STRUCURE ANALYSYS OF THE ENZYMATIC REACTION PRODUCTS FROM ANILINE AND A MODEL HUMIC CONSTITUENT

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Introduction

It is well known that substituted anilines are easily integrated into the soil structure and the covalent binding of aromatic amines to humic substances is known to be one of the most important immobilization mechanisms. Thus the analysis of this polymeric product is important to research about this mechanism and also the fate of the contaminated soil. We proposed pyrolysis-GC/MS, a convenient analysis method for the polymers, for this purpose. It requires samples of less than 1mg with no or little need for pre-treatment. It is not easy to arrive at a total structure, based on information about the pyrolysis products as unit elements, however recently this method is being shown to be effective in the structure analysis of natural organic matters by the accumulation of the knowledge about fractionation by pyrolysis. In this paper an enzymatic reaction product from aniline and protocatechuic acid (a humic constituent) was examined by pyrolysis-GC/MS and also compared with the ^{15}N-NMR spectrum of the same reaction product.

Methods and materials

Incubation: An aqueous solution of 100mL containing 0.5mM 3,4-DCA, 0.5mM protocatechuic acid and 1U/mL HRP (horse radish peroxidase), was prepared. Incubation was initiated by adding H_2O_2 (2mM in reaction solution). The solution was shaken at 28 ^{o}C for 30min. 3,4-DCA decrease as the substrate was monitored by HPLC. After 30min incubation, the reaction solution was ultrafiltered by 1000dalton-membrane filter. The polymer fraction (>1000dalton) was directly measured by pyrolysis GC/MS. Another solution mixture (500mL), that contained 4.26mM ^{15}N-aniline and 5.2mM protocatechuic acid, was also treated in the same way with the increased concentration of 2.8U/mL HRP and 36mM H_2O_2. Besides the above procedure, the larger fraction (>1000dalton) was freeze dried, dissolved in DMSO-D6 and measured by ^{15}N-NMR.

Analytical methods: [HPLC] A Jasco PU-980 (Japan Spectroscopic, Tokyo, Japan) provided with a reverse phase column, Cosmosil 5C18-SL (Nacalai tesque, Japan), was used with a 40:60 methanol/0.08 % phosphate buffer mobile phase. [Pyrolysis-GC/MS] The sample (ca. 100μg) was pyrolyzed at 500^{o}C, 4sec. in a JHP-3 curie point pyrolyzer (Japan Analytical Industry, Japan). The pyrolyzate was measured by an HP5972 / HP5890 II GC/MS (Hewlett Packard, USA) with a Quadrex MS fused silica capillary column. Column oven temperature was held at 50 ^{o}C for 1 min and then raised at 5 deg min^{-1} up to 300 ^{o}C. [NMR] A liquid phase ^{15}N-NMR spectrum was recorded on a JNM-LA500 FT-NMR spectrometer (JEOL ltd., Japan). An INEPT spectrum was recorded using 45454.5-Hz spectral window, 0.5-s acquisition time, and 2.0-s proton relaxation

delay. The polarisation transfer time and refocusing delay were set equal to 1/4J(2.78ms).

Results and Discussion

3,4-DCA was incubated with protocatechuic acid as a simple, model humus constituent. Protocatechuic acid itself is not abundant in the soil but a catechol like structure with two hydroxyl groups in ortho position often appears in humic substances. For this reason, it is often used as a model phenolic monomer of a constituent of humic substances. Peroxidase was used as the enzyme since it is common in nature and readily catalyzes the reaction of aromatic substances.

The presence of protocatechuic acid clearly accelerates 3,4-DCA transformation. About 80% of the 3,4-DCA disappeared within 30 min, when incubated with protocatechuic acid, while only a 50% conversion when incubated alone.

Fig.1 Pyrogram of the enzymatic reaction product from 3,4dichloroaniline and protocatechuic acid

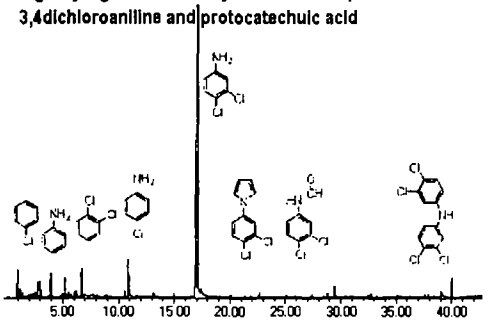

Fig.1 shows the pyrogram of the fraction (>1000daltons) for a 30min reaction solution. The majority of the pyrolysis product was 3,4-DCA itself. However, a number of small aromatic peaks that included nitrogen or chlorine were evident.

To evaluate the side effects of pyrolysis and to clarify the origin of each of the pyrolysis products, the pyrogram was compared with ^{15}N-NMR spectrum of the same reaction product. ^{15}N-aniline was used in order to obtain sufficient signal strength for this time. About 90% of the ^{15}N-aniline had disappeared during the first 5min and only 7% remained after a 30min incubation.

