

## Proteolytic activity under nitrogen or sulfur limitation

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### Abstract

Sand cultures were used to evaluate the effect of C, N, and S ratio on protein degradation by soil microorganisms. Sand was inoculated with soil and amended with defined nutrient media to produce limitation for C, N, or S. Limitation for N or S resulted in reduced biomass (total protein) and increased proteolytic activity as indicated by measurements of dye released from a commercial protease substrate (azocoll). Carbon limitation had little effect on proteolytic activity. As expected, utilization of carbon (glucose) was dependent upon the availability of N or S. Protein synthesis inhibitors (chloramphenicol and cycloheximide) suppressed proteolytic activity, suggesting a need for new gene expression in the response of organisms to N or S stress. Correlations of proteolytic activity and biomass among treatments revealed distinctly different relationships depending upon the availability of C, N, or S. The results of this experiment support a role of proteolytic activity in response of microorganisms to N or S deprivation and suggest that protease activity in soil is more strongly influenced by regulatory signals than by standing biomass. © 2002 Published by Elsevier Science B.V.

**Keywords:** Enzyme regulation; Azocoll; Nutrient cycling

### 1. Introduction

It has long been recognized that soil microbial communities respond to limitations of major nutrient elements, such as C, N, or S (Waksman and Starkey, 1931). Examples of such responses include release of N or S exceeding the needs of a population (mineralization), or acquisition of supplemental N or S from inorganic sources when substrates are deficient of these elements (immobilization). Protein constitutes a significant pool of organic C, N, and S in soil. Important processes affecting turnover of soil protein have been reviewed by Loll and Bollag (1983). In pure culture, extracellular proteases can be induced by exposure of microorganisms to protein (Gill and

Modi, 1981) or starvation for C, N, or S in the presence of traces of protein (Hanson and Marzluf, 1975). In the present work, an experiment was conducted to determine if a soil microbial community would respond to C, N or S starvation by degrading exogenous protein, and whether proteolytic activity was inducible or positively related to microbial biomass.

### 2. Materials and methods

#### 2.1. Chemicals

Reagents for glucose determinations as well as azocoll, a chromogenic protease substrate composed of collagen labeled with azo dye, were purchased from Sigma (St. Louis, MO). Azocoll was sterilized by washing with 95:5 (ethanol:water), which also served to remove unreacted azo dye and soluble labeled

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peptides. These impurities must be removed from azocoll in order to obtain linear kinetics and reduce background in protease assays (Chavira, 1984). Use of azocoll for protease measurement has been described elsewhere (Caplan and Fahey, 1980; Fahey and Caplan, 1981; Chavira, 1984; Rinderknecht, 1968; Will, 1984). Pronase (a proteolytic enzyme) was purchased from Calbiochem Corporation, San Diego, CA. All other chemicals used were ACS reagent grade unless otherwise specified.

## 2.2. Experimental system

Sand cultures were prepared in duplicate by the addition of 5.5 g of air dry Wooster silt loam soil (fine, mixed, mesic Typic Fragiudalf) to 16.5 g of washed, autoclaved, oven dry sand (0.5–1 mm). These cultures were contained in 90 ml glass vials sealed with gas permeable polyethylene film. Each vial received 3.3 ml of deionized water and 1.0 ml of a complete mineral salts medium (Sims et al., 1986) lacking C, N, or S. The basal medium was supplemented with C (1000 mg glucose-C kg<sup>-1</sup>), N (100 mg NH<sub>4</sub>Cl-N kg<sup>-1</sup>) or S (10 mg K<sub>2</sub>SO<sub>4</sub>-S kg<sup>-1</sup>) alone or in combinations to produce C, N, or S limitation. Upon each of these treatments was superimposed, the presence or absence of added protein (gelatin to produce 0.1 mg protein kg<sup>-1</sup>) and the presence and absence of a mixed inhibitor (cycloheximide, 50 mg kg<sup>-1</sup>; chloramphenicol, 25 mg kg<sup>-1</sup>). Samples were pre-incubated for 1 week after the addition of water, and prior to the introduction of treatments to minimize the initial flush of proteolytic activity caused by addition of water. Water content (19% w/w) was maintained throughout the study by the addition of sterile water as necessary. Sand cultures were incubated at 25 °C and subsamples (0.5–1.0 g) were removed for analysis at 0–5 and 7 days after treatment. Analyses included proteolytic activity, glucose concentration (where added) and extractable inorganic-N (NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>). Treatments not receiving gelatin were also analyzed for biomass (protein) on each of the above sampling dates.

