Mechanism of the desmutagenic effect of humic acid

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Summary

The mechanism of an apparent desmutagenic effect of humic acid was investigated. Firstly, components of humic acid (resorcinol, vanillin, vanillic acid, ferulic acid, protochatechuic acid and benzoic acid) were tested and were not found to show a desmutagenic effect. By contrast, lignin did show a desmutagenic effect. The desmutagenic effect of humic acid was decreased by ozone treatment, and the degree of decrease corresponded with a decrease in KMnO₄ consumption. Benzo[a]pyrene and humic acid were incubated at 37°C for 1 h and extracted by ethyl acetate and the extract was investigated by gas chromatography (GC). The peak of the decomposition product did not appear, but the amount of benzo[a]pyrene was decreased. This suggests that the desmutagenic effect of humic acid was caused by adsorption of benzo[a]pyrene by humic acid rather than by decomposition of benzo[a]pyrene. Humic acid had the largest adsorption activity at its critical micelle concentration (CMC), while adsorbed benzo[a]pyrene could be released by ultrasonication. Fulvic acid and water-soluble humic substance showed a slight inhibitory effect on the mutagenicity of benzo[a]pyrene.

In a previous report (Sato et al., 1986), the authors reported an apparent desmutagenic effect of humic acid. Humic acid inhibited the mutagenicities of benzo[a]pyrene, 2-aminoanthracene (+S9 mix), 2-nitrofluorene and 1-nitropyrene (−S9 mix), but not the mutagenicities of 4NQO, AF-2 and MNNG (−S9 mix). It was also shown that the effect appeared to be desmutagenic rather than antimutagenic and heat-stable, and that the effect increased with increased molecular weight of the humic acid. In this report, we have examined the mechanism of the desmutagenic effect of humic acid.

Materials and methods

1) Humic acid components and lignin

Resorcinol was obtained from the Kishida Company (Osaka, Japan). Vanillin, vanillic acid, ferulic acid, protochatechuic acid, benzoic acid and lignin were obtained from the Nakarai Company (Kyoto, Japan).

Vanillin, vanillic acid, ferulic acid, protochatechuic acid and benzoic acid were dissolved in
dimethyl sulfoxide (DMSO). Lignin and resorcinol were dissolved in M/15 phosphate buffer (pH 7.0).

(2) *Humic acid and fulvic acid*

Leaf mould was collected from the brown forest at Gifu City and extracted with 0.5 N sodium hydroxide. The pH of the extract was adjusted to 1.0 by conc. hydrochloric acid and centrifuged. After the precipitate was freeze-dried, it was washed with a small amount of distilled water to remove salts and freeze-dried again. This fraction was humic acid. The supernatant was dialyzed and freeze-dried. This fraction was fulvic acid.

Both fractions were dissolved in distilled water and the pH was adjusted to 7.0. The undissolved portions were removed by centrifugation at 1000 × g for 30 min and the supernatants were used. The amount of KMnO$_4$ consumption by the supernatant was determined (Pharmaceutical Society of Japan, 1980).

(3) *Water-soluble humic substance*

400 g of mould was extracted twice on successive days with 3–4 l of distilled water. The extract was concentrated in a rotary evaporator at less than 40°C and freeze-dried, and treated in the same way as humic and fulvic acids.

(4) *Test of the inhibitory effect on benzo[a]pyrene-induced mutations*

The inhibitory effects of humic acid components and related substances on benzo[a]pyrene-induced mutations were investigated using *Salmonella typhimurium* TA100 and TA98. 59 mix was added. The numbers of surviving cells were determined on B-2 medium, and the frequency of revertants per survivors was calculated. The details were described in the previous paper (Sato et al., 1986).

(5) *Treatment of humic acid by ozone*

500 mg/l of humic acid was treated by ozone for 0, 1, 5, 10, 20, 60 and 120 min. After 120 min ozone treatment, the humic acid was bleached completely. The conditions of ozone treatment were as follows.

Apparatus, Ozone generator 0-3-2 (Japan Ozone Company); voltage, 80 V; electric current, 3 A; oxygen flow, 1.5 l/min.

After ozonation, they were concentrated 80 times and the inhibitory effect on benzo[a]pyrene-induced mutation was investigated. A part of each sample was used for measurement of KMnO$_4$ consumption.

