*Journal of Soil Science*, 1991, **42**, 17–23

# Influence of natural and synthetic humic substances on the activity of urease

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## SUMMARY

The activity of a purified urease, obtained from *Bacillus pasteurii*, was inhibited by humic and fulvic acids obtained from an agricultural soil. Enzyme kinetic studies showed that the humic substances affected the affinity of the enzyme for its substrate ($K_m$) and the maximum velocity of the reaction ($V_{max}$). The $V_{max}$ was inhibited to the same extent by both humic (HA) and fulvic (FA) acids, the precise effect depending on the pH and concentration of humic substance. At pH 4.0, HA concentrations of 25 μg cm$^{-3}$ and 10 μg cm$^{-3}$ inhibited the $V_{max}$ by 38.5% and 20% respectively. HA and FA had similar effects on the $K_m$, but in this case the lowering of the affinity of the enzyme for its substrate was not concentration dependent in the range 0–25 μg cm$^{-3}$ of humic substance. Typically, the affinity was decreased from a $K_m$ of 50 mM in the control to 67 mM in the presence of HA and FA.

The effects were not due primarily to the ash or N contents of the humic substances because de-ashed humic acid and synthetic model humic (made from catechol, guaiacol, pyrogallol, resorcinol and protocatechuic acid) and fulvic acid (made from polymaleic acid), containing virtually no ash or N, were equally as effective. The effect was not related to the phenolic monomers which, before polymerization, had no effect on urease activity.

## INTRODUCTION

Urease is unique among soil enzymes because it affects the fate and performance of an important fertilizer, urea (Bremner & Mulvaney, 1978). It is understandable, therefore, that a considerable amount of work has been directed towards measuring urease activity in soils (Bremner & Mulvaney, 1978; Ladd, 1985). There is also substantial information available on interactions between urease and soil mineral components (Theng, 1979). Such interactions are important in that they can confer substantial stability on urease by decreasing the activities of degrading proteolytic enzymes, or by forming a barrier between the enzymes (Burns *et al.* 1972b; Bremner & Mulvaney, 1978).

Interactions between soil organic matter and urease have also been thoroughly investigated. Urease has been extracted from the soil as an enzyme–humus conjugate (Burns *et al.*, 1972a,b; McLaren *et al.*, 1975) and this conjugate represents 20–40% of total activity, but not necessarily total amount of enzyme, in the soil. Similar yields from the soil were reported by Nannipieri *et al.* (1978). The enzyme–humus complexes were fractionated and during this procedure the enzyme activity increased, probably owing to a progressive separation of the enzyme from its humus inhibitor (Ceccanti *et al.*, 1978). There are many reports (see Vaughan *et al.*, 1985) that show that humic substances inhibit a range of enzymes such as invertase, phosphatase, peroxidase, indole-3-acetic acid oxidase, choline esterase and some proteolytic enzymes.

Despite this, there is a lack of information available on the direct effects of humic substances on the kinetics of urease activity *per se*. This paper reports the influence of humic and fulvic acids on the activities of urease prepared from *Bacillus pasteurii*. The choice of the urease source was based

on convenience, since the precise contribution that microorganisms and/or plants make towards exocellular ureases in soil is still unknown.

## MATERIALS AND METHODS

*Preparation of humic substances*

Humic (HA) and fulvic (FA) acids were prepared from a well-drained soil of the Countesswells series derived from granitic gneiss parent material (Glentworth & Muir, 1963). This soil had been under grass for 20 years and contained 9.4% (w/w) organic matter. Freshly collected soil (20.6% w/w moisture content) was passed through a sieve (2 mm mesh) and extracted (100 g) with 1 $dm^3$ 0.5 M NaOH for 18 h at 20°C. The alkaline extract was decanted from the residue, centrifuged at 2500 ***g*** for 30 min and the resulting supernatant solution was acidified to pH 1.0 with 6 M HCl. The HA precipitate was washed three times with 0.05 M HCl (100 $cm^3$ per wash) and then once with 500 $cm^3$ water, centrifuging at 2500 ***g*** for 20 min between each step, then frozen at −16°C for 48 h. After thawing, the HA granules were filtered and dried at 50°C yielding 1.8 g HA $(100\ g)^{-1}$ dry soil.