## 2.3. Measurement of glucose

Subsamples of sand culture (1 g) were extracted with 10 ml of 10 mmol K<sub>2</sub>HPO<sub>4</sub> l<sup>-1</sup> (pH 7.0) buffer

for 15 min, after which particulates were removed by centrifugation at 10,000 × g for 30 min. Glucose was measured by a glucose oxidase method as described by Coody et al. (1986) for determination of glucose in soil extracts.

## 2.4. Inorganic-N measurements

At daily intervals throughout the incubation time, inorganic-N was determined by a standard microdiffusion method using commercially available three-chamber diffusion cells. The base and catalyst employed were 45% K<sub>2</sub>CO<sub>3</sub>, and Devarda's alloy, respectively and the procedure was performed as described by Keeney and Nelson (1982).

## 2.5. Proteolytic activity

Protein degradation rates were estimated using a modification of a dye release procedure adapted for use on soils (Caplan and Fahey, 1980) and activated sludges (Fahey and Caplan, 1981). The chromogenic substrate, azocoll, is readily hydrolyzed by non-specific (trypsin, collagenase, protease, and pronase) as well as specific (subtilisin) proteases (Chavira, 1984) and has been employed for the assay of proteolytic enzymes in natural samples and culture media.

Samples of sand culture equivalent to 0.5 g oven dry weight were added to 50 ml centrifuge tubes which then received 5 ml of 0.1 M phosphate buffer at optimum pH (7.5, determined previously) and 2.5 mg azocoll ml<sup>-1</sup>. This procedure provided excess azocoll to assure zero-order kinetics. Samples were agitated on a reciprocating shaker at 180 cycles min<sup>-1</sup> for 10 min and were then incubated for 2 h at optimum temperature (35 °C, determined previously). The 2 h incubation time produced linear increases in absorbance as a function of either time or sample size. Longer incubation times did not produce proportional increases in absorbance due to interference from sorption to the soil inoculum. Recovery of added azo dye (released from azocoll with pronase) from sterile sand/soil mixtures exceeded 90% for up to 2 h, but declined with increasing equilibration time. After incubation, tubes were then centrifuged for 30 min at 10,000 × g, and the azo dye released by proteolytic activity was analyzed spectrophotometrically (A<sub>520</sub>). Data were reported in absorbance units attained after 2 h of

incubation. For our sample of azocoll, an absorbance value of approximately 1.0 was equivalent to 0.75 units of pronase activity (one unit of pronase will liberate a product equivalent to 25 mg tyrosine  $\text{min}^{-1}$  at 40 °C, pH 7.5).

### 2.6. Biomass

Biomass was estimated as total protein content of sand culture digests. Sand culture samples equivalent to 1.0 g oven dry weight were digested for 10 min in 10 ml of 1 M NaOH in 25 mm  $\times$  150 mm screw-capped tubes in a boiling water bath. Aliquots (1.0 ml) were transferred to 16 mm  $\times$  100 mm screw-capped test tubes, and protein content was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard for protein determinations, therefore protein data reported herein were expressed relative to bovine albumin. Protein measurements were not converted to cell mass values owing to the possibility of variability in conversion factors among populations and within populations as a result of nutritional status. Biomass measurements were not performed on samples from treatments receiving gelatin.

## 3. Results

### 3.1. Glucose degradation

Glucose degradation was, as expected, stimulated by the inclusion of N or S in sand cultures (Fig. 1). Glucose degradation kinetics were first-order in the CNS treatment, whereas a residual glucose pool was apparent when N or S was withheld. Addition of the mixed inhibitor suppressed glucose degradation in all three treatments, resulting in linear kinetics in treatments lacking N (0.26 mmol glucose  $\text{kg}^{-1}$  sand mix per day) or S (0.46 mmol glucose  $\text{kg}^{-1}$  sand mix per day).

