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DIFFERENTIAL STABILITIES OF SOIL ENZYMES

ASSAY AND PROPERTIES OF PHOSPHATASE AND ARYLSULPHATASE

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Summary

Methods have been refined for the assay of phosphatase and arylsulphatase activities in soil, based on the chromogenic *p*-nitrophenyl ester substrates. Basic assay conditions have been defined, and pH optima and kinetic parameters have been determined. The enzymes follow Michaelis-Menten kinetics; this conclusion is based on three methods of analysis of data determined over a wide range of substrate concentrations. The enzyme activities are very stable to storage of wet soil for up to 4 weeks at soil temperatures and above. For example, phosphatase had a half-life of approximately 2 weeks at 50°C; arylsulphatase was rather less stable. Both enzymes retained 80% of activity after incubation with pronase for 1 week at 25°C. On the basis of this work and studies on other soil enzymes, it is concluded that remarkable stability is a general feature of soil enzymes.

Introduction

In recent years the study of enzyme activity in soil has expanded significantly [1—3]; an expansion stimulated by curiosity concerning the fundamental biochemistry of the soil microenvironment as well as the more applied demands of agricultural science. As a result, more than fifty soil enzymes have been examined, many of them crucial to the cycling of carbon, nitrogen, phosphorus and sulphur.

Perhaps the most detailed studies have concerned urease, an hydrolytic enzyme responsible for the decay or urea from both synthetic (fertilizer) and natural (metabolic waste) sources. Much has been written about the origin, location and kinetic properties of this ubiquitous enzyme [4—13]. It is apparent that a good deal of soil urease (although strictly speaking an intracellular enzyme) has been released from lysed cells and has become associated with the

inorganic (clay) and organic (humus) colloids. This process imparts to it a resistance to degradation and denaturation and means that soils have an indigenous 'background' level of urease independent of immediate microbial proliferation. It has been estimated that this component contributes between 45% and 90% of the total activity [8,12,14—17]. The presence of a recalcitrant catalytic fraction, spacially and temporally removed from its cellular source, is a common feature of soils.

Soil phosphatases and sulphatases have recently been reviewed [18]. They are collective terms for a variety of enzymes concerned with the decay of a number of different substrates. An high proportion of soil phosphorus is associated with organic compounds whose nature is poorly understood [19—21]. They include esters such as inositol, nucleotides, and phospholipids (phosphatidyl choline, phosphatidyl ethanolamine). In addition, traces of phosphorus exist in the soil in phosphoprotein, glucose 1-phosphate and glycerophosphate. Phosphatase enzymes found in soil belong to two principal classes: the phosphoric monoester hydrolases (EC 3.1.3.-) and the phosphoric diester hydrolases (EC 3.1.4.-). Adenosine triphosphatase (EC 3.6.1.3) and pyrophosphatase (EC 3.6.1.1) activities have also been reported.

Modern methods of phosphatase measurement depend upon artificial substrates which, unlike their naturally-occurring counterparts, undergo rapid hydrolysis and have an easily estimated organic moiety. These substrates include β-napthyl phosphate [22], phenylphosphate [23] and *p*-nitrophenyl phosphate [24]. The products are assayed either spectrophotometrically or fluorimetrically.

Very little is known concerning the structure of soil organic sulphur which has been broadly described as being connected by a mixture of oxygen and carbon bonds [20]. The former group (estersulphates) may include sulphated polysaccharides, choline sulphate and ethereal sulphates.

Investigations into the breakdown of sulphur-containing organic substrates in soil have been restricted to arylsulphatase (EC 3.1.6.1) and were initiated by Tabatabai and Bremer [25]. They chose *p*-nitrophenyl sulphate as the substrate and measured spectrophotometrically the *p*-nitrophenol produced. The method has been generally adopted by other workers [26,27].

The work described in this communication has two aims. Firstly the refinement of the phosphatase and arylsulphatase assays using the substrates *p*-nitrophenyl phosphate and *p*-nitrophenyl sulphate respectively. Secondly the application of these techniques to examine the stability of these two enzyme to treatments which inactivate most enzymes in solution. In addition, the kinetic properties of phosphatase and arylsulphatase are examined and critically compared with the reports of other workers.