The original acidified supernatant solution was evaporated to dryness at 35°C under reduced pressure, freed from NaCl by extraction into 100 $cm^3$ ethanol, filtered using Whatman No. 540 filter paper and evaporated to dryness at 35°C under reduced pressure. This process was repeated three times. Finally, the resulting FA was dissolved in 50 $cm^3$ water and freeze-dried yielding 0.5 g FA $(100\ g)^{-1}$ dry soil.

*De-ashing humic acid*

To de-ash HA, 500 mg of solid (prepared as described above) were treated for 48 h with 150 $cm^3$ 10% (w/v) HF and centrifuged at 2500 ***g*** for 10 min. The debris was washed three times with 150 $cm^3$ water. All the debris (483 mg) was dissolved in 20 $cm^3$ 0.5 M NaOH and the solution shaken vigorously for 2 h with 5 $cm^3$ acetyl acetone. After phase-separation, any remaining acetyl acetone in the aqueous phase was removed by three washings with di-ethyl ether, 10 $cm^3$ each wash. Humic acid was precipitated by acidifying to pH 1.0 with HCl and washed three times with 0.05 M HCl, 150 $cm^3$ each wash, to remove all the ether. The HA was dissolved in 0.5 M NaOH, the pH was adjusted to 6.0 with 0.1 M HCl and the final volume made up to 50 $cm^3$ with water. The solution was passed twice through a chelating resin column (Serva chelating Resin Al, 500-100 mesh, dimensions 2 × 25 cm, $Na^+$ form) to remove trace elements. The HA was precipitated by acidification with 1 M HCl, washed four times in 0.05 M HCl, 150 $cm^3$ each wash, and dried at 50°C, yielding 462 mg de-ashed HA.

The ash contents of humic substances were measured after ashing at 450°C for 18 h.

*Preparation of model humic substances*

Model humic acids were prepared by oxidizing 200 $cm^3$ solutions containing 5 g phenolic substance (catechol, guaiacol, protocatechuic acid, pyrogallol or resorcinol) with an equal volume of 5% (w/v) sodium iodate in 0.1 M NaOH for 7 d at 20°C after initially heating the whole solution at 80°C for 2 h to ensure a substantial yield of final product. The model HAs were precipitated, washed and dried as described above for de-ashed HA. All phenolic substances were obtained from BDH Ltd., Poole, Dorset, UK.

A model FA (polymaleic acid) was obtained through the polymerization of maleic anhydride in pyridine (Anderson & Russell, 1976).

The percentage ash contents and elemental analyses of the fulvic, humic and model humic substances are given in Table 1.

*Preparation of solutions for enzyme assays*

Solutions of humic substances, and synthetic models, were prepared by dissolving in NaOH (100 mg sample in 1 $cm^3$ 1.0 M NaOH), adjusting to the appropriate pH with 0.1 M HCl and diluting to a final volume of 100 $cm^3$ with water to give stock solutions containing 1000 mg $dm^{-3}$ humic substance. These solutions were diluted when necessary with 10 mM NaCl to maintain a constant concentration of the salt. In some cases, FA and polymaleic acid were dissolved directly in water.

**Table 1.** Chemical analyses of natural and synthetic humic substances

| Substance | Ash (%) | C | H (mg g$^{-1}$) | N | C:N ratio |
|---|---|---|---|---|---|
| Humic acid (HA) | 2.92 | 50.9 | 4.9 | 3.3 | 15.4 |
| De-ashed HA | 0.12 | 51.3 | 5.1 | 3.4 | 15.1 |
| Fulvic acid (FA) | 6.81 | 42.2 | 4.7 | 3.9 | 10.8 |
| Pyrogallol HA | 0.11 | 47.3 | 5.1 | 0.1 | 473 |
| Resorcinol HA | 0.09 | 52.3 | 5.2 | 0.1 | 523 |
| Protocatechuic HA | 0.08 | 52.3 | 5.4 | 0.1 | 523 |
| Polymaleic acid | 0.12 | 45.4 | 3.6 | 1.2 | 37.8 |

