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POLYACRYLAMIDE AS A SUBSTRATE FOR MICROBIAL AMIDASE IN CULTURE AND SOIL

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Summary—High molecular weight, linear polyacrylamide (PAM) with anionic charge is added to agricultural soils as an anti-erosion additive. Research indicates that soil microorganisms are able to utilize PAM as a source of N and that inorganic N pools are altered in some PAM-treated soils. The potential role of hydrolytic amidase activity in the microbial utilization of PAM for N was investigated. Intracellular and extracellular amidase activity was measured over time in enrichment cultures which used PAM as sole N source. Enzyme activity increased concomitant with cell growth and N removal from PAM. Cell growth, N removal and amidase production were dependent upon readily-available C in the medium. Amidase activity and substrate specificity were determined for PAM-utilizing enrichment cultures exposed to various N sources. Polyacrylamide-specific amidase activity appears to be inducible, and not constitutive, based on the lack of amidase activity in cultures supplied with only ammonium nitrate for N versus substantial activity when PAM was added as an amendment with or without ammonium nitrate. Cultures amended with propionamide exhibited amidase activity largely specific for this small amide substrate, while cultures supplied with PAM as sole N source exhibited amidase activity specific for formamide, propionamide and PAM. Amidase activity and substrate specificity were determined for PAM-treated and untreated agricultural field soils. Polyacrylamide-specific amidase activity was higher in PAM-treated soil (14.86 ± 14.0 μg NH_4^+ released g^{-1} soil) than in untreated soil (1.02 ± 2.3 μg NH_4^+ released g^{-1} soil); activity specific for low molecular weight amides was slightly elevated or unchanged in PAM-treated soil as compared with untreated soil.

INTRODUCTION

High molecular weight, linear polyacrylamide (PAM) is added to agricultural soils as an anti-erosion additive in irrigation water. The type of PAM found to be most effective in reducing erosion has a very high molecular weight (1 to 2×10^7 MW) and a linear (non-crosslinked), anionic configuration. It is a copolymer made up of approximately 82 mol% acrylamide subunits and 18 mol% acrylate subunits (Lentz *et al.*, 1992). The resulting polymer consists of repeating ethylene units in the backbone, with amide and carboxylic acid substituents as side chains. The amount of N present in amide linkages in this form of PAM is approximately 164 g N kg^{-1} PAM. Given the effectiveness of the polymer at reducing erosion, which has been documented to exceed 94% (Lentz *et al.*, 1992), and the magnitude of the effects of soil erosion on agricultural fields, wide-spread application of 1 to 10 kg ha^{-1} y^{-1} is expected.

Although considerable information is available regarding the use of PAM as an anti-erosion soil additive, relatively little is known about microbial biotransformation of the polymer or the effects of PAM treatment on the microbial ecology of agricultural soils. There are reports citing increased growth response of aerobic bacteria grown in the presence of PAM (Nadler and Steinberger, 1993) and increased microbial biomass in soil treated with PAM (Steinberger *et al.*, 1993), but the causes of these effects are not known.

Experiments have demonstrated that bacterial enrichment cultures derived from agricultural soil are able to gain N benefit from PAM when it is provided as the sole N source and that amidase enzyme activity can be detected in PAM-utilizing enrichment cultures (Kay-Shoemake *et al.*, 1998). In addition, some PAM-treated agricultural field soils exhibited elevated bacterial counts and inorganic N concentrations, compared to untreated soils (Kay-Shoemake *et al.*, 1998). These observations could result from the activity of indigenous soil bacteria capable of accessing N from PAM via amidase activity.

Amidase (acrylamide amidohydrolase, EC 3.5.1.4) catalyzes hydrolysis of the C–N portion of

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the amide bond of amides, resulting in the production of ammonia and carboxylic acid (Fig. 1). This group of enzymes has been demonstrated in animals, plants, fungi, and bacteria. Extracellular amidase active in the soil matrix has been described in detail by Frankenberger and Tabatabai (1980a,b, 1981a,b, 1985). Soil enzyme activity, in general, is considered to originate from secreted extracellular enzyme or enzyme released upon cell lysis and immobilized in active form on soil particles (Skujins, 1976).

