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CHEMICAL STUDIES ON SOIL HUMIC ACIDS

IV. Amino Acid, Phenol, and Sugar Composition in the Acid Hydrolysable Fraction of Humic Acids

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The levels of amino acids, phenolic substances, sugars, and total hydrolysable organic matter of 37 humic acids obtained from various types of soils were determined after acid-hydrolysis.

The levels of amino acids, hydrolysable phenols and hydrolysable organic matter increased from Rp(2) type to Rp(1) type, and decreased with increasing *RF* values among Rp(1), P_0, B, and A type humic acids. Very significant positive linear correlations were found among the levels of these acid hydrolysates. Hexose an duronic acid contents decreased with increasing *RF* values. The linear correlations between the hexose and uronic acid contents and *RF* values were very high for calcareous soil humic acids but low for the humic acids from other soils. Amino acids, phenols, and sugars were the main constituents in the acid hydrolysable fraction in humic acids. It was found that absorbance at 210 nm of the acidic supernatant of the hydrolysate was useful for determining the total organic matter content.

Additional Index Words: humic acid, acid-hydrolysis, calcareous soil, humification.

Though humic acid has been investigated extensively as an important fraction in soil organic matter (*3, 5*), its chemical structure and composition have been elucidated little, because it consists of various organic substances most of which are dark colored amorphous high molecules. We have studied the compositions of C, H, N, and O (*6*), oxygen-containing functional groups (*8*), and nitrogenous constituents (*9*) of humic acids and found that these compositions were closely related to the degree of humification and soil types. In this study, amino acid, phenol, and sugar compositions in the acid hydrolysable fraction in humic acids were investigated in order to find their relationships to the degree of humification and soil types.

MATERIALS AND METHODS

1. Humic acids. Thirty-seven humic acids obtained from various soils (Table 1) were analyzed. The origin, method of isolation and purification, elementary and functional group compositions, and nitrogen distribution of these samples have been reported in our previous papers (*6, 8, 9*).

Table 1. Carbon distribution in acid-hydrolysable constituents of humic acids.

Type	No.	Sample	Symbol	*RF*	C % total carbon in humic acid						Uronic acid
					Amino acid (A)	Hydrolysable phenol (P)	Hexose (H)	Uronic acid (U)	(A+P+H+U)	Hydrolysable organic matter	Hexose (U/H)
A	2	Temmondai	●	144	7.49	1.42	1.45	0.612	10.97	9.19	0.454
	3	Nagara-P	■	140	4.12	0.995	0.401	0.344	5.86	7.13	0.925
	4	Inogashira-1	●	138	5.11	1.82	2.31	0.864	10.10	11.9	0.402
	5	Jaana-P	■	132	7.38	1.11	0.603	0.348	9.44	5.83	0.623
	6	Inogashira-2	●	115	6.00	1.68	1.94	0.839	10.46	9.95	0.466
	7	Kinshozan-P,	■	115			1.32	0.553			0.451
	8	Shitara-black	●	114	5.06	1.35	1.43	0.709	8.55	6.92	0.533
	9	Nagara-F	■	102	6.13	1.40	0.89	0.655	9.08	6.08	0.793
	10	Fujinomiya-3	●	97	8.45	2.79	1.91	0.866	14.02	13.6	0.490
	12	Kinshozan-P	■	92	6.97	1.54	1.06	0.474	10.04	8.60	0.480
	13	Komagahara	●	89	11.0	2.32	2.15	0.827	16.3	14.5	0.412
	14	Nagara-OH	■	89	9.32	2.70	0.918	0.779	13.7	13.6	0.915
	15	Fujinomiya-2	●	82	11.3	2.74	1.73	0.772	16.5	16.1	0.482
B	16	Shitara-brawn	◑	76	16.2	5.13	3.62	1.36	26.3	18.7	0.403
	40	Hichiso	◑	51	13.6	3.37				15.8	
	18	Kuragari-1	◑	61	9.79	2.82	2.28	0.820	15.7	14.3	0.386
	19	Kinshozan-F	◪	60	13.0	3.36	1.74	1.09	19.2	14.9	0.677
	20	Anjo black	⊕	57	13.7	3.99	1.90	0.777	20.4	17.5	0.442
	21	Higashiyama (A)	◑	47	11.1	3.06	1.51	0.584	16.3	15.1	0.418