**Table 2.** Effect of different concentrations of humic substances on urease activity in universal buffer at pH 4.0

| Humic conc. (μg cm$^{-3}$) | Inhibition (%) of urease activity | | |
|---|---|---|---|
| | HA | De-ashed HA | FA |
| 100 | 81±4.3 | 80±45.5 | 83±5.2 |
| 50 | 78±4.2 | 80±4.2 | 81±4.9 |
| 25 | 68±3.5 | 56±3.8 | 50±3.8 |
| 10 | 35±2.3 | 32±3.1 | 32±3.4 |
| 1 | 11±2.1 | 9±1.6 | 8±2.1 |
| 0.1 | 2±1.2 | 2±1.3 | 0±2.4 |

Values are means of three independent determinations ± 2 SD.

**Table 3.** Effect of different concentrations of model humic substances on urease activity assayed in universal buffer at pH 4.0

| Model humic substance | Inhibition (%) of urease activity at | | |
|---|---|---|---|
| | 100 μg cm$^{-3}$ | 10 μg cm$^{-3}$ | 1 μg cm$^{-3}$ |
| Protocatechuic acid* HA | 69±4.2 | 43±3.2 | 3±1.1 |
| Pyrogallol* HA | 78±4.2 | 37±3.3 | 4±1.1 |
| Guaiacol* HA | 74±4.4 | 38±3.3 | 7±1.7 |
| Resorcinol* HA | 81±4.7 | 41±3.7 | 8±1.5 |
| Catechol HA | 77±4.5 | 46±3.7 | 7±1.4 |
| Polymaleic acid FA | 74±5.1 | 35±3.0 | 3±1.4 |

*These phenolic starting materials had no effect on urease activity.
Values are means of three independent determinations ± 2 SD.