Hydrolytic amidase activity specific for small amides has been demonstrated in a variety of soils (Frankenberger and Tabatabai, 1980a), as well as in many common soil bacteria and fungi (Kelly and Clarke, 1962; Thalenfeld and Grosswicz, 1976; Friedrich and Mitrenga, 1981; Nawaz *et al.*, 1994). The mechanism by which soil bacteria degrade acrylamide, one of the monomeric constituents of PAM, also involves an amidase enzyme (Shanker *et al.*, 1990). In addition, Abdelmagid and Tabatabai (1982) reported that acrylamide-treated soils exhibited elevated amounts of inorganic N due to release of N from acrylamide. A similar increase in inorganic N pools has been reported for some PAM-treated agricultural soils (Kay-Shoemake *et al.*, 1998). Amidase activity has also been implicated in the degradation of amide-containing pesticides in culture and soil (Bollag and Liu, 1990). Grula *et al.* (1994) reported that certain *Pseudomonas* sp. were able to utilize a type of PAM as a N source and that transient amidase activity was associated with growth of those cultures. However, the PAM utilized in these experiments was not the type used as an anti-erosion additive (lower MW and different charge configuration). These observations led to our investigation of the potential role of amidase in the microbial biotransformation of PAM.

Identifying and characterizing the mechanism by which soil bacteria can access N from PAM is a critical step toward understanding the potential environmental effects of repeated PAM application to agricultural soil systems. Our specific objectives were to (1) assess amidase production by PAM-utilizing cultures and determine if amidase production correlates with cell growth and PAM degradation, (2) characterize enzyme activity and substrate specificity for intracellular and extracellular amidase produced in PAM enrichment cultures, and (3) determine whether PAM application to field soils alters soil amidase activity or the substrate specificity. We are unaware of previous comprehensive examinations of the microbial biotransformation of this agrochemical. The approach we describe provides substantial evidence regarding the microbiological fate of PAM in soil, even in the absence of usable techniques for intact PAM extraction from soil.

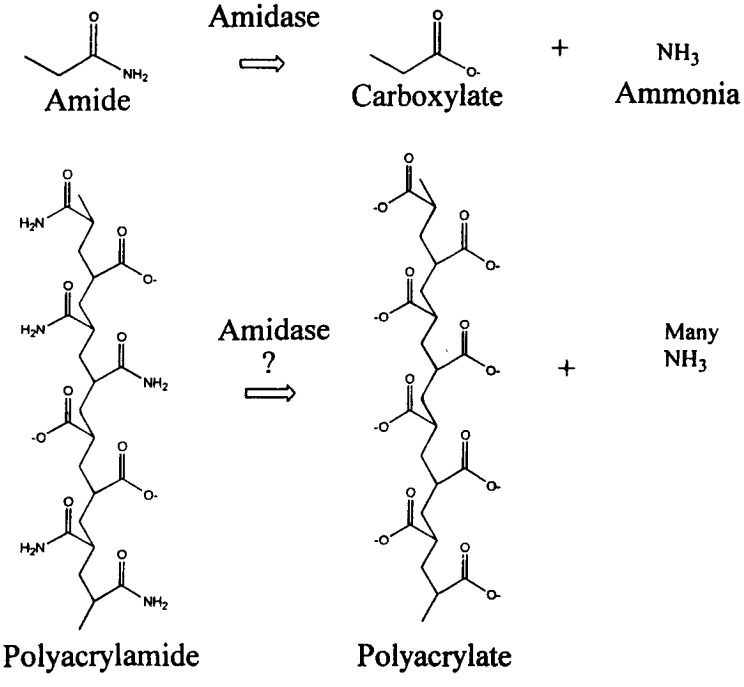

Fig. 1. Mechanism of amidase catalyzed hydrolysis of a small amide and potential hydrolysis of PAM

MATERIALS AND METHODS

Site description and soil sampling

The PAM study site consists of a 2 ha area divided into discrete experimental plots. The soil is Portneuf silt loam (coarse-silty, mixed, mesic, Durixerollic Calciorthid), with a pH of approximately 7.8, and a 2 to 8% $CaCO_3$ equivalent in the A horizon. Two PAM experimental fields, identified as "assimilation" and "long-term", had been established at the time of our field sampling. Furrows in each plot were assigned treatments in random order with three replicate furrows for each treatment.

