphatase activity of 19 μmol *p*-nitrocatechol sulphate h^{-1} mg^{-1} solid (pH 5.0, 37 °C); 30 mg of soil enzyme was dissolved in 100 ml 0.5 *M* acetate buffer solutions of pH 4.0–8.0.

A 0.2-ml aliquot of the enzyme preparation was added to 2 ml of soil organic matter extract and the mixture was then incubated for 2 h at 37 °C. The sulphate released (hydrolysis of soil ester sulphate) during the incubation was determined by the high performance liquid chromatography system described above. Control samples were prepared by incubating the mixture of acetate buffer solution and soil organic matter extract.

The effect of pH on the enzymatic hydrolysis of soil ester sulphate was determined by incubating the soil organic matter extracts with the sulphatase preparations at pH values ranging from 4.0 to 8.0. The effect of incubation time on enzymatic hydrolysis was tested by determining the sulphate released after 2 and 25 min, and 1, 2, 4, and 24 h of incubation. Chloride, phosphate, and sulphate are common anions in soil organic matter extracts and therefore their effects on the enzymatic hydrolysis of soil ester sulphate are of particular interest. Toluene has been used to inhibit microbial growth in the traditional assay of soil arylsulphatase activity (Tabatabai and Bremner 1970). However, it is not clear whether toluene affects the enzymatic hydrolysis of soil ester sulphate. To assess the effect of toluene and of the common anions, 20 μg Cl (as KCl), P (as KH_2PO_4), S (as K_2SO_4), or 0.1 ml toluene was added to the mixture (2.2 ml) of soil organic matter extracts and sulphatase preparations before incubation.

Results and discussion

Choice of buffer

Although 0.5 *M* sodium acetate buffer was used in the traditional assay of soil arylsulphatase activity (Tabatabai and Bremner 1970), our preliminary study showed that in the presence of acetate at this concentration, a quantitative measurement of sulphate by the high performance liquid chromatography-conductivity detector system was impossible. The blocking effect of acetate was minimised when the concentration of acetate was reduced to 0.05 *M*. The buffering capacity of the mixtures of 0.05 *M* acetate and soil organic matter extracts was so high that the maximum pH shift during the enzymatic hydrolysis was only 0.01–0.06 pH units. A phosphate buffer was not used because phosphate inhibits the activity of some sulphatase enzymes (Dodgson and Rose 1975) and interferes, at high concentrations, with the determination of sulphate by high performance liquid chromatography, as shown by our preliminary study.

Effect of buffer pH and incubation time

The effect of the buffer pH on the enzymatic hydrolysis of ester sulphate in soil organic matter extracts is shown in Fig. 1. The activity of the arylsulphatase from *Helix pomatia* towards soil ester sulphate was sensitive to pH, especially when a large amount of ester sulphate was hydrolysed (Fig. 1). The optimum pH for the hydrolysis

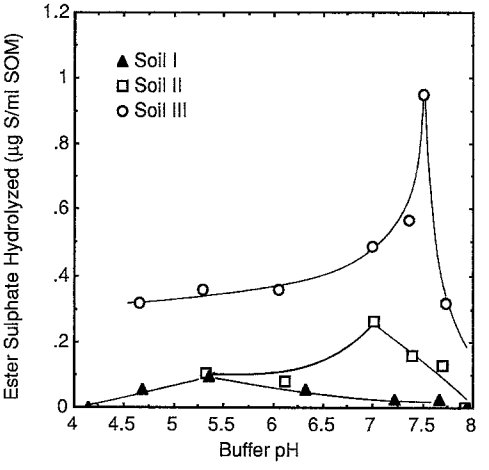

Fig. 1. Effect of buffer pH on the enzymatic hydrolysis (37 °C, 2 h) of ester sulphate in organic matter extracted from three Podzolic sandy loam soils

of ester sulphate extracted from soils I, II, and III was 5.3, 7.0, and 7.5, respectively. Thus, the optimum pH for the enzymatic hydrolysis increased with the increasing concentration of hydriodic acid-reducible organic sulphate in the extracts (Table 1) and the amount of ester sulphate hydrolysed (Fig. 1). When simple substrates, such as *p*-nitrocatechol sulphate, are hydrolysed by the *Helix* enzyme, the optimum pH for the hydrolysis shifted upwards as the concentration of substrate was increased (Dodgson and Rose 1975). It appears that the same effect of substrate concentration on optimum pH holds for the hydrolysis of soil ester sulphate (Fig. 1, Table 1).