Materials and Methods

Soil. A silt loam soil with the following characteristics was used for these experiments: sand 16%; silt 64%; clay 20%; organic matter 6.4%; cation exchange capacity 14.8 mequiv. · 100 g soil^{-1}; pH 5.4; water-holding capacity (w.h.c.) 0.72 ml · g^{-1}. It is fully described elsewhere [28].

Substrates. p-Nitrophenyl sulphate, *p*-nitrophenol and pronase were obtained

from Sigma (London) Ltd; *p*-nitrophenyl phosphate from Aldrich Chemical Co., and all other reagents from Fisons Ltd.

Assay. All enzyme assays were performed in Universal bottles using 1 g of air-dried (48 h at 20° C) and sieved (<2 mm) soil. The assays were modifications of those described by Tabatabai and Bremner [24,25] and are summarised in Table I. The soil was pre-incubated for 1 h with 1 ml of 0.2% aqueous sodium azide prior to the addition of buffer and substrate. The function of the sodium azide was to prevent substrate turnover due to microbial proliferation and de novo enzyme synthesis. In the case of arylsulphatase (16 h incubation) the presence of NaN_3 is essential, for phosphatase assays (1 h) this precaution was probably unnecessary but the inhibitor was included for uniformity. It had no inherent effect on enzyme activity. The reaction was terminated by cooling to 0° C in a salt/ice bath, prior to centrifugation.

The *p*-nitrophenol product was detected in the supernatant, after suitable dilution, by addition of sodium hydroxide in the ratio of 3 ml *p*-nitrophenol solution : 2 ml NaOH. Absorbance was read at 400 nm and the amount of *p*-nitrophenol was calculated after reference to a calibration curve relating 1—5 $\mu g \cdot ml^{-1}$ *p*-nitrophenol to absorbance. Although the actual colour development is stable, the residual substrate is both unstable when in solution and light sensitive. Therefore the absorbance of the supernatant was read as soon as possible (<3 h) after centrifugation.

pH Profiles. The activity curves of the two enzyme were measured over a pH range of 4.0—9.0. The buffers used were sodium acetate, sodium maleate, Tris/maleate and Tris · HCl (all 0.1 M), chosen because they extract minimal amounts of soil organic matter which would otherwise interfere with the subsequent spectrophotometry. The inherent buffering capacity of the soil affects the pH of the buffer/soil mixture, such that the true reaction pH may be different from that of the buffer. For phosphatase the pH of the reaction was taken

TABLE I

THE ASSAY FOR SOIL PHOSPHATASE AND ARYLSULPHATASE

Phosphatase	Arylsulphatase
1 g air dried soil + 1 ml aqueous 0.2% NaN_3 Preincubate for 1 h at 20° C	
+ 4 ml 0.1 M Tris/maleate pH 6.9 + 1 ml aqueous 50 mM *p*-nitrophenol *P*	+ 4 ml 0.5 M sodium acetate pH 5.8 + 1 ml aqueous 200 mM *p*-nitrophenyl sulphate
Agitate in the dark at 25° C	
1 h	16 h *
Reaction stopped by cooling to 0° C in a salt/ice bath for 10 min To the controls 1 ml substrate added Soil removed by centrifugation at 20 000 × *g* for 10 min Activity determined by reading the absorbance of the yellow product *p*-nitrophenol after dilution and colour development with 0.5 M NaOH.	

* Now being assayed over 1 h period.

as that recorded at the completion of the 1 h assay (average shift during incubation = −0.3 pH units) whilst for arylsulphatase the mean of the zero and 16 h pH values (no pH shift during incubation) was used. A *p*-nitrophenol calibration curve was constructed for each buffer.

Kinetics. The kinetic constants, K_m and V were determined by analysis of the direct plot [29], Lineweaver-Burk and Eadie-Hofstee plots. In the case of the direct plot the points of intersection giving the best K_m and V values were computed whilst in the other instances best-fit lines were derived from computed least squares analyses.

Thermal stability. Soil was brought to and maintained at 65% w.h.c. (with water containing 0.2% NaN_3) for 4 weeks at 5 temperatures (−20, 4, 25, 30 and 75°C and the activities of both enzymes followed. In addition the effect of wetting the soil followed by lyophilization was measured.