Rp(1)	28	Ishimakisan-F	□	37			1.53	0.773			0.546
	29	Sanage-1	⊕	32	17.2	4.88	2.83	0.700	25.6	22.8	0.266
	31	Jaana OH	□	30	26.0	5.42	2.09	1.15	34.7	24.3	0.590
	32	Kinshozan OH-1	□	29	21.6	5.20	2.03	1.20	30.0	21.7	0.635
	34	Ishimakisan-OH	□	22	17.7	5.00	2.09	0.888	25.7	21.5	0.456
	35	Gifu	⊕	22	21.2	6.67	3.00	0.815	31.7	26.6	0.293
	36	Anjo	⊕	21	16.2	5.13	2.51	0.789	24.6	21.5	0.338
	39	Kinshozan-OH-2	□	16	22.2	6.42	3.17	1.05	32.8	24.4	0.355
Rp(2)	30	Higashiyama (F-H)	○	31	9.06	3.14	1.90	0.739	14.8	13.9	0.419
	33	Higashiyama (L)	○	23	10.6	3.32	2.40	0.792	17.1	15.7	0.354
	37	Yamamomo	○	21	3.11	1.55	1.02	0.347	6.03	8.17	0.367
	38	Kisokoma (F)	○	20	12.1	3.68	2.25	0.793	18.8	17.5	0.379
P_0	23	Ishimakisan-P	◪	43			0.978	0.489			0.540
	24	Kuragari-2	◐	43	11.4	3.33	1.71	0.754	17.2	15.6	0.476
	26	Onobaru	◐	39	12.0	4.37	3.30	1.36	21.0	21.7	0.446
	27	Sanage-2	⊕	38	13.1	5.66	2.48	0.876	22.1	26.2	0.381
P_{+++}	1	Tsubame-Pg	◎	202			0.112	0.089			0.856
P_{+}	11	Tsubame-4	◎	94	7.69	2.66	0.677	0.353	11.4	11.9	0.562
P_{++}	22	Tsubame-1	◎	69	12.5	3.39	2.41	1.28	19.6	17.9	0.573
P_{++}	25	Shishi	◎	42	13.9	3.21	3.48	1.04	21.6	18.5	0.321

Symbols: kuroboku soil A ● P_0 ◐, calcareus soil A ■ B ◪ Rp(1) □ P_0 ◐, A_0 layer of forest soil ○, paddy soil ⊕, $P_{+\sim+++}$ ◎.

2. Determination of amino acid, phenol, and total organic matter contents in the 8 N *H_2SO_4 hydrolysable fraction.* Methods of 8 N H_2SO_4 hydrolysis and the determination of total amino acid content were reported in detail in a previous paper (*9*). Humic acid sample was hydrolyzed with 8 N H_2SO_4 at 110°C for 20 hr. The supernatant of the hydrolysate was analyzed to determine the contents of amino acids, phenols, and total organic carbon. The contents of phenolic substances were determined as follows. A 0.5 ml portion of the supernatant was taken into a test tube, and 3 ml of Folin's reagent diluted with 5 volumes of water and 1 ml of 20% Na_2CO_3–4% NaOH mixture were added in this order. After leaving to stand for 30 min at 30°C, the color was measured at 750 nm. The calibration curve was made with standard solutions of phenol. The contents of total organic carbon were determined according to TATSUKAWA's method (*7*). One ml of the supernatant solution was taken into a test tube, and 2 ml of 0.5 N $K_2Cr_2O_7$ in conc. H_2SO_4 was added. After 30 min, the color was measured at 645 nm. The calibration curve was made with standard solutions of glucose.

3. UV spectra. A 0.5 ml portion of the supernatant solution was diluted with 2.5 ml of water and the UV spectrum was measured between 210 nm and 400 nm with Hitachi-124 spectrophotometer.