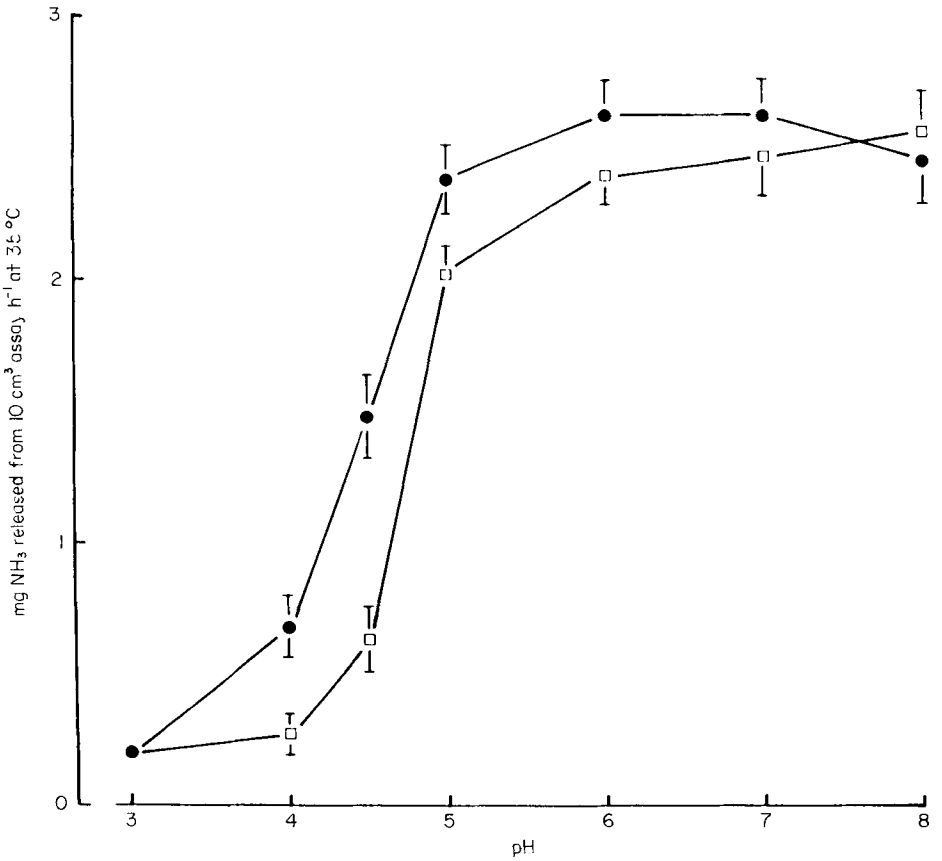


**Fig. 1.** Effect of HA (25 μg $cm^{-3}$), □, on *Bacillus pasteurii* urease activity (control values, ●) at different pH values using a universal buffer.

Aqueous stock solutions of CuII ions contained 10 μg cation $cm^{-3}$ as $CuSO_4$ or $CuCl_2$. Urease (EC 3.5.1.5) Type X was obtained from *Bacillus pasteurii* and contained 200 μmolar units $mg^{-1}$ (Sigma Chemical Co.). The solid was dissolved in water to give a stock solution of 250 μg $cm^{-3}$.

*Urease assays*

Urease activities were determined by adding 5 $cm^3$ 2 M urea to a solution comprising 1 $cm^3$ humic substance or 10 mM NaCl, 1 $cm^3$ urease, 1 $cm^3$ Cu II solution or water, and 2.0 $cm^3$ 0.1 M universal buffer. After a 20 min incubation at 35°C, the 10 $cm^3$ assay solution was added to 40 $cm^3$ water, followed immediately by 1 $cm^3$ 5 M NaOH. The ammonia released into the constantly stirred solution was determined using an ammonia-selective electrode (Orion Research Ltd., UK) to measure the change in potential (−mV) with a Beckmann model 3550 digital pH meter. Solutions of $NH_4$ Cl, containing 0.001, 0.01, 0.1, 1.0 and 10 mM $NH_3$, were used as standards and calculations were carried out using semi-logarithmic graph paper.

Except for the chemical analyses in Table 1 (one experiment in duplicate, because of the scarcity of the materials), the results are the mean values of three separate experiments (with duplicate samples in each experiment) ± SD of the means.

## RESULTS AND DISCUSSION

At pH 4.0, humic substances inhibited the activity of urease prepared from *B. pasteurii*. The inhibition increased with increasing concentrations of humic substance (Table 2). HA and FA were

