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NOTE

Sources and Variations of Extracellular Enzymes in a Wetland Soil

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A wetland soil was sterilised by two methods and changes in microbial enzyme activities were assessed. The short-term effects were determined by toluene addition, while the longer-term effects of elimination was monitored by γ-radiation. The changes in β-glucosidase, β-xylosidase, cellobiohydrolase, phosphatase, arylsulphatase, and N-acetylglucosaminidase activities were determined by using methylumbelliferyl model substrates and comparing with the activities of control samples. Toluene addition induced different responses of enzymes. For example, phosphatase activity increased by the treatment while β-glucosidase and arylsulphatase activities decreased. In contrast, γ-radiation decreased all enzyme activities compared to control by 40-80%. The overall results of the toluene and γ-radiation experiments indicate that the large amounts of enzymes are stabilised outside of living cells, at least in the short term, but that the persistence of enzymes is maintained by *de-novo* synthesis of microbes.

Key words : soil enzyme, sterilisation, peatland, and bacteria

Microorganisms are known to produce and release various types of enzymes into their environments. Some of those extracellular enzymes play a key role in decomposition of large organic molecules and hence affect nutrient cycles in a substantial way (Chróst, 1992). For example, phosphatase activities have intensively studied in relation to the regeneration of inorganic phosphorus and eutrophication in aquatic ecosystem (Ahn and Choi, 1992). In wetland ecosystems, roles of extracellular enzymes in biogeochemical reactions have recently been revealed (Freeman *et al*., 1997). However, the origin and the status of enzymes in wetland matrix have rarely been elucidated.

Unlike the aquatic ecosystems, the origin and longevity of enzymes in soils are more complicated due to the stabilising effects of the soil matrix on enzymes released from microbial or plant cells. Many studies have been conducted to determine the origin and state of soil enzymes (e.g., Burns, 1982). The simplest approach involves eliminating enzyme sources from the soil, and chasing the change of soil enzymes. For this purpose, toluene (Frankenberger and Johanson, 1986), γ-radiation (Lensi *et al*., 1991), antibiotics or fungicides (Nakasa *et al*., 1987) or autoclaving (Frankenberger and Tabatabai, 1991) have been applied. The ideal method should eliminate target sources without affecting any other components in the soil. Each method adopted to-date, however, has shortcomings. For example, toluene addition can eliminate microorganisms by changing the permeability of their membranes, but could also be used as a carbon source for certain species of bacteria (Kaplan and Hartenstein, 1979). Therefore toluene is not an appropriate treatment for a long-term study. Likewise, γ-radiation, which could terminate all living organisms in soil, has been reported to change

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the physicochemical structure of soils (DaSilva *et al*., 1997). The radiation could produce various types of chemically active free radicals, which may react with other chemicals resulting in substantial changes in humic compositions. Antibiotics or fungicides have been widely adopted in soils, but the efficiency of the sterilisation is questionable (e.g., Nowak and Wronkowska, 1991). Finally, although autoclaving is an efficient and simple way to sterilise soils, the method changes the physical structure of the soil, and hence destabilises the associated immobilised enzymes.

Our aims were to determine what proportions of enzyme activities may 1) persist after microorganisms had been eliminated and 2) be affiliated with microbial cells. For this, enzyme activities were determined after wetland soil was sterilised by toluene or γ−radiation. The toluene addition was used to determine what proportion of enzymes are affiliated with intact microbial cell walls or stabilised outside the microbes. Secondly, the γ−radiation was applied to assess how long enzyme activities may persist without the source (i.e., microbes).

Soil samples were collected from a wetland in Ogwen valley in North Wales, UK. The site is domimated by *Sphagnum* spp. and *Juncus* spp., of which pH is 3.8−4.3. Soil organic matter content is 94.4% and bulk density is 0.029 g cm^{-3}. The samples were collected to 10 cm depth from the surface and transferred on ice. Ten cm3 of soil was gently homogenised for 30 seconds with 50 mL of deionised water in a Seward Colworth model 400 stomacher to obtain homogeneous slurry samples.

To find out the origin of the enzymes, toluene was applied to determine short−term effects of microbial elimination. For this, 0.3 ml of toluene (i.e., *ca*. 3% final concentration) or deionized water (for control) was added to 10 ml of peat slurry (1 : 10 v/v) in 30 minutes prior to the enzyme assay. This concentration of toluene has been reported to be sufficient to disrupt cells but with minimal interference to immobilised enzymes (e.g., Burton and McGill, 1989). Since toluene dissolves the membrane of microorganisms and changes their permeability, the differences between the control and the treatment may represent enzyme components, which require intact microbial cells.

