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An *in vitro* effect of soil organic matter fractions and synthetic humic acids on the generation of superoxide radicals

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Summary Humic acid, and the acid-extracted residue obtained from it, stimulated the production of superoxide radicals ($O_2^{\cdot -}$) generated in the xanthine-xanthine oxidase system. Several synthetic humic acids, prepared by the oxidation of simple phenolic substances, also stimulated the production of $O_2^{\cdot -}$ but the degree of stimulation depended on the initial phenol. Fulvic acid and water-extractable soil organic matter were less effective in stimulating $O_2^{\cdot -}$ production than was humic acid. The activity of superoxide dismutase, an enzyme which destroys $O_2^{\cdot -}$, was also enhanced by HA. In contrast, fulvic acid and water-extractable soil organic matter had little effect on the activity of the dismutase.

Introduction

The superoxide radical ($O_2^{\cdot -}$) is formed in several aerobic biochemical reactions and is highly toxic in biological systems[5,6,7]. This free radical initiates lipid peroxidation and is involved in the destruction of cell membranes and in the inactivation of membrane-bound enzymes[2,6]. Indeed bacteria exposed to $O_2^{\cdot -}$ are rapidly killed[10]. Production of $O_2^{\cdot -}$ is probably responsible for the effects of bipyridyl herbicides[3]. Hence under normal conditions plants need to protect themselves against superoxide radicals[2]. Any substances which are taken up by a plant (for example soil organic matter[14]) and which enhance the production of superoxide radicals are, therefore, likely to have a deleterious effect on plant metabolism unless their actions can be counteracted.

As part of a continuing investigation on the influence of humic acid on enzyme activities we now report the effects of soil organic matter fractions on the production of $O_2^{\cdot -}$ during the oxidation of xanthine to uric acid in the presence of xanthine oxidase *in vitro*. This system generates $O_2^{\cdot -}$ which can be measured by the reduction of cytochrome C[9]. Because the enzyme superoxide dismutase catalyzes the destruction of superoxide radicals[11], the interaction between this dismutase and soil organic matter was also investigated.

Materials and methods

Humic (HA) and fulvic (FA) acids, and water-extractable organic matter (WEM) were extracted from a soil of the Countesswells series (National Grid Ref. NJ 905045) as described previously[12,13]. A sample of the HA was refluxed with 6M HCl to yield acid-soluble (ASHA) and acid-insoluble (AIHA) fractions[12]. Synthetic humic acids were prepared by the oxidation of catechol, resorcinol or guaiacol, in alkaline solution using $NaIO_3$[12]. A synthetic HA containing N was also prepared from catechol by including alanine in the alkaline oxidizing solution[12]. In tests on enzyme activity, the organic matter fractions were dissolved in NaOH (100 mg solid: 1 cm^3 1 *M* NaOH) and the pH adjusted to 7.8. All media, with or without organic material, contained 8 m*M* NaCl.

To mesure xanthine oxidase activity, stock solutions were prepared containing xanthine and cytochrome C (Cyt C) of concentrations 5×10^{-5} *M* and 4×10^{-5} *M* respectively. Xanthine oxidase of activity 4 EU cm^{-3} (BDH Ltd.) was diluted with 0.1 *M* Na phosphate buffer, pH 7.8, to give a stock solution containing an activity of 0.15 EU cm^{-3}. The enzyme activity was determined in a solution containing 1 cm^3 aliquots of xanthine, Cyt C, buffer or soil organic matter fraction, and

finally xanthine oxidase. The rate of reduction of the Cyt C was measured by recording the increase in absorbance at 550 nm min^{-1}($\triangle A_{550}$) using a Pye-Unicam SP 1800 spectrophotometer at a scale expansion of 0–0.2.