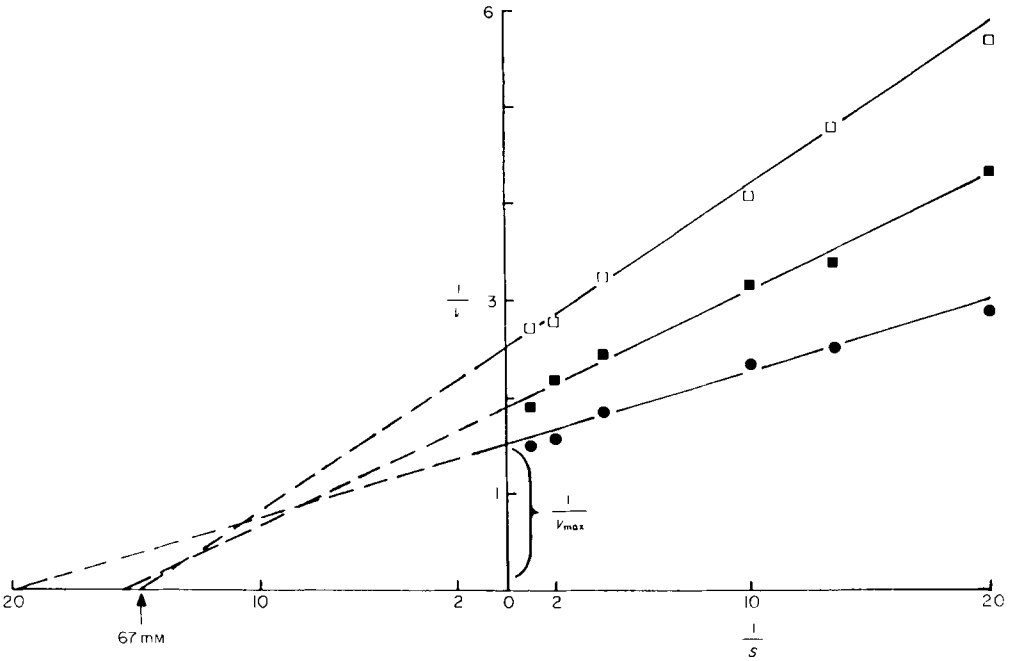


**Fig. 2.** Effect of HA (25 μg cm$^{-3}$), □, and FA (10 μg cm$^{-3}$), ■, on enzyme kinetics of urease at pH 4.0, expressed as a Lineweaver–Burk double reciprocal plot. $1/S$ values are calculated as reciprocals of urea concentrations, from 1.0 M to 0.05 M giving values of 1 to 20 respectively. $1/V$ values are calculated as reciprocals of the rate of reaction at pH 4.0 expressed as mg $NH_3$ liberated per assay h$^{-1}$.

generally equally as effective as inhibitors throughout the concentration range used. The order of addition of enzyme, humic substance and substrate had little effect on the inhibition. This contrasts with clay–enzyme–substrate systems where the order of addition can markedly influence enzyme activity (Theng, 1979). The solutions of humic substances also contained 10 mM NaCl, but at this concentration the salt had no effect on urease activity.

Urease inhibition was not due to the inorganic components of the humic material because HA, treated to lower its ash content from 2.92% (w/w) to 0.12% (w/w) was as effective an inhibitor as the HA before treatment (Table 2). Furthermore, the model humic substances, which contained virtually no ash or nitrogenous substances, inhibited urease activity to the same extent as HA and FA (Table 3).

Most of the original phenolic starting materials (pyrogallol, resorcinol, guaiacol and protocatechuic acid) had no effect on urease activity. Catechol, however, at a concentration of 10 μg cm$^{-3}$ inhibited enzyme activity by 40% and this inhibition increased with increasing concentration of catechol giving an 84% inhibition at 100 μg cm$^{-3}$. Similar results have been reported for the influence of phenolic substances on soil ureases (Bremner & Douglas, 1971; Mulvaney & Bremner, 1978). These workers showed that catechol, hydroquinone and *p*-benzoquinone were effective inhibitiors, whereas pyrogallol, resorcinol, phoroglucinol, protocatechuic acid and gallic acid were without effect.

The inhibition of purified urease by humic substances suggests that under natural conditions, soil ureases may also be inhibited by these substances. The observations by Ceccanti *et al.* (1978), that during the purification of an extracted soil urease the removal of humic substances from the enzyme–humic complex leads to an enhanced enzyme activity, adds weight to this interpretation. The percentage inhibition of urease activity produced by humic substances is pH dependent (Fig. 1).

**Table 4.** Influence of humic and fulvic acids on the effect of cupric ions on urease activities at pH 7.1, expressed as positive (+) or negative (−) percentage effects

| Copper concentration ($\mu g\ cm^{-3}$) | Control | Urease activity (percentage effect) | | | |
|---|---|---|---|---|---|
| | | HA conc. ($\mu g\ cm^{-3}$) | | FA conc. ($\mu g\ cm^{-3}$) | |
| | | 10 | 100 | 10 | 100 |
| 0 | * | +7±1.4 | +11±1.8 | 0±1.2 | +8±1.4 |
| 0.05 | −71±3.4 | −28±3.2 | −5±1.4 | −76±3.2 | −2±0.4 |
| 0.10 | −88±3.9 | −86±4.3 | −11±1.7 | −88±4.7 | −27±2.9 |
| 0.50 | −90±4.2 | −89±4.2 | −69±3.4 | −87±3.9 | −78±4.3 |

*Control, free from CuII ions or humic substances, had an activity of 3.4 mg $NH_3$ liberated from 10 $cm^3\ h^{-1}$ at 35°C in 0.01 M tetrasodium pyrophosphate at pH 7.1

The pH optimum of *B. pasteurii* urease lies in the range 6–7, but the greatest relative effect of HA on the enzyme is in the range pH 4–5. There is an indication that in the presence of HA, the pH activity profile is displaced to more alkaline regions; an observation also commented on for the urease-humate complexes extracted from soil (Ladd, 1985).