Proteolysis resistance. This was measured over a period of 7 days by incubating soil (65% w.h.c., 0.2% NaN_3) with pronase (3 mg · ml^{-1}) at 25°C. The persistence, and thus efficacy, of the pronase itself was determined using benzoyl arginine amide as a substrate and a Conway micro-diffusion dish [30].

Results and Discussion

The problems to be considered when establishing reliable spectrophotometric assays for these soil hydrolases are principally: (i) selection of suitable substrate concentrations and periods of assay to satisfy the normal criteria of $[S] >> K_m$, insignificant decline of [S] during the assay, and measurable product formation; (ii) development of an effective means of terminating the reaction at a defined time; (iii) accounting for non-enzymic hydrolysis and any contribution of soil humates to the final absorbance; (iv) correction for any soil adsorption of products.

Development of assay. Tabatabai and Bremner [24,31] chose reaction mixture concentrations of 25 mM and 1 mM *p*-nitrophenyl sulphate respectively for their assays of phosphatase and arylsulphatase. We found that 8.3 mM *p*-nitrophenyl sulphate was a satisfactory excess but that for the arylsulphatase assay 33.3 mM *p*-nitrophenyl sulphate was required. It is difficult to account for Tabatabai and Bremner's choice of 1 mM *p*-nitrophenyl sulphate when they quote K_m values for arylsulphatase of 1.37—5.69 mM.

The immediate termination of the reaction after 1 h or 16 h was not, unlike that described by other workers, achieved by filtration or centrifugation as both these operations took some minutes to perform. We therefore used rapid cooling to 0°C (suspension reached 0°C in 7 min) which halted the reaction. Subsequent centrifugation at 4°C clarified the supernatant for assay.

It has been reported [32] that *p*-nitrophenol is adsorbed by soil. We found that 18.5 ± 5.6% of added *p*-nitrophenol was adsorbed throughout the range of concentrations employed (30—300 μg *p*-nitrophenol · ml soil $suspension^{-1}$) and all subsequent results were corrected accordingly. This partial adsorption isotherm suggests that the adsorptive capacity of soil for *p*-nitrophenol is in excess of 300 $\mu g \cdot ml^{-1}$.

pH profiles. The pH activity curves for both enzyme are shown in Figs. 1 and 2. For phosphatase 0.1 M Tris/maleate was used buffered at pH 6.9 (reaction

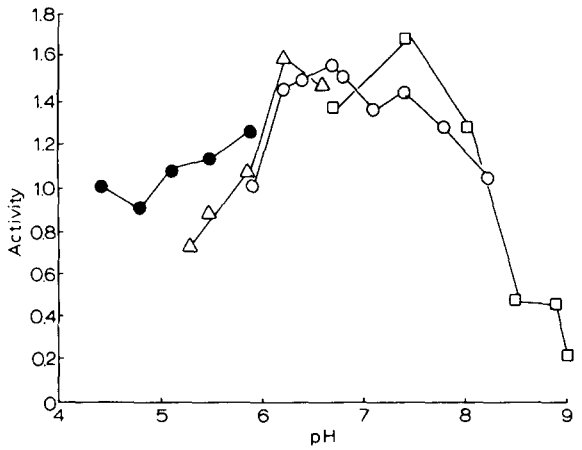


Fig. 1. Effect of pH on soil phosphatase activity. ● = sodium acetate buffer; △ = sodium maleate buffer; ○ = Tris/maleate buffer; □ = Tris · HCl buffer. Activity expressed in μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

mixture pH 6.7). The possibility of a second peak of activity at pH 7.4 should not be discounted due to the occurrence of various forms of acid, alkaline and neutral phosphatases [33,34]. Arylsulphatase gave a peak at pH 5.8 in 0.5 M acetate buffer (reaction mixture also pH 5.8). Guidelines concerning the choice of buffer in soil enzyme determinations have recently been presented elsewhere [35].

Both enzyme assays revealed a stoicheiometric relationship between activity and quantity of soil (i.e. amount of enzyme) trom 0.3 to 2.0 g (Fig. 3). A time study (Fig. 4) showed that phosphate ester hydrolysis increased linearly up to 5 h, and sulphate ester hydrolysis up to 32 h. These simple relationships confirm that the reaction is due to accumulated enzymes and not complicated by microbial growth or the assimilation of metabolites.

