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COMPARISON OF THE EFFECT OF VARIOUS HUMIC SUBSTANCES ON THE ENZYMATIC TRANSFORMATION OF PCP

Kengo Morimoto[1], Hiroyasu Ichikawa[1], Kenji Tatsumi[1], Ken-ichi Kuroda[2] and Teruo Higashi[2]

1. Agency of Industrial Science and Technology, Onogawa 16-1, Tsukuba, Ibaraki, JAPAN 305-8569 2. University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki, JAPAN, 305-8572

Introduction

Humic substances or soil organic matters are known to readily incorporate chlorinated phenols and anilines by the catalysis of phenoloxidizing enzymes such as peroxidase. This transformation is considered one of the self-purification processes of these xenobiotics. Application of this reaction for the remediation of contaminated soil has been investigated.

A large amount of research has been done on the enzymatic transformation of chlorophenols and anilines with model humic constituents[1] and also some researches with humic acid extracted from the soil[2]. However little research has been done to show how this reaction changes with the use of humic acids of various characteristics.

Humic acids are classified by the optical absorbance of their alkaline solutions based on the principle that they become darker with humification[3]. In this study we compared the effects of seven humic acids with various degrees of humification on the enzymatic transformation of pentachlorophenol (PCP).

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Methods and Materials

Sample soils were collected mainly from the Kanto area of Japan. Characteristics of the sample soils are listed in Table.1.

Determination of the degree of humification:

The soil sample (approx. 200mgC) was put into 60mL of 0.5% NaOH solution and heated 30min in a boiling water bath. After cooling it was centrifuged at 4000rpm for 20min. Twenty mL of the supernatant was mixed with 0.2mL-concentrated sulfuric acid and stirred vigorously for 30seconds. This solution was filtered to obtain the humic acid, which remained as a residue on the filter. The humic acid was washed out into a 100mL volumetric flask by poring 0.1% NaOH on to the filter and the optical absorbance of the result was measured at 400nm (K_{400}) and 600nm (K_{600}). Also the chemical oxygen demand (COD), an index of organic carbon content, of was measured and expressed in 0.1N $KMnO4$ consumption (mL) for 30mL of the sample (Q).

The indices of the degree of humification were calculated as follows.

$$RF = K_{600}*2247/Q$$

$$\Delta\log K = \log K_{400} - \log K_{600}$$

(2247is multiplied for the normalization as RF of a standard humic acid to be 100.)

The types of the humic acids were determined using the values shown in fig.1

Humic acid extraction:

The Nagoya method was used to extract humic acid from the sample soils as follows: A soil sample was mixed into 0.1M NaOH to make an aqueous solution whose volume was 300 times (V/W) that of the organic carbon content of the sample soil. After shaking this solution for 48 hours, 30mg/l NaCl was added to the solution and centrifuged at 1500g for 15min. The sediment was then washed with the same volume of NaOH solution and centrifuged. Both supernatants were mixed together and acidified to pH 1.0 by adding 3M HCl and allowed to settle for one night. Most of the supernatant was then discarded by decantation and the solution was centrifuged

at 1500g for 10min. This sediment, humic acid, was neutralized by 2M NaOH and dissolved again into 0.1M NaOH whose volume was the same as the initial solution and centrifuged at 6000g for 20min to wash out the impurities. The supernatant was acidified, settled and centrifuged as before. This cleaning cycle was repeated three times. The acidified sediment finally obtained was suspended in a 0.1M HCl: 0.3M HF (1:1) solution, stirred for 24 hours and centrifuged. The supernatant was discarded. This procedure was repeated three times. The resultant sediment was dialyzed to remove inorganic minerals and freeze dried. The obtained particles were humic acids.
CHN elemental analysis was done with Perkin-Elmer 2400 CHN elemental analyzer at Chemical Analysis Center, University of Tsukuba.

Enzymatic reaction:

20μM of PCP, 25mg/l of a humic acid and 0.5U/ml of horseradish peroxidase (HRP) were dissolved in a Mcllvaine buffer at pH 5.5 (648mg/l sodium phosphate dibasic and 288mg/l citric acid). The reaction was initiated by adding H_2O_2 to make 150μM and the solution mixture was shaken at 28C in a water bath shaker. PCP concentration in the reaction solution was monitored by HPLC using a Jasco PU-2080 (Japan Spectroscopic, Tokyo, Japan) provided with an UV detector (Jasco UV-2075). A reverse phase column, Cosmosil 5C18-AR(4.6 mm i.d. x 25 cm, Nacalai tesque, Kyoto, Japan), was used. The mobile phase (flow rate 1.0 mL/min) consisted of methanol and a 0.08 % phosphate buffer and the ratio of methanol to the buffer was 85:15.