(6) *The measurement of benzo[a]pyrene treated by humic acid*

100 µg of benzo[a]pyrene and several amounts of humic acid were incubated at 37°C for 1 h and extracted by ethyl acetate. On one occasion, ultrasonication (Ultrasonic cleaner Jtn USC-48, Kimura Co.) was used for extraction. The amounts of benzo[a]pyrene in the extracts were measured by GC. The conditions of GC were as follows.

Apparatus, Shimazu GC-4BM; detector, hydrogen flame ionization detector; column, glass (3 mm × 2 m); stationary phase, 2% silicone OV-17; support, Uniport HP, 80–100 mesh; column temperature, 310°C; injection and detector temperature, 315°C; carrier gas, nitrogen (2.0 kg/cm$^2$). An internal standard method was used, with chrysene as the standard.

**Results and discussion**

(1) *Inhibitory effects of humic acid components and lignin on the mutagenicity of benzo[a]pyrene*

Various substances have been identified as the components of humic acid (Schnitzer and Khan, 1972, 1978; Lias et al., 1982; Sato et al., 1985). To investigate the desmutagenic effects of some of these components, resorcinol, vanillin, vanillic acid, ferulic acid, protocatechuic acid and benzoic acid were selected and their inhibitory effects on the mutagenicity of benzo[a]pyrene were investigated. The results obtained for resorcinol are shown in Fig. 1.

An inhibitory effect was not observed. Vanillin, vanillic acid, ferulic acid, protocatechuic acid and benzoic acid did not show inhibitory effects either. The effect of lignin was also investigated. (Lignin is the high molecular weight component of plants which may be changed to humic acid in soil.) The results are shown in Fig. 2.

An inhibitory effect was observed. For manifestation of the inhibitory effect of humic acid, some high molecular weight structure would therefore appear to be necessary.
Fig. 1. Inhibitory effect of resorcinol on the mutagenicity of benzo[a]pyrene. The dotted line indicates spontaneous revertants. 10 μg/plate of benzo[a]pyrene was added for TA100. S9 mix was used.

(2) Decrease in the inhibitory effect of humic acid on the mutagenicity of benzo[a]pyrene following ozone treatment

Humic acid was treated with ozone, and the inhibitory effect of the treated humic acid on the mutagenicity of benzo[a]pyrene was investigated.

Fig. 2. Inhibitory effect of lignin on the mutagenicity of benzo[a]pyrene. The dotted line indicates spontaneous revertants. 10 μg/plate of benzo[a]pyrene was added for TA100. S9 mix was used.

(3) Decrease of benzo[a]pyrene treated by humic acid

Benzo[a]pyrene was treated by humic acid and extracted with ethyl acetate and investigated by GC. The chromatogram of the extract is shown in Fig. 4.

The peaks of solvent and benzo[a]pyrene only were observed and the peak of decomposition products of benzo[a]pyrene did not appear. It can therefore be considered that humic acid did not cause decomposition of benzo[a]pyrene.

Benzo[a]pyrene was treated with humic acid and extracted by ethyl acetate, in successive stages. The amounts of benzo[a]pyrene in the extracts were determined and the percentages of extracted benzo[a]pyrene were calculated. The results are shown in Fig. 5.

It was clear that total benzo[a]pyrene was extracted by only 2 or 3 times extraction in the case
Solvent

Fig. 4. Gas chromatogram of benzo[a]pyrene treated by humic acid. 100 μg/0.1 ml of benzo[a]pyrene was treated by 2 ml of humic acid solution and extracted in successive stages with 2 ml of ethyl acetate each, and the extracts were combined.

Benz[a]pyrene

Fig. 5. Relation between the recovery of benzo[a]pyrene and the number of extraction. 100 μg/0.1 ml of benzo[a]pyrene was treated by 2 ml of humic acid solution and extracted in successive stages with 2 ml of ethyl acetate each. ○, treatment by water; •, treatment by 3.8 mg/2 ml of humic acid (as KMnO₄ consumption); ▲, treatment by 75 mg/2 ml of humic acid (as KMnO₄ consumption).

Fig. 6. Decrease of benzo[a]pyrene by humic acid treatment. 100 μg/0.1 ml of benzo[a]pyrene was used and humic acid was dissolved to 2 ml of distilled water. 5 ml of ethyl acetate was used for each extraction stage and 3 times extracts were combined.

of water treatment. But, some benzo[a]pyrene was extracted by ethyl acetate after even 8 times extraction in case of humic acid treatment. It was very interesting that the extracted amount of benzo[a]pyrene treated by 3.8 mg of humic acid

(as KMnO₄ consumption) was smaller than that treated by 75 mg of humic acid (as KMnO₄ consumption).