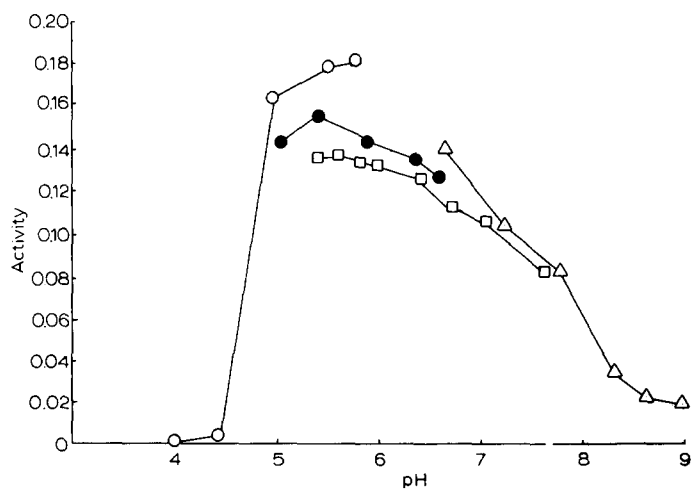


Fig. 2. Effect of pH on soil arylsulphatase activity. ○ = sodium acetate buffer; ● = sodium maleate buffer; □ = tris/maleate buffer; △ = Tris · HCl buffer. Activity expressed in μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

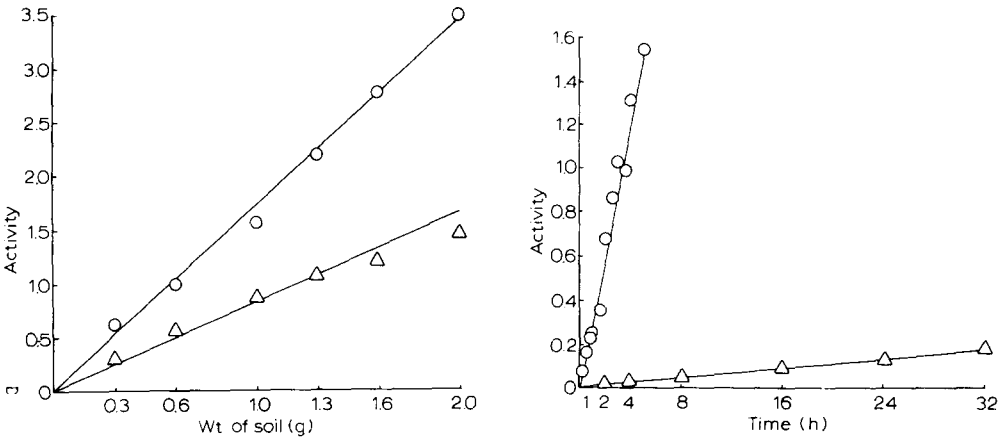


Fig. 3. Stoicheiometry of phosphatase (○) and arylsulphatase (△) in soil. Activity expressed in μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

Fig. 4. Effect of length of incubation upon soil phosphatase (○) and arylsulphatase (△) activities. Activity expressed in μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

Kinetics. Kinetic parameters were derived from determinations of initial velocities at eleven substrate concentrations; for phosphatase the range covered was 0.167—8.33 mM, for sulphatase 0.83—33.3 mM. The enzymes obeyed Michealis-Menten kinetics, as demonstrated by the linearity of conventional Eadie-Hofstee and Lineweaver-Burk plots (Figs. 5, 6, 7, 8).

Cases of deviations from Michaelis-Menten kinetics for soil enzymes have been presented and analysed [36], but such deviations are definitely not