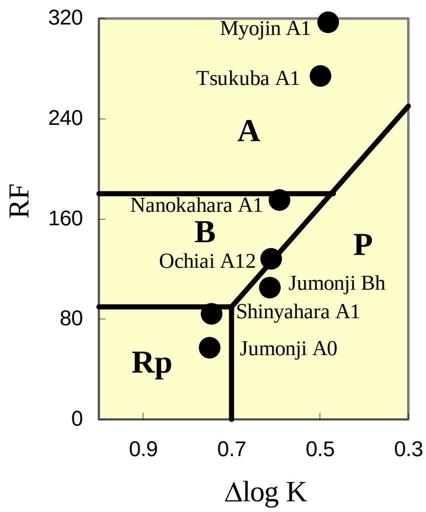
Results and Discussion

Degree of humification and elemental analysis of each soil samples:

Humic acid is known to become darker as humification proceeds and therefore the degree of humification is determined by its optical absorbance which is expressed by two indices: RF and Δlog K. Index RF is the absorbance at 600nm (K_{600}) normalized to the organic carbon concentration of the humic acid solution and the index Δlog K is the slope of the absorbance spectrum. The absorption spectrum of a humic acid is known to decrease linearly with an increase of wavelength. Also, the humic acid type is defined by these same indices and is classified according to which of the four sectors of Fig. 1 the indices of a humic substance fall.

Index RF increases and Index Δlog K decreases with humification. The RF and Δlog K indices of the soil samples used in this study are listed in Table1.We used two soil samples each of the A, B, and Rp types and one soil sample of the P type and their spectral values are shown in Fig.1. Results of the elemental analysis are shown in Table.2.

Figure.1 RF-Δlog K diagram and the definition of humic acid types



Carbon and oxygen content increased with humification and hydrogen decreased with humification, thus the degree of unsaturation increased with humification.

Enzymatic reaction:

Fig.2 shows the reduction of PCP concentration by enzymatic reaction catalyzed by HRP. In the first stage, co-existence of the humic acids somehow decelerated enzymatic reaction.

However, the humic acids retained reaction activity during a longer incubation period giving a larger decrease of PCP after more than one-hour incubation. When we used a larger amount of humic acid, this tendency became stronger. HRP catalyzes the radical formation of phenolic hydroxyl groups in humic acid and this process must be in competition with radical formation of PCP. This is most likely the reason why the reactivity decreased in the first stage of incubation with the coexistence of the humic acid. However, the reactive sites such as quinones or radicals that were enzymatically formed in the humic acid seemed to continue reactivity with PCP even after HRP activity decreased.

Fig.3 shows the decrease of PCP with three different humic acids. PCP decrease was greater when it was incubated with Rp type humic acid and least with A type humic acid, a highly humified type. To explore the relation between the degree of humification and the enzymatic reactivity with PCP, another four humic acids were also tested and the amount of PCP remaining after 30min incubation was plotted against its RF and Δlog K values, the degree of humification indices (fig.4). A clear correlation is shown and the lower humified humic acids had greater reactivity with PCP. The decrease of PCP after 30min was also plotted against the result of elemental analysis (fig.5) and also showed this correlation. PCP decreased more when the humic acid had smaller carbon and larger hydrogen content, and thus a smaller degree of unsaturation.

The reactive or easily transformable sites in a humic acid are thought to be degraded with humification and so it becomes more stable or resistive against chemical reactions. This is speculated to be one of the reasons why the reactivity of humic acid decreased with humification. Also there was a clear correlation between reactivity and the degree of unsaturation. Aromaticity of the humic acid is known to increase with humification, which results in an increase of unsaturation. This is also one of the ways humic acids become stable with humification.