Furrows in the "assimilation" study plot were 91 cm apart and 114 m long, and treatment consisted of a one time application of PAM of 1120 kg ha^{-1} (1000 lb $acre^{-1}$) at the beginning of the growing season, followed by PAM application (10 mg l^{-1}; 22.6 l min^{-1}) during each irrigation throughout the growing season; there were paired non-treated control furrows. This field was planted to mixed cover including potatoes, sugar beets, corn, and beans and will be referred to hereafter as the mixed-cover field.

The "long-term" study plot has been previously described (Kay-Shoemake *et al.*, 1998) and will be referred to hereafter as the bean field, based on its planted cover. During 1995–1996 we collected soils from these fields to use in laboratory assays.

Soil (approximately 100 g) was collected from the upper 3 cm of each sampled furrow bottom, at a distance of 0.5 m, 1.0 m and 1.5 m from the irrigation inlet. Samples from each furrow were combined, sieved (4 mm), and stored for no more than 1 week at 4°C. Three composite samples were obtained for each treatment and crop cover. Field soil samples were analyzed directly and also served as inoculum for enrichment cultures.

Chemicals

The polyacrylamide (PAM) preparation used in enrichment cultures, E-4103, was provided by Cytec Industries, Stamford CT. The polymer has a molecular weight of $1–2 \times 10^7$, with 18 mol% anionic charge, and is free of contaminant N. All other chemicals used were of at least reagent grade quality.

Enrichment cultures

Soil samples (1.0 g) from PAM-treated furrows were inoculated into 25 ml of a mineral salts medium (Kay-Shoemake *et al.*, 1998). Polyacrylamide was provided as the sole source of N to give a final concentration of 0.05% in the medium. The medium was supplemented to contain 0.05% acetate and 0.05% mannitol for carbon, yielding a C-to-N ratio of 6:1.

Enrichment cultures were generated as described by Kay-Shoemake *et al.* (1998). Enrichment cultures able to utilize PAM as a sole source of N were maintained on mineral salts medium supplemented with 0.05% mannitol, 0.05% acetate and 0.05% PAM.

Culture amidase determination

Amidase activity was measured in a 2-month old enrichment culture able to use PAM for N. Subcultures were grown in 25 ml of mineral salts medium supplemented with either NH_4NO_3 (0.024%), propionamide (0.04%) or PAM (0.05%) as N source and 0.05% mannitol +0.05% acetate for C. These concentrations yielded a final C-to-N ratio of approximately 6:1. Cultures were inoculated, incubated and analyzed for amidase activity as described by Kay-Shoemake *et al.* (1998), except culture supernatants and cell free extracts were assayed separately. Protein determinations for the enzyme preparations were conducted using the method described by Lowry *et al.* (1951). All assays were done in triplicate.

Amidase production and activity during culture growth

Inoculum for amidase production experiments was obtained by centrifuging 10 ml of a 24 h PAM-utilizing enrichment culture at 8000 g for 10 min at 4°C, discarding the supernatant and washing the cells once in 10 ml of 75 mM phosphate buffer to remove any residual extracellular amidase. The enrichment culture used had been growing for approximately 4 months. The washed cells were resuspended in buffer (1 ml) and added to 100 ml of fresh, supplemented mineral salts medium identical to that on which the enrichment had been established. The culture was incubated as described above, and culture growth was monitored every 24 h by absorbance at 520 nm using a Bacharach Coleman model 35 spectrophotometer. After 72 h, when C apparently became limiting, 0.5 ml of 10% mannitol solution +0.5 ml 10% acetate solution was added to the culture.