### 3.2. Inorganic-N

Inorganic-N was depleted rapidly (40–62 mg N  $\text{kg}^{-1}$  sand mix per day) in the presence of C, whereas little loss occurred when C was absent (<8 mg N  $\text{kg}^{-1}$  sand mix per day). These results suggest N-immobi-

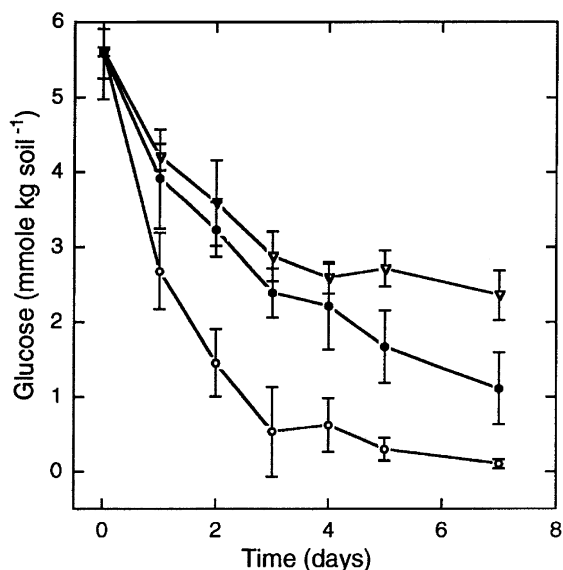


Fig. 1. Degradation of glucose in sand cultures. Symbols: open circles, CNS; closed circles, CN; open triangles, CS. Vertical bars on data symbols indicate 95% confidence intervals; where absent, bars fall within symbols.

lization resulting from added C, and demonstrate N limitation in the CS treatment.

### 3.3. Proteolytic activity

In the absence of added inhibitors or protein, proteolytic activity increased when N (and to a lesser extent S) was withheld (Fig. 2). Withholding C had no effect on proteolytic activity. The effect of gelatin was masked by variability in the protease measurements, resulting in no significant increase in proteolytic activity for the first 24 h of incubation. Addition of the mixed inhibitor significantly decreased proteolytic activity ( $P = 0.05$ ) for the first 6 days of incubation when N was withheld. During this 6-day period, proteolytic activity did not differ significantly from the CNS treatment. Proteolytic activity in other treatments was unaffected by inhibitor. Addition of gelatin had no effect on protease activity of inhibited cultures for the first 6 days of incubation (data not shown). The results of the protease measurements with and without protein synthesis inhibitors suggest that N limitation resulted in increased synthesis of proteolytic enzymes.

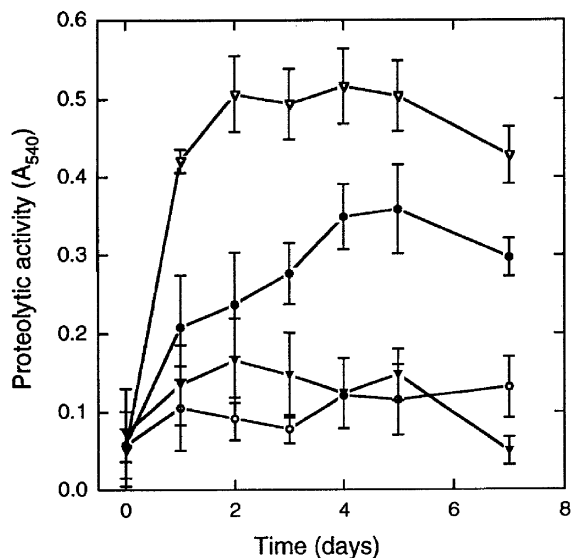


Fig. 2. Proteolytic activity in sand cultures. Symbols: open circles, CNS; closed circles, CN; open triangles, CS; closed triangles, NS. Vertical bars on data symbols indicate 95% confidence intervals; where absent, bars fall within symbols.