Table 1. Sample soils used in this study

Soil type	Soil name and horizon	Type of humic acid	RF[a]	logK[a]	Sampling location (town or city, pref.)
Kuroboku soil	Myojin A1	A	317	0.481	Imaichi, Tochigi
Kuroboku soil	Tsukuba A1	A	274	0.498	Tsukuba, Ibaraki
Volcanogenous regosol	Nanokahara A1	B	175	0.591	Zao, Miyagi
Brown forest soil	Ochiai A12	B	128	0.582	Tama, Tokyo
Podozol	Jumonji Bh	P_{+++}	105	0.613	Jumonji, Saitama
Red and yellow soil	Shinyahara A1	Rp(1)	84	0.745	Iwata, Shizuoka
Podozol	Jumonji A0	Rp(2)	57	0.749	Jumonji, Saitama

Table 2. Chemical composition of the sample soils and their extracted humic acids.

Soil name and horizon	HA type	Carbon content of the whole soil	Elemental analysis of HA				Degree of unsaturation
			C	H	N	O	
Myojin A1	A	16.4%	44	34	2	21	63
Tsukuba A1	A	4.2%	41	35	2	23	59
Nanokahara A1	B	5.1%	38	40	3	19	50
Ochiai A12	B	5.7%	37	43	2	18	45
Jumonji Bh	P_{+++}	5.9%	34	41	3	21	44
Shinyahara A1	Rp(1)	5.2%	35	47	2	16	37
Jumonji A0	Rp(2)	41.2%	36	45	2	17	40

Conclusions

We found we could obtain greater PCP reduction when humic acid samples had a lower degree of humification. This characteristic may be useful in choosing soils for remediation and estimating the pollution duration of various soil types.

Figure.2: Effect of the co-existence of humic acids on the enzymatic transformation of PCP (Shinyahara Rp-type humic acid was used)

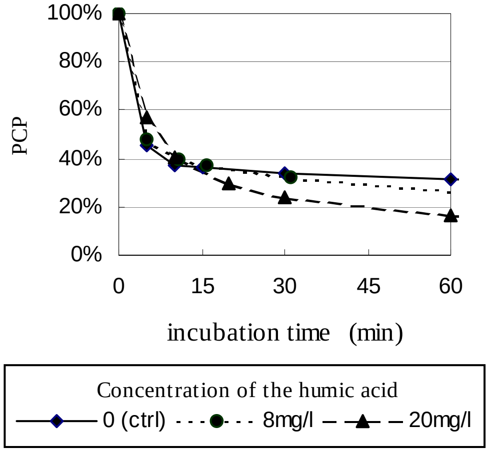


Figure.3: Variation of the rate of PCP decrease among different types of co-existing humic acids.

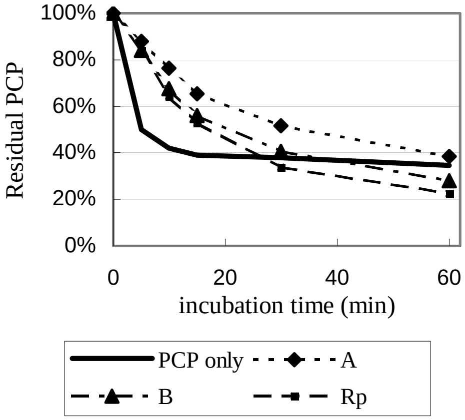


Figure 4: Relation ship between PCP transformation rate and the degree of humification of co-existing humic acids.

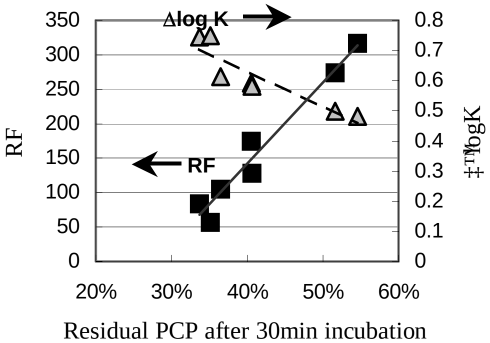
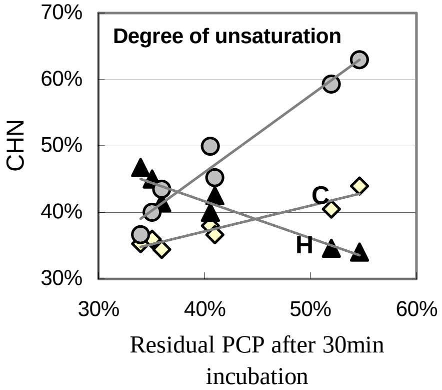


Figure 5: Relation ship between PCP transformation rate and CHN elemental constitution of co-existing humic acids



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