Periodically, aliquots (15 ml) were removed from the culture and centrifuged to separate cells from supernatant. Protein content and intracellular and extracellular amidase activity were determined as described above using PAM as amide substrate. In addition, separate supernatant samples were analyzed periodically to estimate N removal from PAM and the amount of free NH_4^+–N contained in the medium. At each sampling time an aliquot (3 ml) of cell-free supernatant was acidified to pH 5.5, placed on ice, and analyzed for free NH_4^+–N via ion chromatography to determine the amount of free NH_4^+–N in the supernatant. To determine the amount of N remaining on the PAM in the medium, a supernatant subsample (1.5 ml) was hydrolyzed by adding 0.5 ml 12 N HCl and incubating at 100°C for 30 min (Challis and Challis, 1970). The

preparation was cooled to room temperature, neutralized with 6 N NaOH, and filtered (0.45 μm). Ammonium–N was determined using Nessler's reagent and absorbance at 440 nm. The concentration of NH_4^+–N measured after hydrolysis was used to calculate the N remaining within the PAM polymer by subtracting the free NH_4^+–N quantified in the medium prior to hydrolysis. The PAM–N concentrations measured in the supernatant periodically throughout the experiment were subtracted from the value measured at zero time to give estimates of cumulative N removal from PAM. Uninoculated culture medium, incubated and analyzed for NH_4^+–N and PAM–N as described above, provided an abiotic control.

Soil amidase assay

Soil amidase activity in PAM-treated and untreated soils was determined using a modification of the method described by Frankenberger and Tabatabai (1980a). Soil samples (5.0 g) were treated with 0.2 ml toluene and incubated with amide substrate (formamide at 50 mM or PAM at 0.05%) in Tris (hydroxymethyl) aminomethane (THAM) buffer (100 mM, pH 8.5). Incubations were conducted for 2 h when using formamide as the substrate and for 24 h when testing for PAM-specific activity. Following incubation, 50 mM KCl in deionized H_2O was added to yield a total soil slurry volume of 50 ml. The slurries were agitated on a rotary shaker for 30 min and allowed to settle for an additional 15 min. Supernatant was collected, filtered through 0.45 μm filter and placed on ice. The extracts were diluted in deionized water and analyzed via ion chromatography for NH_4^+–N released due to amidase activity. Control preparations consisted of soil + buffer were incubated as described above; each test amide was added following incubation and immediately prior to KCl addition to estimate the amount of NH_4^+–N present in the test preparation independent of amidase action.

Ion chromatography

Ammonium was quantified using a Dionex 100 ion chromatograph, as described by Kay-Shoemake *et al.* (1998). The detection limit of this method is 10 μg l^{-1}.

RESULTS

Amidase production and N removal during enrichment culture growth

Enrichment cultures, which utilized PAM as the sole N source, produced intracellular and extracellular amidase during batch incubation (Fig. 2). Production of intracellular and extracellular amidase specific for PAM (Fig. 2a) increased with culture density initially, then growth and amidase production levels plateaued. PAM-specific amidase

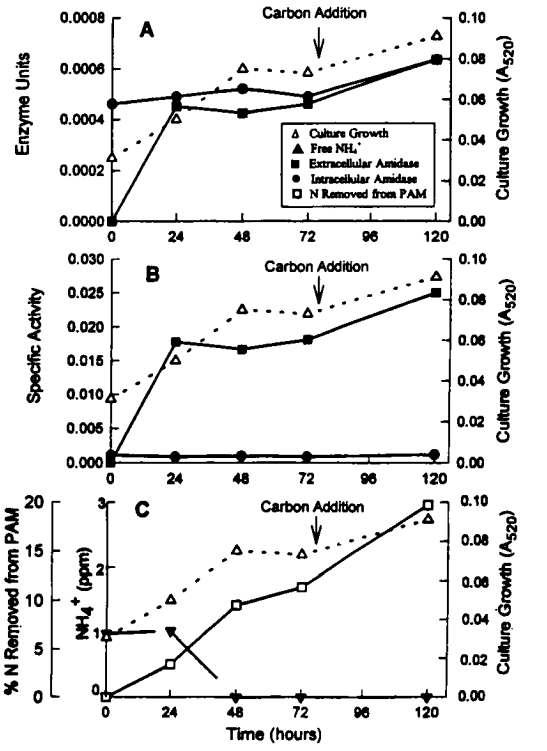

Fig. 2. Culture growth, amidase production, and PAM degradation over time for a soil-derived enrichment culture. (a) Intracellular (•) and extracellular (■) amidase in enzyme units during culture growth (Δ). (b) Intracellular (•) and extracellular (■) amidase specific activity during culture growth (Δ). (c) Removal of N from PAM (□) and residual, free NH_4^+–N (▼) during culture growth (Δ)

production and cell growth increased at 72 h following the addition of 3.42 mmol of C in the form of mannitol + acetate to the culture.