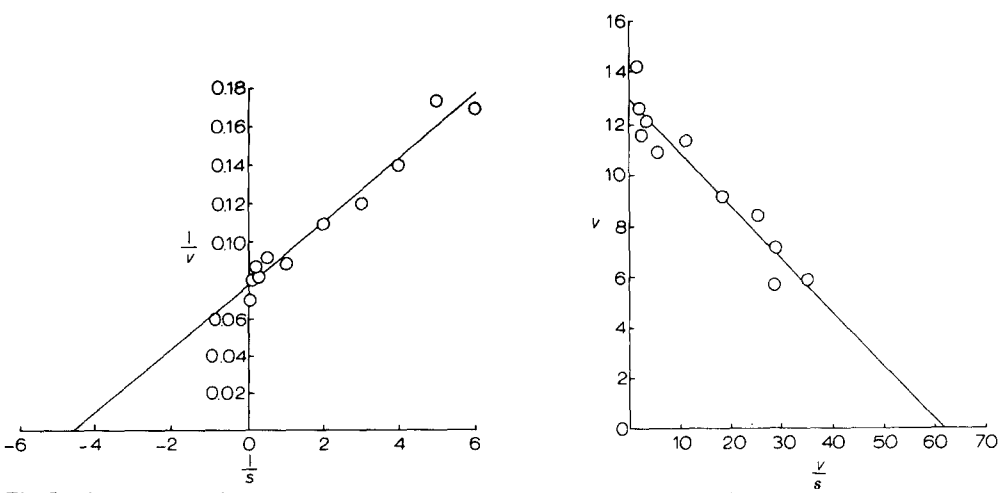


Fig. 5. Lineweaver-Burk plot of soil phosphatase activity in Tris/maleate buffer. v = μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

Fig. 6. Eadie-Hofstee plot of soil phosphatase activity in Tris/maleate buffer, v = μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

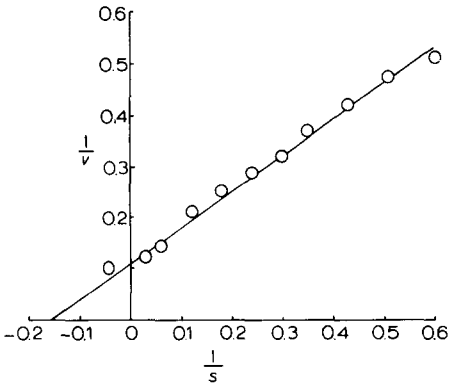


Fig. 7. Lineweaver-Burk plot of soil arylsulphatase activity in sodium acetate buffer. v = μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

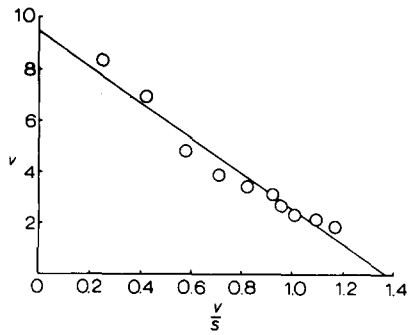


Fig. 8. Eadie-Hofstee plot of soil arylsulphatase activity in sodium acetate buffer. v = μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

observed in the present cases. The consistency with Michaelis-Menten kinetic behaviour is presented on the following grounds, (i) Eadie-Hofstee plots, which have been claimed to be the most sensitive linear transformation for displaying deviations from Michaelis-Menten kinetics [37], were linear over a wide substrate range and showed correlation coefficients of 0.91—0.98 (phosphatase) and 0.97—0.99 (arylsulphatase), (ii) values of K_m and V derived from Eadie-Hofstee plots (Table II) corresponded closely to those obtained from Lineweaver-Burk plots (correlation coefficients 0.96—0.99 (phosphatase) and 0.98—0.99 (sulphatase)), and to those obtained from the direct linear plot, which is the method of parameter estimation least sensitive to errors [38]. Any systematic deviation from Michaelis-Menten kinetics would lead to corresponding differences in the derived values of K_m and V, since the three methods weigh experimental values differently. The values quoted in Table II are means and standard deviations derived from five separate determinations for each enzyme.

A separate question is whether, in the case of enzyme in heterogeneous systems, such as soil enzymes and membrane-bound enzymes, determined values of K_m can be given a simple physical interpretation, as corresponding approximately to the dissociation constant for the enzyme-substrate complex.

TABLE II

THE KINETIC CONSTANTS FOR SOIL PHOSPHATASE AND ARYLSULPHATASE

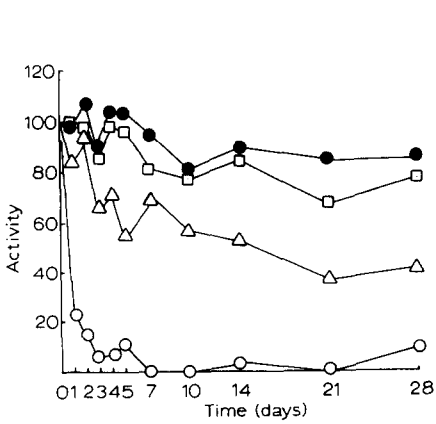
	Direct plot		Lineweaver-Burk plot		Eadie-Hofstee plot	
	K_m (mM)	V	K_m (mM)	V	K_m (mM)	V
Phosphatase	0.33 ± 0.09	1.56 ± 0.20	0.32 ± 0.08	1.50 ± 0.16	0.32 ± 0.09	1.53 ± 0.16
Arylsulphatase	4.99 ± 1.13	0.88 ± 0.06	4.98 ± 1.18	0.89 ± 0.07	5.46 ± 1.31	0.94 ± 0.08