Superoxide dismutase (SOD) was extracted from fronds of *Lemna gibba* L. by essentially the method of Baker[1]. Fronds (1 g fr. wt.) were homogenized in 50 cm^3 0.1 *M* Na phosphate buffer, pH 7.8, using an all-glass homogenizer. The homogenate was centrifuged at 2,000 *g* for 10 min to remove the debris then at 20,000 *g* for 15 min to remove mitochondria. All procedures were carried out at 2–4°C. The 20,000 *g* supernatant was used as a source of SOD activity. The assay method involved the measurement of the rate of Cyt C reduction in the Xanthine-Xanthine oxidase system described above and the inhibition of this reduction by SOD[11]. The SOD extract from Lemna was diluted to give a 50% inhibition of xanthine oxidase activity corresponding to 1 unit of SOD[11]. Stock solutions were used at concentrations described above and 1 cm^3 aliquots were added in the order xanthine, Cyt C, buffer or soil organic matter fraction, SOD extract and finally xanthine oxidase. The rate of increase in A_{550} ($\triangle A_{550}$) was measured as described above.

Results and discussion

Under our conditions the rate of Cyt C reduction (xanthine oxidase activity) was linear over the first 75 s of the reaction and HA stimulated xanthine oxidase activity. This stimulation increased with an increase in HA concentration but the response was not linear (Table 1). FA also stimulated xanthine oxidase activity but this was considerably less than for HA, while the WEM fraction produced a response similar to that of FA.

The effect of HA on xanthine oxidase activity was probably not due to the presence of N because synthetic HA's prepared by oxidizing phenolic starting materials, and containing essentially no N, also enhanced xanthine oxidase activity. Pertinent to this, a synthetic HA prepared from catechol and alanine (final N content 1.9%) produced the same stimulation of xanthine oxidase as the HA prepared from catechol alone (Table 1). The degree of stimulation produced by the synthetic HA's depended on the initial phenolic material, the preparation from resorcinol being the least effective

Table 1. Effect of different concentrations of soil organic matter fractions, synthetic humic acids and phenolic substances on xanthine oxidase activity. Effects expressed as percent stimulation and inhibition

Substance in assay medium	Final concentration (μg cm^{-3}) 1	2	4	8	12	16
*Stimulation**						
Humic acid (HA)	31.1	76.5	105.2	160.3	208.9	217.3
Acid-insoluble HA	29.8	81.2	110.9	175.3	219.6	243.7
Fulvic acid	0	9.6	17.3	28.2	39.1	46.3
Water extractable material	0	8.1	16.4	27.5	36.3	40.5
Resorcinol HA	17.0	43.2	70.3	70.3	48.6	43.0
Catechol HA	60.3	116.2	207.3	208.1	170.3	152.6
Catechol & Nitrogen HA	58.7	121.3	215.4	210.3	179.2	149.7
Guaiacol HA	75.1	156.8	183.8	208.4	272.7	290.6
Inhibition						
Resorcinol	2.7	11.1	16.3	24.2	25.2	23.7
Catechol	0	13.5	19.2	22.3	26.3	32.6
Guaiacol	9.1	19.6	21.7	32.6	42.6	51.1

* Xanthine oxidase activity in the Control was 0.035 $A_{550\,nm}$ min^{-1}.

while that obtained from guaiacol was even more effective than natural HA. In contrast, the three phenolic substances resorcinol, guaiacol and catechol all inhibited xanthine oxidase, although the magnitude of these inhibitions were usually in a different order from the stimulations produced by the synthetic HA's prepared from them.

The data of Table 1 also show that the insoluble debris remaining after HA has been refluxed with HCl (AIHA) stimulates xanthine oxidase activity to a somewhat greater extent than the original HA. In contrast, the acidsoluble HA fraction (ASHA) produced a stimulation of xanthine oxidase activity in the same order as FA. It has been suggested[8] that HA comprises a 'core material' to which is attached proteins, carbohydrates, phenols, amino acids and mineral salts. The debris remaining after HA has been refluxed with 6 *M* HCl comprises only the 'core material' and hence the HA 'core' contributes to the greatest extent towards the stimulation of xanthine oxidase by HA.