Specific activities determined over time for intracellular and extracellular forms of PAM-specific amidase exhibited distinct patterns (Fig. 2b). Specific activity of the intracellular amidase fraction was low and relatively constant, while the extracellular enzyme specific activity varied over time. The extracellular enzyme activity increased during the period of logarithmic cell growth, followed by a plateau period prior to the culture reaching stationary phase. Intracellular amidase specific activity remained relatively constant after C addition, whereas the activity of the extracellular fraction increased.

Cumulative removal of N from PAM, determined for the same batch culture (Fig. 2c), increased concomitant to cell growth, levelled off somewhat during stationary phase, and increased again after the addition of supplemental C. To assess the possibility that cells were surviving on contaminant NH_4^+, this compound was quantified in free form in the medium throughout the experiment; approximately 1.0 mg NH_4^+–N l^{-1} was detected initially as

a contaminant in the culture medium. Concentrations of free NH_4^+-N declined to non-detectable within approximately 48 h and showed no subsequent increase during the duration of the experiment. The batch culture experiment was repeated in its entirety (data not shown), and strikingly similar results were obtained.

Amidase activity and substrate specificity

No intracellular or extracellular amidase activity was detected in enrichment cultures provided with NH_4NO_3, an easily assimilated N source (Table 1). Enrichments provided with propionamide as an N source produced both intracellular and extracellular amidase forms. The intracellular amidase exhibited marginal activity specific for PAM. No extracellular amidase specific for PAM was detected.

Enrichment cultures grown with PAM for N exhibited both intracellular and extracellular amidase activity capable of hydrolyzing all amide substrates tested. Other than marginal activity detected in the propionamide-amended culture, only the PAM-supplied enrichment exhibited substantial amidase activity specific for PAM. When the culture was grown on PAM, extracellular activity specific for the smaller amides was an order of magnitude greater than the intracellular activity. For PAM-specific amidase activity, however, similar amounts were detected in these two fractions.

Although no intracellular or extracellular amidase activity was detected in enrichment cultures provided with NH_4NO_3, enrichment cultures grown with PAM and NH_4NO_3 produced the PAM-specific amidase (data not shown).

Soil amidase activity

Amidase activity assays of field soils (Table 2) detected PAM-specific amidase activity in both PAM-treated and untreated soils. PAM-specific amidase activity of the PAM-treated furrow soils was 4 to 15 times higher than that of untreated furrow soils. Similarly, formamide-specific amidase activity in bean field soil was higher in PAM-treated soils than in untreated soils. In the mixed-cover field however, PAM-treated and untreated soil amidase activities specific for formamide were no different ($P = 0.05$). Comparisons between the different substrates for a given soil were not possible due to differences in the duration of the assay incubation.

Table 2. Soil amidase activity toward various amide substrates in PAM-treated and untreated soil samples.

	NH_4^+-N released ($\mu g\ g^{-1}$ soil dry weight)	
Soil treatment	Formamide	PAM
Mixed cover plot		
Untreated	218.0 ± 7.6	36.7 ± 11.1
PAM	161.3 ± 15.1	146.6 ± 24.8*
Bean plot		
Untreated	89.3 ± 10.1	10.2 ± 2.3
PAM	135.7 ± 6.1*	148.6 ± 14.0*
Mixed cover plot (7 months post harvest)		
Untreated	ND	6.47 ± 1.26
PAM	ND	28.87 ± 1.95*

*Significantly different from untreated soil ($P = 0.05$) as determined by the student's t-test; ND = Not determined

Soils collected from the mixed-cover field 7 months after harvest and tilling still exhibited significantly higher amounts of PAM-specific amidase activity than did soils collected at the same time from untreated areas.