To assess the long−term effect of bacterial removal, the peat sample was treated with 4 MGy of γ−radiation (Isotron PLC, Swindon, Wiltshire, UK), and then maintained in airtight plastic bags at 4°C for 2 months. This radiation dose has been reported to be sufficient to sterilise peat (Brown, 1981). The two−month incubation after the radiation was applied to stabilise the sample (Ramsay and Bawden, 1983) and to provide enough time for degradation of the non−stabilised enzymes. This experiment was to determine the importance of the microorganisms as an enzyme source, and to estimate how much enzyme activities could exist without the source in the long−term.

Methylumbelliferyl (MUF) compounds were employed as a model substrate for enzyme assay (Kang and Freeman, 1998). MUF−glucopyranoside, MUF−xyloside, MUF−cellobiose, MUF−phosphate, MUF−sulphate, and MUF−N−acetylglucosamine were employed to assess β−glucosidase, β−xylosidase, cellobiohydrolase, phosphatase, arylsulphatase, and N−acetylglucosaminidase, respectively. In brief, 10 mL of slurry was added with 5 mL of each specified concentration of MUF substrates. After a 60−minute incubation at 15°C, 1.5 mL of reactant was pipetted into a centrifuge vial and centrifuged at 10,000 rpm for 5 minutes. Fluorescence in the supernatant was determined with a Perkin−Elmer LS50 fluorometer at 450 nm emission and 330 nm

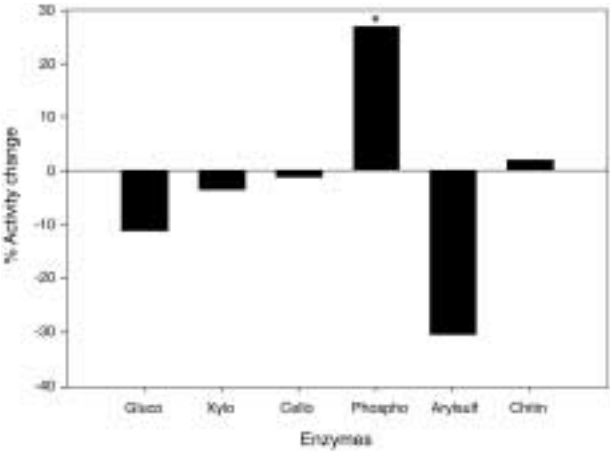

Fig. 1. Effects of toluene on the enzyme activities. *represents significant differences from the control at $P < 0.05$ (n = 3). Gluco: β−glucosidase, Xylo: β−xylosidase, Cello: cellobiohydrolase, Phospho: phosphatase, Arylsulf: arylsulphatase, and Chitin: N−acetylglucosaminidase.

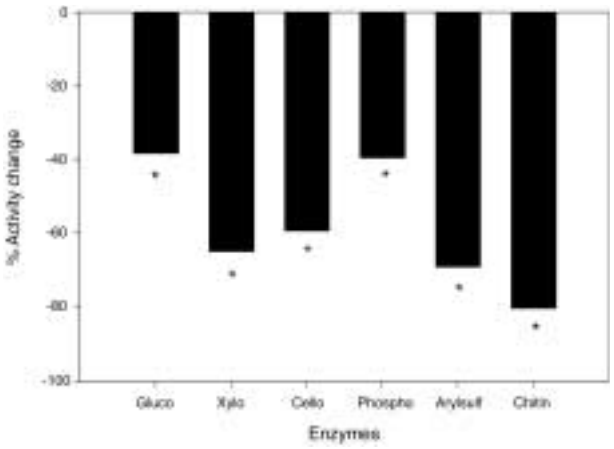

Fig. 2. Effects of γ−radiation on the enzyme activities. *represents significant differences from the control at $P < 0.05$ (n = 3). Gluco: β−glucosidase, Xylo: β−xylosidase, Cello: cellobiohydrolase, Phospho: phosphatase, Arylsulf: arylsulphatase, and Chitin: N−acetylglucosaminidase.

excitation wavelength with slit setting 2.5. A calibration curve was prepared to using 0−100 μM of MUF free acid solution in soil slurry to correct interference of phenolics and adsorption of product to soil matrix. Differences between control and treatment were determined by two sample t−test at $P < 0.05$ level.