Although the hydrolysis of soil ester sulphate is dependent on buffer pH, and the optimum pH for the hydrolysis varies with soil ester sulphate levels, a routine analysis of hydrolysable ester sulphate may be carried out at a single pH. Figure 1 indicates that soils with different ester sulphate levels may be compared at a pH within the range 6–7. Blair and Lefroy (1991) reported that S uptake by crops was better correlated with the soil hydriodic acid-reducible organic sulphate (ester sulphate) removed by extraction with KCl (40 °C, 3 h) than with that extracted by $Ca(H_2PO_4)_2$ or $NaHCO_3$. The results may be interpreted to some extent in terms of pH effect. The acidic and alkaline conditions provided by the latter two extractants were apparently unfavourable for the enzymatic hydrolysis of soil ester sulphate.

Figure 2 shows the effect of incubation time on the enzymatic hydrolysis of ester sulphate extracted from the soil with a high and a medium level of hydrolysable ester sulphate. There was a rapid increase in sulphate release during the first 2 h, but no further increase in sulphate concentration was observed (Fig. 2). No additional hydrolysis of ester sulphate was detected, even after incubation for 24 h (not shown). The decrease in the reaction rate may be caused by (1) the limited substrate, (2) the instability of the *Helix* enzyme, and (3) the inactivation of the *Helix* enzyme by soil proteases in soil organic matter extracts. However, the same pattern of reaction rate shown in Fig. 2 was observed for the hydrolysis of a soil organic matter extract in an immobilized *Helix* enzyme reactor. The activity of the enzyme reactor was stable after being constantly used for a soil organic matter study for a period of 4 weeks (authors' unpublished data, 1992). Thus, the decrease in reaction rate is unlikely to have been a result of the loss of enzyme activity during a 2-h incubation. The substrate concentration, therefore, was most likely the limiting factor in the present study. Figure 2 suggests that an equilibrium was reached after the first 2 h of incubation, and it is appropriate to sample sulphate at this point.

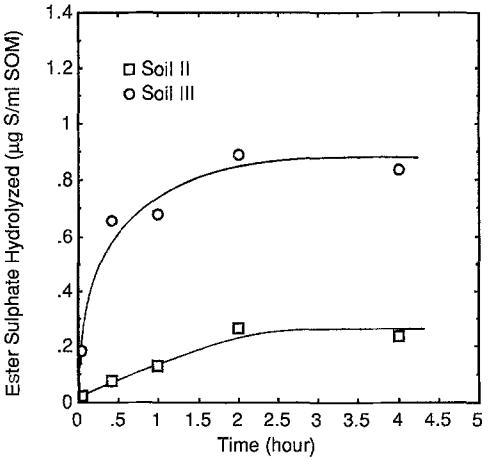

Fig. 2. Effect of incubation time on the enzymatic hydrolysis of ester sulphate in organic matter extracted from two Podzolic sandy loam soils (37 °C; buffer pH: soil II, 7.0, soil III, 7.5)

Effect of chloride, phosphate, sulphate, and toluene

The addition of 20 µg Cl (as KCl) ml^{-1} soil organic matter extract to bring the final Cl^- concentration to 26 ppm had no significant effect on the release of sulphate from soil ester sulphate (Table 2). However, the additional 20 µg P and S (as KH_2PO_4 and K_2SO_4, respectively) ml^{-1} extract reduced the hydrolysis by about 50% and 40%, respectively (Table 2). Thus, phosphate and sulphate inhibited the activity of the *Helix* enzyme towards soil ester sulphate.

Toluene appeared to inhibit the hydrolysis of soil ester sulphate by the *Helix* enzyme (Table 2), although it apparently did not interfere with the assay of soil arylsulphatase activity using *p*-nitrophenyl sulphate as a substrate (Tabatabai and Bremner 1970; Sarathchandra and Perrott 1981). Further study showed that the inhibitory effect of toluene on the hydrolysis of soil ester sulphate was not due to inactivation of the *Helix* enzyme by toluene because toluene did not affect the activity of this enzyme when *p*-nitrophenyl sulphate was used as a substrate. The reduction in the hydrolysis of soil ester sulphate in the presence of toluene may be attributed to the adsorption of toluene onto the surfaces of soil humic polymers, forming a steric barrier between the enzyme and the soil substrates.