DISCUSSION

We successfully established enrichment cultures that utilized PAM as sole N source. Under these growth conditions, the cultures produced intracellular and extracellular amidase which exhibited activity specific for PAM. Nitrogen was removed from the PAM polymer concomitant to amidase production, even in the absence of trace inorganic N. Abiotic controls indicated that NH_4^+ was not spontaneously released from PAM under these incubation conditions. Amidase production, N removal, and growth under these conditions appeared to be C limited in these cultures. This was not unexpected since we had reported that soil enrichment cultures similar to these were unable to

Table 1. Intracellular and extracellular amidase activity of PAM utilizing bacterial enrichment provided with different N sources

	Amidase specific activity for given amide (units mg^{-1} protein)		
N source/amide in growth medium	Formamide	Propionamide	PAM
Intracellular			
NH_4NO_3	0	0	0
Propionamide	0	0.479 ± 0.007	0.005 ± 0.001
PAM	0.075 ± 0.024	0.099 ± 0.007	0.268 ± 0.034
Extracellular			
NH_4NO_3	0	0	0
Propionamide	0	0.332 ± 0.024	0
PAM	0.744 ± 0.224	0.720 ± 0.061	0.244± 0.022

utilize PAM for C (Kay-Shoemake *et al.*, 1998). Polyacrylamide-specific amidase activity was also detected in field soils, and soils with a history of PAM treatment exhibited significantly higher activity. This is the first report of an amidase, in culture or in soil, capable of attacking a polymer in this size range ($1–2 \times 10^7$ MW).

Amidase activity has been identified in numerous genera of bacteria, including *Rhodococcus* (Nawaz *et al.*, 1994), *Bacillus* (Thalenfeld and Grosswicz, 1976), *Mycobacterium* (Draper, 1967), *Brevibacterium* (Maestracci *et al.*, 1984), *Alcaligenes* (Friedrich and Mitrenga, 1981), and *Pseudomonas* (Kelly and Clarke, 1962; Clarke, 1970; Ciskanik *et al.*, 1995). In addition, amidase activity has been demonstrated in several fungi, including *Aspergillus* (Hynes and Pateman, 1970) and *Fusarium* (Reichel *et al.*, 1991). Some of these organisms exhibit amidase activity with relatively limited substrate specificity, while others appear to produce amidase enzyme able to hydrolyze a comparatively broad range of substrates. Furthermore, some of these genera, when grown with a single amide substrate, acetamide, have been found to produce a single amidase, while others appear to produce numerous isoenzymes (Clarke, 1980). Many of these genera are common in soils, and because the enrichment cultures we used consisted of a bacterial consortium derived from soil, it is possible that some of the amidases reported in the literature were represented here.

Our data suggest that cells grown on PAM rapidly produce a intracellular and extracellular PAM-specific amidase that utilizes the polymer amide N; that the extracellular fraction is likely responsible for N extraction; and that production and secretion of the enzyme depends on C availability. The following support this concept: (1) it is unlikely that the huge PAM molecule is transported into the cell; (2) cells produced intracellular and released extracellular PAM-specific amidase enzyme rapidly upon transfer into fresh medium; (3) while intracellular activity remained fairly constant during all phases of batch culture growth, amounts of extracellular activity varied according to culture age and growth, increasing during exponential growth (presumably prior to exhaustion of available C in the medium); and 4) extracellular activity, together with cell growth and N removal from PAM, was stimulated by further addition of available C.

It appears that cells enriched for growth on PAM for N maintain a low amount of intracellular amidase and can alter production and secretion of the enzyme, depending on C availability. Microbial uptake of NH_4^+–N released from PAM is quite rapid. After initial depletion of contaminant NH_4^+–N in the culture medium, NH_4^+–N remained undetectable for the duration of the batch experiment.