4. Determination of the contents of hexose and uronic acid. Humic acid (10–50 mg) dissolved in 5 ml of 0.1 N NaOH was taken into a test tube with a reflux condenser, and 1 ml of 6 N H_2SO_4 was added. The mixture was heated on a boiling water bath for 2 hr. The hydrolysate was made to 10 ml in a volumetric flask and filtered through dry filter paper. The filtrate was analyzed for the contents of hexose and uronic acid by colorimetry. Hexose content was determined by the method of ATAKE and SENO (*1*) except that the optical density was measured at 625 nm instead of 620 nm. Correction was made for background color. Calibration curve was made with standard solutions of glucose. Uronic acid content was determined by the method of BITTER and MUIR (*2*). Correction was made for background color. Calibration curve was made with standard solutions of glucuronic acid.

RESULTS AND DISCUSSION

1. Amino acid content

Total yields of amino acid by 8 N H_2SO_4 hydrolysis of humic acids ranged from 400 to 2800 μmol/g (*9*), and increased from Rp(2) type humic acids which were isolated from A_0-layer humus or rotted plants to Rp(1) type humic acids which were isolated from A-layer soils or plowed layer soils, and decreased in the order of Rp(1), P_0, B and A type humic acids with increasing degree of humification. Amino acid-carbon is supposed to be the main fraction of the acid-hydrolysable-carbon in humic acids (Table 1).

2. Phenolic substance content

Very significant positive linear correlations were found between the yields of total phenolic substance and total amino acid by 8 N H_2SO_4 hydrolysis ($r=0.883^{***}$) and between the yields of the former and the acid-hydrolysable organic matter ($r=0.967^{***}$). Tyrosine and tryptophan react with Folin's reagent, and are estimated into the yield of phenolic substance. Tyrosine, however, was only 1.56±0.55% of the total amino acids in molar quantity and tryptophan was not detected in the supernatant of the hydrolysate (*9*), and the total yields of amino acid ranged from 400 to 2800 μmol, so that the yields of tyrosine ranged from 6 to 45 μmol/g, which accounted for less than one tenth of the total hydrolysable phenolic substances ranging from 100 to 500 μml/g. Though tyrosine and tryptophan are degraded considerably during acid-hydrolysis, most of the hydrolysable phenolic substances in humic acids are presumed to be phenolic substances other than tyrosine and tryptophan.

Similar to the trends observed for amino acids, the total yields of hydrolysable phenolic substances increased from Rp(2) type (obtained from A_0-layer humus and rotted plants) to Rp(1) type humic acids (obtained from A-layer and plowed layer soils), and then decreased in the order of Rp(1), P_0, B and A type humic acids with increasing *RF* values (Fig. 1). Because of their similar trends, the proteinous substances and hydrolysable phenolic substances in humic acids are presumed to be combined with each other and to have similar stabilities, though sufficient proofs are needed to certify whether they are combined or not. It is also presumed that the combination of the two substances takes place during the first stage in humification (from Rp(2) type to Rp(1) type), and the degradation takes place during the next stage (among Rp(1), P_0, B, and A type humic acids).

The levels of hydrolysable phenolic substances varied with *RF* value quite differently from the way the total phenolic hydroxyl group contents of humic acids did. Total phenolic hydroxyl group contents decreased from Rp(2) type to Rp(1) type humic acids

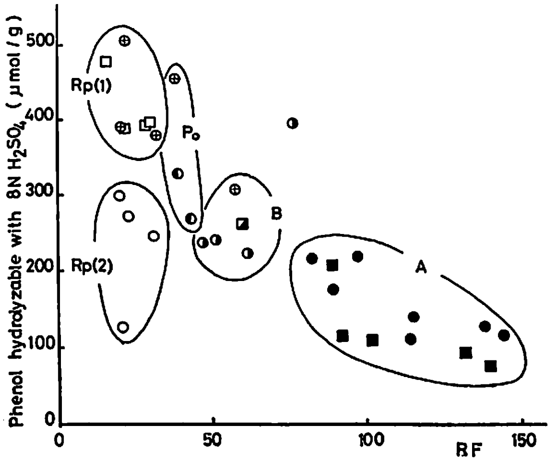

Fig. 1. Relationship between the level of acid-hydrolysable phenol and *RF* value of humic acids.