Effect of soil inorganic and organic sulphate levels

For soils I, II, and III, the maximum amount of ester sulphate hydrolysed was 0.09 (±0.03), 0.26 (±0.00) and 0.95 (±0.01) µm S ml^{-1}, respectively. It is noteworthy that the level of hydriodic acid-reducible sulphate found in the organic matter extracted from these soils increased in the same order (Table 1). A plot of the ester sulphate hydrolysed versus hydriodic acid-reducible organic sulphate shows a typical enzyme activity-substrate concentration relationship (Fig. 3). Therefore, the hydrolysis of soil ester sulphate depends on the concentration of

Table 2. Effect of chloride, phosphate, sulphate and toluene on enzymatic hydrolysis of soil ester sulphate

Treatment	SO_4^{2-} release (µg S ml^{-1} SOM)
Control	0.95 (±0.06)
Chloride (26 ppm Cl)	1.00 (±0.07)
Phosphate (40 ppm P)	0.49 (±0.06)
Sulphate (60 ppm S)	0.61 (±0.06)
0.1 ml toluene	0.58 (±0.06)

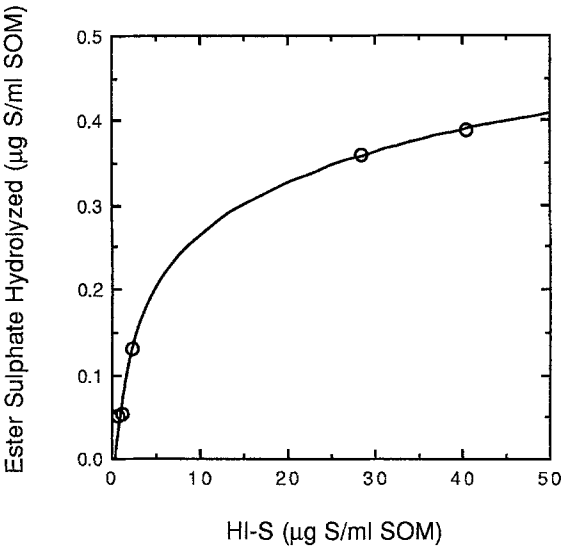

Fig. 3. Effect of hydriodic acid-reducible organic sulphate (*HI-S*) level on the enzymatic hydrolysis of ester sulphate in soil organic matter (*SOM*) extracts (37 °C, 2 h; buffer pH 6.4)

hydriodic acid-reducible organic sulphate, which is believed to be composed mainly of ester sulphate. It appeared that the level of hydriodic-reducible organic sulphate in the organic matter extracts was largely affected by levels of soil sulphate and soil pH. In a recent study on a Podzolic soil the phosphate-extractable soil sulphate was negatively correlated with soil pH ($r^2 = 0.69$, $P = 0.0001$; authors' unpublished data, 1991). A low pH soil has more positive charges and hence a higher capacity to retain sulphate. Since the soil sulphate level is positively correlated with ester sulphate (Adams and Lowe 1984) and inorganic sulphate can be incorporated into soil organic matter by forming ester sulphate (Fitzgerald et al. 1983), a high level of hydriodic acid-reducible organic sulphate is apparently buffered by a high inorganic sulphate concentration. Furthermore, inorganic sulphate may also inhibit the hydrolysis of soil ester sulphate by soil sulphatases, although it does not affect the hydrolysis of simple substrates such as *p*-nitrophenyl sulphate as used in the traditional assay of soil arylsulphatase activity. The synthesis of microbial sulphatases in pure systems is known to be repressed by inorganic sulphate (Dodgson and Rose 1975). Moreover, the activity of soil arylsulphatase towards ester sulphate is expected to be inhibited at a low soil pH, which may also contribute to the accumulation of soil ester sulphate.

The present study revealed the occurrence of a biochemically unstable fraction of ester sulphate in soil organic matter. Since this fraction was not found in the "humic acid" extracted with NaOH (Houghton and Rose 1976), it was apparently altered under highly alkaline conditions during the extraction with NaOH. Therefore, in order to recover the biochemically unstable fraction of soil ester sulphate, a milder extraction method, such as the chelating resin extraction procedure, is required. The observation that this fraction of ester sulphate tends to accumulate in soil with a high inorganic sulphate level suggests that the ester sulphate fraction may serve as a temporary store of soil sulphate. The biochemical instability of this organic fraction ensures its potential availability to plants when soil inorganic sulphate level is lowered. Therefore, the contribution made by the enzymatic hydrolysis of this fraction to the overall mineralisation of soil organic S merits attention.

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