### 3.4. Biomass

Background protein concentration in the sand mix was  $0.43 \text{ mg protein g}^{-1}$  sand mix prior to the addition of the treatment solutions. Over the course of the study, biomass production was least when C or N was withheld. Presence of inhibitors resulted in significantly reduced biomass only in cultures receiving all three major nutrients. Protease data was clustered according to treatment when regressed with biomass data (Fig. 3). When populations grew in response to added C, an increase in biomass was detected. The range of biomass values obtained over the course of the study provided a basis for correlation to proteolytic activity. Growth was not observed in the absence of added C, thus biomass data for the NS treatment were clustered at the origin. There was no significant relationship between biomass and proteolytic activity when all three major nutrients were present ( $R^2 = 0.1733$ ), or when carbon was withheld ( $R^2 = 0.1102$ ), whereas a significant positive relationship was observed when N ( $R^2 = 0.5534$ ) or S ( $R^2 = 0.9005$ ) was withheld in the presence of carbon. Nitrogen-deprived cultures displayed the highest proteolytic activities, and the

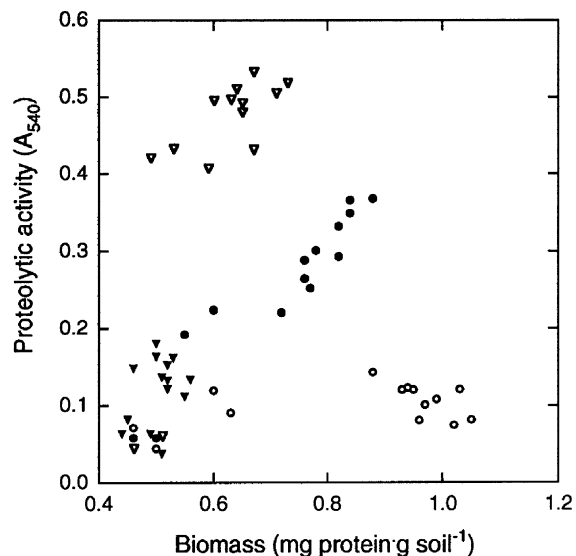


Fig. 3. Relationships between biomass (protein) and proteolytic activity among treatments. Symbols: open circles, CNS; closed circles, CN; open triangles, CS; closed triangles, NS.

steepest slope in the regression line for dye release versus biomass.

## 4. Discussion

The results suggest regulation of proteolytic activity by N (and to a lesser extent, S) status. Analogous responses of *Neurospora crassa* in culture were reported by Hanson and Marzluf (1975), although they reported a stronger induction signal from added protein. This may be explained by a higher background protein content in our experimental system. Hanson and Marzluf (1975) proposed that a common non-specific protease, encoded by a single structural gene, was activated by control signals from any one of three separate regulatory circuits (e.g. C, N, or S starvation). Similar regulation of proteolytic activity has been reported for *Candida* (Ogrydziak et al., 1977), *Mucor* (Lasure, 1980), and *Aspergillus* (Cohen, 1973). Lindberg and Drucker (1984) reported an analogous regulation of extracellular RNase activity by C, N, or P starvation. It is likely that non-specific extracellular hydrolases are involved in C, N, S, or P metabolism in other microorganisms as well.

Little attention has been given to the regulation of enzymes in soil experiments, though most enzymatic functions in microorganisms are subject to some form of regulation. The results presented herein strongly support dependence of proteolytic activity upon conditions that should favor induction (N or S starvation), and may be applicable to other hydrolytic enzymes supplying substrates for fueling reactions, such as RNase, cellulase, amylase, xylanase, etc. Analogous to results reported herein, Watanabe and Hayano (1995) observed no correlation between soil protease and total bacterial numbers during a 4-month field study. Such results cast doubt on the utility of inducible enzyme activities as indicators of population size or biomass, particularly when conditions affecting induction status may not affect biomass in the same manner.

### Acknowledgements

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval to the exclusion of other products that may also be suitable.

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