and then increased gradually with increasing *RF* value among Rp(1), P_0, B and A type humic acids (*8*). The high levels of phenolic group in Rp(2) type humic acids may be attributed to the phenolic plant constituents, such as lignin, phenolic acids, and flavonoids remaining in humic acid structures. From the alkaline degradation of humic acids, it was found that the contribution of lignin in humic acid structure was high but that of tannin and flavonoid was very low (*10*). Contrary to the total phenolic hydroxyl contents, the yields of acid-hydrolysable phenolic substances from Rp(2) type humic acids were lower than those from Rp(1) type humic acids. The yield was the lowest for the humic acid (No. 37) which was isolated from rotted wood of *Myrica rubra* containing high levels of flavonol (Myricitrin). Lignin is also stable and degraded little by acid-hydrolysis. Therefore, it is presumed that the phenolic substances detected in the acid-hydrolysates of humic acids are mainly due to phenolic substances other than lignin and flavonoid. These phenolic substances may be due to microbial metabolites and chemical degradation products of residual constituents of plants. Therefore, they increased during the first stage of humification. The higher levels of hydrolysable phenolic substances in Rp(1) type humic acids than in Rp(2) type humic acids were also attributed to the vegetations. In this study the vegetations of the soils from which the Rp(1) type humic acids were taken all happened to be gramineous, on the other hand, those for the Rp(2) type humic acids were wood. It was reported that lignin in gramineous plants contains more phenolic acids in the form of esters compared with lignin from other sources (*4*). But among Rp(1), P_0, B, and A type humic acids, the levels of hydrolysable phenolic substances decreased with increasing *RF* values, and the difference in vegetation did not affect this trend.

3. Hexose content

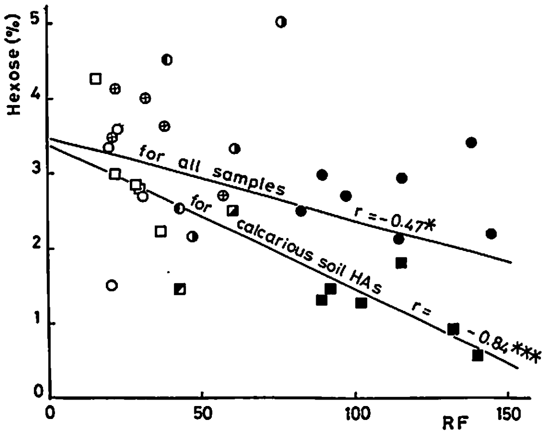

Fig. 2. Relationship between the level of hexose and *RF* value of humic acids.
Calcareous soil humic acids are denoted by ■, ◪, ◩, and □ for A, B, P_0, and Rp type, respectively.

The levels of hexose in humic acids ranged from 0.5% to 5% (dry and ash-free basis), and tended to decrease with increasing *RF* values (Fig. 2). A significant negative linear correlation ($r=-0.472^{*}$) was found between the hexose level and *RF* value of all the humic acids except those belonging to $P_{+\sim\#}$ type. Compared with other humic acids of similar *RF* values, the humic acids extracted from calcareous soils contained lower levels of hexose. Especially among A type humic acids, there were obvious differences between the hexose contents in the humic acids from calcareous soils and those from kuroboku soils. The humic acids obtained from calcareous soils were isolated by successive extraction with different solvents. Nevertheless, considerable amounts of hexose still remained in the second and third extractions. A negative linear correlation between the hexose content and *RF* value was found to be much more significant for the humic acids obtained from calcareous soils ($r=-0.842^{***}$) than for all the humic acids ($r=-0.472^{*}$). In calcareous soils organic matters are thought to be oxidized so readily on account of their high pH values that the levels of hexose in humic acids rapidly reach certain values corresponding to the degree of humification.