Enzyme kinetic studies revealed that humic substances lowered the maximum velocity ($V_{max}$) of the reaction, the precise effect depending on the concentration of the humic material. Thus, HA and FA at concentrations of 25 $\mu g\ cm^{-3}$ and 10 $\mu g\ cm^{-3}$ inhibited the $V_{max}$ by 38.5% and 20% respectively (Fig. 2). Although the data are not shown, HA and FA give similar results throughout the range 0–25 $\mu g\ cm^{-3}$. The Michaelis constant ($K_m$) is a measure of the affinity of an enzyme for its substrate, the lower the $K_m$ the higher the affinity (Dawes, 1962). At pH 4.0, *B. pasteurii* urease had a $K_m$ of 50 mM, but in the presence of both HA and FA concentrations (10–25 $\mu g\ cm^{-3}$) this was increased to 67 mM. In contrast to the effect on $V_{max}$, $K_m$ is less sensitive to concentrations of humic substances in the range 10–25 $\mu g\ cm^{-1}$. Concentrations of HA and FA up to 100 $\mu g\ cm^{-3}$ only increased the $K_m$ to 72 mM. Our kinetic data show the inhibition to be of a mixed type (both $K_m$ and $V_{max}$ affected) and it is likely that humic substances cause conformational changes in the enzyme activity by acting on the tertiary structure and/or the active enzyme sites rather than by interacting primarily with the urea substrate (Vaughan *et al.*, 1985).

Humic substances cause metals to complex, some of which, such as CuII, HgII and Ag, inhibit soil urease activity (Hughes *et al.*, 1969; Bremner & Mulvaney, 1978). Copper, used as $CuSO_4$ or $CuCl_2$, inhibited *B. pasteurii* urease activity, the effect increasing with increasing concentration of the cation (Table 4). At the pH used (pH 7.1 in 0.1 M sodium phosphate buffer to avoid complications arising from the chelating agent TRIS, in the universal buffer, complexing copper), the HA and FA had either no effect, or a stimulating effect, on urease activity depending on the concentration of humic substance used. HA and FA to some extent assuaged the inhibitory effects of the CuII, the precise effect depending on the relative proportions of CuII to humic substances (Table 4). Thus, low concentrations of HA (10 $\mu g\ cm^{-3}$) had a substantial ameliorating effect on Cu concentrations of 0.05 $\mu g\ cm^{-3}$, but had no effect on Cu concentrations of 0.1 $\mu g\ cm^{-3}$ and above. High concentrations of HA (100 mg $cm^{-3}$) were even more effective, but the inhibition produced by 0.5 $\mu g\ cm^{-3}$ CuII was reduced by only 23%. FA only exerted an ameliorating effect at 100 $\mu g\ cm^{-3}$.

In soils copper is complexed to humic substances (Sanders & Bloomfield, 1980) and this is probably responsible for the observations that the amounts of Cu, and other heavy metals, needed to effect substantial inhibition of soil urease are much greater than the amounts needed for an equivalent inhibition of purified urease (Bremner & Douglas, 1971). The immobilization of heavy metals and the interactions with urease have considerable implications for their disposal on agricultural land of sewage sludges and effluents containing substantial amounts of heavy metals (Berrow & Burridge, 1980). Our data also suggest that humic substances have least inhibitory effect on

urease activity in fertile agricultural soil of pH 5–6, but in upland and marginal areas, where the pH of the soil is less than 5.0, not only is the urease activity *per se* lower because of a pH effect and lower urease concentrations (fewer ureolytic bacteria), but even that activity is further lowered owing to the presence of large amounts of humic substances found in such soils.

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*(Received 20 March 1990; accepted 2 October 1990)*