Fig.2 a) shows the ^{15}N-NMR spectrum of the reaction product from ^{15}N-aniline and protocatechuic acid. Spectrum pattern of 80-180ppm is quite similar to the published ^{15}N-NMR spectrum[1] of the peroxidase catalyzed reaction product from ^{15}N-aniline and a soil humic acid. Both spectra have large peaks at around 90 and 160 ppm and smaller peaks in between. They assigned 1) the peaks around 90ppm to simple amino linkage like diphenylamine, anilinoquinone or anilinohydro-quinone, derivatives. 2) 160ppm to heterocyclic nitrogen, such as that in indole or pyrrole structures and 3) the smaller peaks in between to be anilide nitrogen.

Fig.2 b) shows a pyrogram of the same product. In contrast to the NMR spectra, the majority of the pyrolysis products from the same enzymatic reaction product was ^{15}N-aniline itself. These results clearly show that the aniline was cleaved from the polymer, as the result of pyrolysis. Small peaks of Phenylpyrrole, diphenylamine, and isocyanobenzene also appeared in the pyrogram. The production of aniline and isocyanobenzene from formanilide was observed in the pyrolysis of the standard chemical. Thus, one of the reaction pathways to produce aniline by pyrolysis could involve anilide. Another major pathway could involve the cleavage of an amino linkage. Diphenylamine itself is not cleavable by a 500 ^{0}C pyrolysis. However, if it is substituted or added, as in the case of anilinoquinone, there are some cleavable cases.

Phenylpyrrole corresponds to the peaks 145ppm downfield on the ^{15}N-NMR spectrum.

The relative abundance of phenylpyrrole in the pyrogram was far smaller than that in ^{15}N-NMR

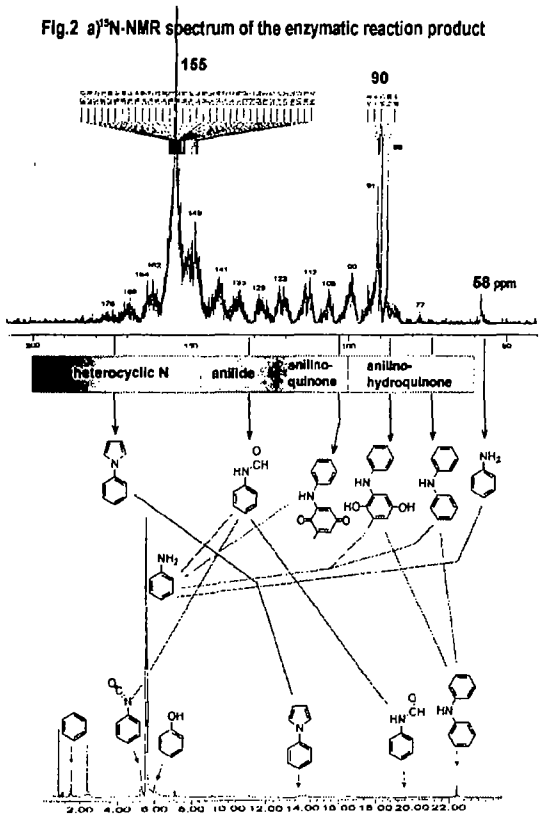

Fig.2 a)^{15}N-NMR spectrum of the enzymatic reaction product
b) 500°C pyrogram of the same reaction product

result suggesting a strong linkage to the polymer. The large peaks around 155ppm were located in the region corresponding to heterocyclic nitrogen, but were not so close to N-phenylpyrrole (175ppm) itself. Possibility is that the signals were shifted as a result of the covalent bond through which they were incorporated into the polymeric structure. This influential linkage is also suggestive of a strong bond to the polymer, which is resistant to pyrolysis. Whereas the small peaks downfield of 160ppm were not significantly affected by linkage and must correspond to the small amount of phenylpyrrole detected by pyrolysis-GC/MS.

It has been reported that Phenylpyrrole is produced by the reaction of aniline and the alkyl chain, including ketone groups[2]. In this experimental system, however, no alkyl chain existed in the initial solution, suggesting the peroxidase catalyzed ring cleavage of protocatechuic acid. These results suggest that the heterocyclic structures, such as phenylpyrrole must be one of the most important structures in the humic substances that are contaminated with substituted anilines.

From these experiments it seems that the aniline derivatives that constitutes the polymeric reaction product shows three types of reactions at pyrolysis. 1) Cleaved and detected as

a cluster including aniline 2) cleaved as a simple monomer like aniline 3) No cleavage and no detection. The difference seems to come according to how they were bound to the polymer. Thus the large peak of the aniline and the small peaks of dimers will together give the information about the polymer structure.

The pyrolysis-GC/MS measurement is an easy method for analyzing a polymer and also requires only a small quantity of the samples without purification. A comparison with NMR result allowed considerable information and revealed that the tiny peaks in the pyrogram were not negligible. A compilation of these findings will contribute to our attempts to make the pyrolysis GC/MS a truly useful method for the analysis of the humic-xenobiotics complex.

1.Thorn,K.A etc. (1996) Chap.19 In Humic and fulvic acids,Amer.Chem.Soc.ISBN0-8412-3468-X
2.Thorn,K.A etc. (1996) Env. Sci. & Technol., vol.30, No.9, 2764-2775