A significant positive linear correlation ($r=0.607^{***}$) was found between the levels of hexose and alcoholic hydroxyl group in humic acids (Fig. 3). The hydroxyl group in polysaccharides amounted to about 15% of the total level of alcoholic hydroxyl group in humic acids. On the other hand, the percentage recovery of hydroxyl group was about 15% for cellulose powder (200 mesh), and 100% for glucose by the quantitative acetylation method used here (*8*). Since most of the hexoses in humic acids are thought to exist in the form of polysaccharides, the hydroxyl group in polysaccharide may be more than 15% of the total level of alcoholic hydroxyl group in humic acids.

4. Uronic acid content

The levels of uronic acid ranged from 0.5% to 2%. Though no significant linear correlation ($r=-0.319$ N.S.) was found between the level of uronic acid and *RF* value for all the sample, very significant negative linear correlation ($r=-0.780^{**}$) was found

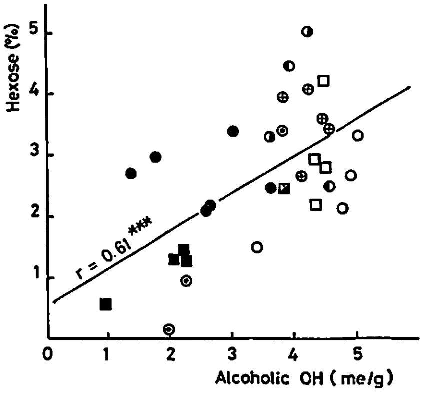

Fig. 3. Relationship between the hexose and alcoholic hydroxyl contents of humic acids.

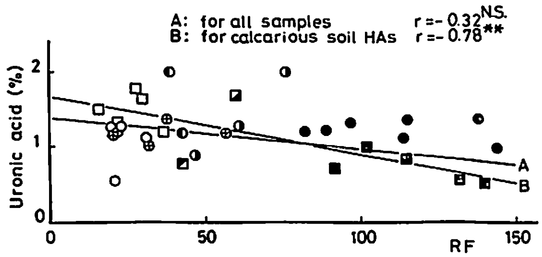

Fig. 4. Relationship between the level of uronic acid and *RF* value of humic acids.

between these values for calcareous soil humic acids (Fig. 4). Moreover, the ratios of uronic acid content to hexose content of the humic acids obtained from calcareous soils were higher than other humic acids having similar *RF* values (Table 1). These results were probably obtained because the organic matters rich in carboxyl groups are stabilized by complex formation with calcium.

5. *Contribution of the carbon contents distributed in amino acids, phenolic substances, and sugars as acid-hydrolysable constituents to the total carbon content of humic acid*

Of the total carbon contents in humic acids, the carbon contents in amino acids accounted for 4–26%, in hydrolysable phenolic substances 1–6.7%, in hexose 0.6–3.5%, and in uronic acid 0.3–1.4%. The sum of the carbon contents of acid-hydrolysable constituents, (A+P+H+U) value in Table 1, accounted for 5.9–35% of the total carbon content in humic acids. On the other hand, the levels of carbon contained in the supernatant solutions of the 8 N H_2SO_4 hydrolysates of humic acids, which were determined directly by TATSUKAWA's method (*7*), accounted for 5.8–27% of the total carbon. These values of hydrolysable organic matter carbon were smaller than the (A+P+H+U) values, and those of Jaana P, Nagara F and Jaana OH humic acids were smaller than the amino acid carbon contents of these humic acids. This discrepancy of the determined values may be because each value of amino acid, hydrolysable phenol, hexose, uronic acid and hydrolysable organic matter contents was determined colorimetrically using different standard materials. These values, as well as the carbon contents determined by colorimetry are approximate values and may not always lead to the real contents of these constituents as already discussed here and elsewhere (*9*). The values of (A+P+H+U) and hydrolysable organic matter were similar in most of the humic acids examined here, however, and a very significant positive linear correlation ($r=0.931^{***}$) was found between them. Therefore, great part of the acid-hydrolysable fraction of humic acids is supposed to be accounted for by amino acids, hydrolysable phenolic substances, and sugars.