V in μmol *p*-nitrophenol · g soil^{-1} · h^{-1}.

Cervelli et al. [39] have shown that when significant adsorption of the substrate to the soil matrix occurs K_m values (defined empirically as the total substrate concentration required for half-maximal activity) can be considerably higher than those obtained after correction for substrate adsorption. While this is a significant point in the interpretation of K_m values, it is evident that systematic determination of substrate-binding isotherms for soils will not become commonplace, and that the directly derived K_m for a soil enzyme, though not of simple physical significance, remains an important definitive characteristic of the soil.

Thermal stability. Soil samples were stored wet at a variety of temperatures, and phosphatase and arylsulphatase were assayed at intervals over 28 days. In soil stored at 4°C, phosphatase activity remained constant over the entire period while arylsulphatase showed a decline in activity to 70%. The greater stability of soil phosphatase is emphasized by the studies at higher temperatures. Fig. 9 shows phosphatase activities as % of those in samples stored at 4°C; the activity disappears over a period of a week at 75°C, but has a half-life of approximately 2 weeks at 50°C, and is reasonably stable at 25°C. For arylsulphatase, however, (Fig. 10) no activity is detectable in samples stored at 75°C, and the activity of sample stored at 50°C declines rapidly. Comparison of these data with those published for urease in the same soil [12] indicates an order of stability phosphatase > urease > sulphatase, for storage in wet soil. Paradoxically, soil phosphatase was rather unstable to storage in frozen solutions and to freeze-drying. Freeze-drying cause a 33% loss of phosphatase activity, but only 11% loss in sulphatase activity. This difference is also evident in the storage data (Figs. 9, 10). These data indicate that the soil phosphatase is in an environment in which it is protected from active degradative influences, but where it is susceptible to denaturation by the phase changes involved in freezing and thawing.

Stability to proteolysis. Phosphatase and arylsulphatase assays were also per-



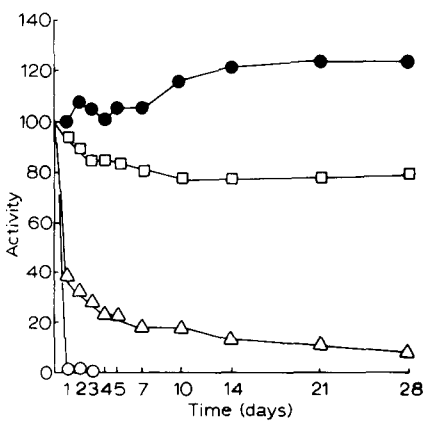


Fig. 9. Stability of soil phosphatase stored at −20°C (●); 25°C (□); 50°C (△) and 75°C (○). Activity expressed as percent of that at 4°C.

Fig. 10. Stability of soil arylsulphatase stored at −20°C (●); 25°C (□); 50°C (△) and 75°C (○). Activity expressed as percent of that at 4°C.

formed on soil samples stored wet in the presence of pronase for up to 7 days. More than 80% of these activities were retained at the end of the period, while the pronase itself retained 60% of its initial activity; this was sufficient to degrade any unprotected enzyme [40]. The resistance of phosphatase and arylsulphatase was predictable, as the indigenous proteolytic activity of the soil was twelve times greater than that caused by the added protease.

Conclusion

The refinement of assay methods for soil phosphatase and arylsulphatase has allowed the kinetics and stability of these enzymes to be studied. It is apparent, from the many previous studies, that soil enzymes are remarkably stable entities, but that there is significant variation in this stability. It is not yet clear whether this variation arises from intrinsic properties of the enzymes, or from differences in their extracellular microenvironments in the soil. Effective methods for the bulk solubilization of soil enzymes will be necessary to resolve this question.

Acknowledgements

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