Addition of toluene decreased β−glucosidase and arylsulphatase activities by 11% and 30%, respectively (Fig. 1). In contrast, phosphatase activity increased by 26%. All these changes were significant at $P < 0.05$. However, cellobiohydrolase, β−xylosidase and N−acetylglucosaminidase activities did not change significantly ($P > 0.05$). Gamma−radiation treatment reduced all enzyme activities, to extents ranging from approximately 40% (phosphatase and β−glucosidase), 60−70% (xylosidase and cellobiohydrolase), and around 80% (arylsulfatase and chitinase) (all significant at $P < 0.05$) (Fig. 2).

The decreased activities of β−glucosidase and arylsulphatase by toluene treatment imply that much of their activities can be attributed to endocellular enzyme or that at least they require intact cell membrane for their activities. Both endocellular and extracelullar β−glucosidase have been reported from soil microorganisms (Burns, 1982), and toluene treatment might decrease or terminate the endocellular β−glucosidase activity. The decrease of arylsulphatase was unexpected as arylsulphatase has been reported as an extracellular or exoenzyme (Speir and Ross, 1978). Even exoenzymes, however, may depend on an intact microbial cells, if they are embedded in outer membrane or glycoproteins (Chrost, 1992). Large quantities of phosphatase are believed to exist in the periplasmic space of Gram−negative bacteria (Burns, 1982), which might be released or exposed to substrates by toluene treatment in this study. Another possibility is that a certain proportion of the phosphatase was released from algae or zooplankton. The absence of an effect of toluene on β−xylosidase, cellobiohydrolase, and N−acetylglucosaminidase activities suggests that these enzyme activities are true exoenzymes. It is concluded from the toluene experiment that a large proportion of the enzymes function outside of microbial cells.

The results, however, do not necessarily mean that the activities of soil enzymes are independent of microbial cells. Even stabilised enzymes are degraded gradually by proteinase or harsh conditions (e.g., pH change, denaturation by high temperature, or lack of cofactors). Therefore, soil enzyme activities would not persist in the long term without continuous *de−novo* synthesis by microbial cells. For example, by using toluene and kinetic analysis, Burton and McGill (1989) have shown that even a relatively stable component of the total soil enzyme activity may gradually decrease, although the rate is generally far slower than a labile component (i.e., enzyme component dependent upon living microbes). This was confirmed by the γ−radiation experiment in this study.

All the activities decreased including phosphatase activity, with a range from 38.5% (β−glucosidase) to 80.4% (N−ase) ($P < 0.05$). Brown (1981) has reported comparable changes of enzyme activities by 4MGy γ−radiation. In that experiment, about 60% of β−glucosidase and phosphatase, and 50% of N−acetylglucosaminidase and arylsulphatase activities remained after the radiation. In a similar time−series experiment with a mineral soil, β−glucosidase activity decreased rapidly in a week after the radiation, then showed a steady rate of disappearance for at least 8 weeks (Lensi *et al.*, 1991). It is suggested that the remaining activities in the present study would represent the true abiontic enzymes (i.e., stable component of soil enzymes,

which is not affiliated with microorganisms) in the soil. The results of the toluene and γ-radiation experiments indicate that the large amounts of enzymes are stabilised outside of living cells, at least in the short term, but that the persistence of enzymes is maintained by *de-novo* synthesis of microbes.

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＜국문적요＞

습지 토양에서 체외효소의 근원과 변화

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습지토양을 두 가지 방법으로 멸균한 후, 미생물 효소활성도의 변화를 알아보았다. 단기 멸균의 효과는 톨루엔을 가하여 알아 보았고, 장기적인 효과는 감마선 조사를 이용하였다. 처리된 시료에서 β-glucosidase, β-xylosidase, cellobiohydrolase, phosphatase, arylsulfatase, N-acetylglucosaminidase 활성도를 methylumbelliferyl 계열의 인공기질을 사용하여 측정한 후 공시료와 비교하였다. 톨루엔을 가한 경우 효소에 따라 다른 반응을 보였다. 예를 들어, 처리에 의하여 phosphatase의 활성은 증가하였으나 반대로 β-glucosidase와 arylsulfatasem의 활성도는 감소하였다. 감마선 조사는 모든 효소의 활성을 40-80% 정도 감소시켰다. 본 연구의 전반적인 결과에 의하면, 적어도 단기적으로는 다량의 효소가 미생물 체외에서 안정적으로 존재하나, 장기적으로는 de-novo 합성이 중요함을 알 수 있었다.