Xanthine oxidase is involved in the oxidation of xanthine by molecular O_2 to produce uric acid and H_2O_2. According to Halliwell[6] the steps involved in the production of H_2O_2 may be represented as:

$$O_2 + e^- + H^+ \rightarrow HO_2 \quad \text{(i)}$$
$$HO_2 + e^- + H^+ \rightarrow H_2O_2 \quad \text{(ii)}$$

The species HO_2 dissociates to give $O_2^{\cdot -}$ and this superoxide radical reduces Cyt C in the above system. Because the five soil organic matter fractions, but particularly HA, enhance the rate of Cyt C reduction, it is probable that these organic matter fractions stimulate the production of $O_2^{\cdot -}$. This supposition was tested using superoxide dismutase (SOD), an enzyme involved in the destruction of superoxide radicals[11].

The volume of the solution containing Lemna SOD was adjusted to give an inhibition of xanthine oxidase activity of about 50% (Table 2) and at this SOD concentration the reaction rate for xanthine oxidase was still linear over the first 75 s of the reaction. A 50% inhibition also corresponds to one unit of SOD activity as defined by McCord and Fridovich[10]. At SOD concentrations giving inhibitions 65%, the reaction for xanthine oxidase was no longer linear. At all concentrations of HA used, SOD reduced the stimulation of xanthine oxidase activity produced by this soil organic matter fraction. Because SOD removes 50% of the $O_2^{\cdot -}$ produced by xanthine oxidase, it might be assumed that this value could be subtracted from the stimulations produced by the various HA concentrations. If this were true, then for the 104.7% stimulation produced by 4 μg cm^{-3} HA for

Table 2. Effect of superoxide dismutase (SOD) on the stimulation of xanthine oxidase in the presence of humic (HA) or fulvic (FA) acids or water-extractable soil organic matter (WEM). Effect expressed in percent

Addition to assay medium	Final concentration of soil organic matter fraction (μg cm^{-3})				
	0	2	4	8	12
HA	0	72.1	104.7	153.6	207.9
HA + SOD	−49.6	1.8	22.1	45.1	75.2
Difference	49.6	70.3	82.6	108.5	132.7
FA	0	8.5	17.9	32.3	40.2
FA + SOD	−49.6	−44.0	−36.8	−24.5	−20.0
Difference	49.6	52.5	54.7	56.8	60.2
WEM	0	6.8	15.7	28.4	35.6
WEM + SOD	−49.6	−44.4	38.4	28.0	23.7
Difference	49.6	51.2	54.1	56.4	59.3

example, the value in the presence of SOD should be about 55% whereas a value of 22.1% was obtained experimentally. Indeed this net stimulation increases with an increase in HA concentration (figures for differences in Table 2). The other alternative that a 50% reduction of xanthine oxidase activity produced by SOD should also result in a 50% decrease in the stimulation produced by HA is also clearly untrue. The most likely explanation is that HA enhances SOD activity. In contrast, SOD activity is only slightly stimulated in the presence of fulvic acid or the water-extractable soil organic matter.

The data of this preliminery *in vitro* investigation show that soil organic matter fractions can affect the production of superoxide radicals and the activity of superoxide dismutase involved in their destruction, although the magnitude of these effects varies according to the soil organic matter fraction used. However, because HA and FA are obtained by relatively harsh chemical extractions their relevance to the true conditions obtaining in the soil is now being questioned by biologists. Although there is no evidence for the presence of HA in the soil solution, there is no doubt that FA is virtually indistinguishable from the brown polycarboxylic acid components present in water-extractable soil organic matter[15]. Hence it is likely that polycarboxylic acids of the FA type could influence the production of superoxide ions in plant roots at the root/soil interface and experiments are now in progress to investigate this possibility.

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