Then, benzo[a]pyrene was treated by various amounts of humic acid and extracted by ethyl acetate, and the amounts of benzo[a]pyrene in the
The amount of extracted benzo[a]pyrene became a minimal value when benzo[a]pyrene was treated with 10–40 mg humic acid as KMnO₄ consumption (in 2 ml of distilled water) which corresponds to 2–9 g/l of humic acid.

Hayase and Tsubota (1983) reported that humic acid or fulvic acid was aggregated to make micelle and aggregation concentration (AGC) of humic acid was 1–10 g/l. This value is about the same as our value which is the range when adsorption ability became maximal. The AGC of humic acid corresponds to its CMC. At a lower concentration than the CMC, humic acid may form a monodisperse system and benzo[a]pyrene may be adsorbed to hydrophobic parts of humic acid and become unextractable with ethyl acetate. But, at higher concentrations than the CMC, humic acid may form a micelle with hydrophobic parts orientated toward the inside and hydrophilic parts orientated outwards; thus the contribution of the hydrophobic parts for adsorption would decrease, and benzo[a]pyrene would become more difficult to adsorb to humic acid. By such phenomena, the adsorption activity of humic acid may become maximal at its CMC.

As shown in the previous report (Sato et al., 1986), the mutagenicity of benzo[a]pyrene was decreased to the level of spontaneous mutation if

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**Fig. 7.** Relation of ultrasonication time and the release of benzo[a]pyrene. First, 100 μg/0.1 ml of benzo[a]pyrene treated with 38 mg/2 ml of humic acid (as KMnO₄ consumption) was extracted twice with 5 ml of ethyl acetate, then 5 ml of ethyl acetate was added to water layer and treated by ultrasonication. The ultrasonication process was repeated and both ethyl acetate extracts were combined.

**Fig. 8.** Inhibitory effects of fulvic acid and water-soluble humic substance on the mutagenicity of benzo[a]pyrene. The dotted line indicates spontaneous revertants. 10 μg/plate of benzo[a]pyrene was added for TA100. S9 mix was used.
humic acid was added at more than about 10–15 mg/plate (KMnO₄ consumption). In this case, more than 50% of benzo[a]pyrene was extracted by ethyl acetate. Some of the benzo[a]pyrene which was adsorbed to humic acid may be extractable by ethyl acetate.

After benzo[a]pyrene was treated with humic acid and extracted with ethyl acetate, another ethyl acetate portion was added to the lower water layer and treated by ultrasonication. The amount of benzo[a]pyrene in the latter ethyl acetate layer was investigated. The results are shown in Fig. 7.

After 2 × 20 min ultrasonication, nearly all of the benzo[a]pyrene which had been adsorbed to humic acid was released. From this result, it is clear that benzo[a]pyrene was absorbed to humic acid rather than decomposed by it. McCarthy and Jimenez (1985) reported that several polycyclic aromatic hydrocarbons bind to dissolved humic materials and that the binding of benzo[a]pyrene to dissolved humic material was completely reversible.

In natural environments, the adsorbed mutagens may be decomposed by bacteria in humic substances after long periods. Shinohara et al. (1980a,b) reported that various PAH were degraded by activated sludge.

(4) Inhibitory effects of fulvic acid and water-soluble humic substance on the mutagenicity of benzo[a]-pyrene

The inhibitory effects of fulvic acid and water-soluble humic substance on the mutagenicity of benzo[a]pyrene were investigated and the results are shown in Fig. 8.

A weak inhibitory effect was observed in both cases. Fulvic acid is a lower molecular weight substance than humic acid, while water-soluble humic substance may also be of a lower molecular weight than the humic acid which was soluble in 0.5 N sodium hydroxide. This result is consistent with the idea that a high molecular weight structure is necessary for the desmutagenic effect of humic acid.

Conclusion

The mechanism of desmutagenic effect of humic acid did not involve decomposition of the mutagen, but rather adsorption of the mutagen. A high molecular weight structure appears to be necessary for the desmutagenic effect of humic acid.

Various mutagens are adsorbed to humic substances and lose their mutagenic activities. After long periods, they may be decomposed by bacteria in humic substances. By this mechanism, humic substances may play an important role in protection against carcinogenesis.

References