Microbial amidase production is generally induced by the presence of an amide, although in some systems certain amides inhibit enzyme production (Clarke, 1980). In *Pseudomonas aeruginosa* the induction of amidase production is under positive control by a regulatory protein (Clarke, 1980). An additional control mechanism identified in some species is that of catabolite repression in the presence of glucose and succinate (Clarke, 1980). Constitutive production of amidase was reported for a *Rhodococcus* sp. (Nawaz *et al.*, 1994), *Bacillus subtilis* and *Thermophilus aquaticus* (Clarke, 1980).

Polyacrylamide-specific amidase activity was not observed when cultures were supplied with an easily assimilated N source, NH_4NO_3, in the absence of PAM. The trace amounts of PAM-specific activity detected in propionamide–N supplied cultures may have been residual from the original PAM-amended enrichment. Substantial amounts of PAM-specific enzyme activity were detected in the propionamide–N and NH_4NO_3–N supplied cultures only when PAM was also supplied. This indicates that the enzyme is not constitutively produced and that synthesis is controlled by induction in this microbial culture.

Significantly higher amounts of PAM-specific amidase activity in PAM-treated soils versus untreated soils also indicate that the enzyme is inducible in the soil environment. However, the cellular mechanism for induction remains unclear; the intact polymer substrate is probably too large (approximately 0.2 μm) to enter the cell and act as a direct inducer. It may be an unidentified metabolite that induces enzyme production. Furthermore, the measurable, albeit low, amounts of PAM-specific activity observed in soils with no PAM exposure history raise questions regarding constitutive production of the enzyme under field conditions. It is possible that certain soil organisms capable of producing the enzyme constitutively may not have been represented or functional in the laboratory enrichments. It is also possible that this amidase, like many inducible enzymes, was produced in low amounts even in the absence of inducer, and gene expression was stimulated in the presence of the appropriate inducer (Lewin, 1994).

An issue related to the enzyme control mechanism is the question of whether the activity measured in our study resulted from a single PAM-specific amidase, a generic high MW amide inclusive amidase, or a suite of amidase isozymes. When we provided PAM as an N-source, both PAM-specific activity and amidase activity specific for low MW amides was exhibited, in both enrichment cultures and soils. Amidases that demonstrate broad substrate specificities have been reported (Draper, 1967; Maestracci *et al.*, 1984), although the enzyme preparations used were either crude or only partially purified, consequently could represent a suite

of amidase enzymes. There is also a report of multiple amidase isozymes, each with a limited substrate specificity, produced simultaneously by *Aspergillus nidulans* when induced by a single small amide (Hynes and Pateman, 1970). It is not clear from our data whether the observed amidase activity on multiple amides was due to the presence of multiple amidase isoenzymes or a single amidase with broad specificity.

It is evident from the soil amidase assays that elevated PAM-specific amidase activity in response to PAM treatment is not a transient phenomenon. Exposure of soil microbial communities to PAM resulted in the production, release and long-term retention of PAM-specific amidase activity.

The effects of long-term PAM application to agricultural soils are not well understood. The N added to agricultural soils via long-term PAM treatment does not appear to be substantial when compared to the amount of N added via fertilization or N_2 fixation in an agricultural setting. The amount of N contained in the PAM added to the soils when applied according to the "best standard practice" of 10 mg l^{-1} in the initial irrigation front (0.2–1.7 kg N ha^{-1} y^{-1}), is a fraction of the N added to the soil via fertilization per season (in the fields examined here, 30 kg N ha^{-1} y^{-1}). The relatively small amount of N added to agricultural soil via PAM-treatment may result in slight shifts in microbial communities and inorganic N concentrations, as reported by Kay-Shoemake *et al.* (1998). However, the overall effect of PAM application appears to be small in agricultural soils, especially when considering the wide range of chemical additives commonly applied to such soils. The relatively low effect observed is particularly remarkable when considering that mixed-cover plot received a far higher dose (1100 kg ha^{-1}) than is typically applied (1–10 kg ha^{-1} y^{-1}).

The effects that elevated soil amidase activity might have on other agricultural additives, particularly amide containing fertilizers and pesticides, is being investigated. Additional research is needed to clarify issues related to substrate specificity, number of isozymes produced and regulation mechanisms of PAM-specific amidase activity.

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