6. *UV spectra*

UV spectra of the supernatant of the acid-hydrolysates (Fig. 5) were very similar among all the humic acids used here except the humic acid (No. 37) isolated from rotted wood of *Myrica rubra*. Moreover, a very significant positive linear correlation

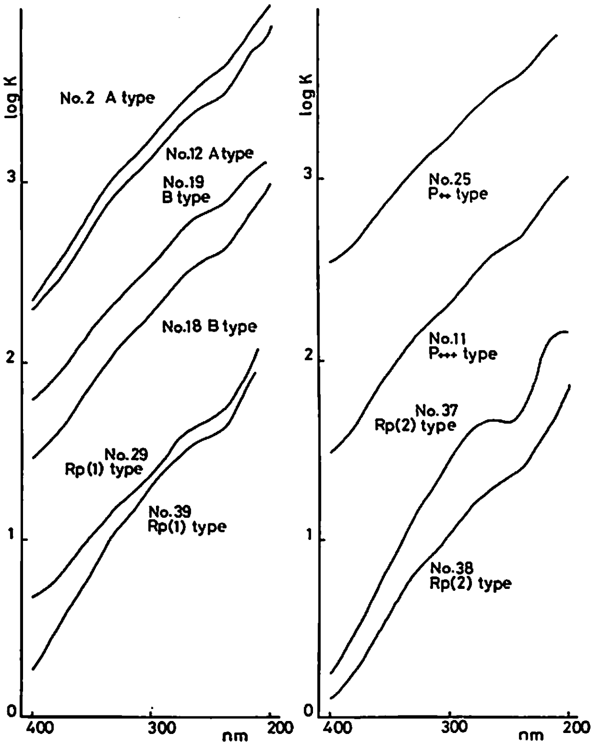

Fig. 5. UV spectra of the acid-hydrolysate solutions of humic acids.

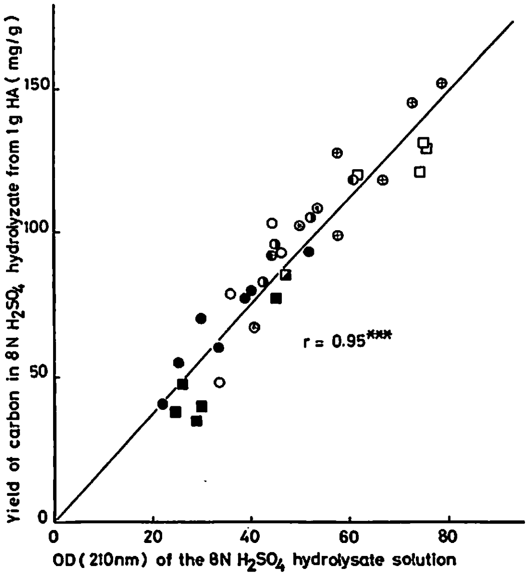

Fig. 6. Relationship between the carbon content and the O.D. at 210 nm of the acid hydrolysate solution.

($r=0.946^{***}$) was found between the absorbance at 210 nm of the supernatant and the level of acid-hydrolysable organic matter. This may be due to the fact that the absorption at 210 nm is common to the organic substances with double bonds, and that the absorption by CO_3^{2-} or HCO_3^- ion does not interfere because the spectra are measured in acidic solution. The supernatant solution containing carbon at 0.1% level gave an optical density of about 60 at 210 nm, while the absorbance of the same solution at 645 nm by TATSUKAWA's method was about 0.68. Because the reproducibility of the optical density at 210 nm depends solely on the error by sampling and dilution, the experimental errors were less than 0.1%. Reproducibility of TATSUKAWA's method is also high and the errors are less than 1%, but it requires sample solutions with relatively high concentrations. Because of their low concentrations, our samples gave optical densities of about 0.1 or less, so that the experimental errors by TATSUKAWA's method were more than 1% in our case. Therefore, the amount of organic matter in the supernatant of the acid-hydrolysate can be determined more easily with higher sensitivity and reproducibility by UV absorbance at 210 nm than by TATSUKAWA's method.

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