Aoyama *Chemical and Biological Technologies in Agriculture* (2015) 2:16
DOI 10.1186/s40538-015-0041-5

Chemical and Biological Technologies in Agriculture
a SpringerOpen Journal

RESEARCH Open Access

Separation of acid-soluble constituents of soil humic acids by dissolution in alkaline urea solution and precipitation with acid

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Abstract

Background: Humic substances are considered to be composed of relatively small, heterogeneous molecules bound by weak linkages. The dissociation of acid-soluble constituents from soil humic acids (HAs) during the preparative polyacrylamide gel electrophoresis in the presence of concentrated urea has been previously demonstrated. Moreover, the dissociation of acid-soluble constituents has been attributed to the action of concentrated urea. The aim of this study was to investigate the effects of concentrated urea on the dissociation of acid-soluble constituents of soil HAs.

Results: Three types of soil HAs were solubilized in 0.1 M NaOH containing 7 M urea and precipitated after 16 h by acidifying the samples to pH 1.0. The acid-soluble constituents were separated from the dark-colored precipitates by concentrated urea treatment and accounted for 16–45 % of the total organic carbon in HAs. Approximately half of the acid-soluble constituents was recovered in the DAX-8-adsorbed fraction. The humification degree of the DAX-8-adsorbed fraction was considerably lower than that of the corresponding unfractionated HA. In contrast, the humification degree of the precipitated fraction increased due to the separation of acid-soluble constituents. The molecular sizes of the DAX-8-adsorbed and DAX-8-non-adsorbed fractions, estimated by high-performance size exclusion chromatography, were similar and smaller than the precipitated fraction. Three-dimensional excitation-emission matrix fluorescence spectroscopy revealed that the acid-soluble constituents exhibited fluorescence similar to that of fulvic acid (FA), added to which the DAX-8-non-adsorbed fraction exhibited protein-like fluorescence. Diffuse reflectance infrared Fourier transform spectroscopy showed that the DAX-8-adsorbed fraction contained proteinous moieties and the DAX-8-non-adsorbed fraction was rich in proteinous and polysaccharide moieties.

Conclusions: The present findings suggest that soil HAs are formed by the molecular associations between dark-colored acid-insoluble constituents, FA-like acid-soluble constituents, protein-like constituents, and polysaccharides bound by weak linkages.

Keywords: Acid-soluble constituents; Concentrated urea; Degree of humification; Fluorescence; Humic acid

Background

Soil organic matter is a complex, heterogeneous mixture resulting from the decomposition of plants, animals, and microorganisms in the soil environment. Soil humic acids (HAs) are usually extracted from soil using an alkaline solution and precipitated by acidification; therefore, these are considered to be heterogeneous in composition. Recent research has suggested that humic substances are formed due to the association between heterogeneous, relatively small molecules derived as a result of the degradation and decomposition of biological material. It is considered that the constituent molecules are bound by weak linkages, such as hydrogen-bonding and hydrophobic interactions [1–3].

Our previous study on two-dimensional (2-D) electrophoresis of HAs in the presence of 7 M urea showed that the HAs were separated into their constituents with different charge characteristics and molecular sizes [4]. In that study, the 7 M urea was used to facilitate the dissociation of HA constituents by disrupting the hydrogen-

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bonding and hydrophobic interactions [1, 5, 6]. However, we observed a low recovery of organic carbon in the precipitates after acidification to pH 1.0, indicating the dissolution of a significant quantity of HAs in the acid solution upon being subjected to electrophoresis [4].

We further subjected the HAs to polyacrylamide gel electrophoresis (PAGE) in the presence of 7 M urea, using a preparative electrophoresis system [7]. Acidification of the electrophoretic fractions resulted in the separation of acid-soluble constituents from the dark-colored precipitates. A part of the acid-soluble constituents could be recovered by adsorption onto DAX-8 resin. The degree of humification was higher in the precipitates and lower in the acid-soluble DAX-8-adsorbed constituents, when compared to the corresponding whole HA. Thus, our previous studies have demonstrated that acid-soluble HA constituents can be dissociated by electrophoresis in the presence of concentrated urea.

Concentrated urea enhances the solvation capacity of alkaline solution, leading to the use of an alkaline concentrated urea solution in the extraction of humin from soils [8]. Concentrated urea could also be used in the fractionation of HAs into constituents displaying different chemical properties, using size exclusion chromatography [1, 9, 10]. Furthermore, soil HA constituents with different fluorescent properties were separated by ultrafiltration in the presence of concentrated urea [11, 12]. The acid-soluble constituents can be dissociated by acidification after dissolution in alkaline medium [13]. However, the abovementioned findings suggested that the dissociation of acid-soluble constituents from soil HAs was attributable to the action of concentrated urea.

The purpose of this study was to investigate the effect of concentrated urea on the dissociation of acid-soluble constituents from soil HAs. Three soil HA samples were solubilized in 0.1 M NaOH containing 7 M urea and precipitated by acidification to pH 1.0. The supernatant solutions were further separated into the DAX-8-adsorbed and DAX-8-non-adsorbed fractions. The fractionated constituents were characterized by degree of humification, high-performance size exclusion chromatography (HPSEC) with UV and fluorescence detections, three-dimensional excitation-emission matrix (3-D EEM) fluorescence spectroscopy, and diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy.

Materials and methods

Preparation of HAs

The HAs used in this study were prepared from a Fluvisol (Fujisaki HA) and an Andosol (Takizawa HA) and purchased from the Japanese Humic Substances Society (Dando HA). The Fujisaki and Takizawa HAs were extracted twice from the soil samples containing 500 mg of organic carbon with 150 mL of 0.1 M NaOH under N_2 by intermittent shaking for 24 h and were separated by acidification to pH 1.0 with HCl. The HA precipitates were obtained by centrifugation at 10,000×*g* for 30 min and dissolved in 150 mL of 0.1 M NaOH containing 0.3 M KCl, then precipitated again by acidification to pH 1.0 with HCl. The dissolution-precipitation cycle was repeated twice. The resultant precipitates were suspended in a 0.1 M HCl/0.3 M HF solution and shaken overnight. This step was repeated three times to minimize the ash content. The suspension was dialyzed against ultrapure water and freeze-dried. The Dando HA is the standard HA provided by the Japanese Humic Substances Society, and the details were reported elsewhere [14]. The elemental compositions of the HA samples used are presented in Table 1.

Fractionation with an alkaline concentrated urea solution

Forty milligrams of the HA samples was dissolved in 40 mL of 0.1 M NaOH containing 7 M urea and left standing for 16 h at 25 °C under N_2, then acidified with HCl to reach a pH of 1.0. The dark-colored precipitates were obtained by centrifugation at 10,000×*g* for 10 min and washed four times with 0.01 M HCl and once with ultrapure water. The resultant precipitates were freeze-dried and designated as the precipitated fraction. The supernatant and the washings were combined and passed through a column containing DAX-8 resin (Supelco, Bellefonte, PA, USA). The column was washed with 0.1 M HCl followed by ultrapure water. The pass-through solution of the DAX-8 column and the washings were combined and dialyzed in a Spectra/Por 7 membrane tubing (nominal molecular weight cutoff of 1000 Da; Spectra/Por, Rancho Dominguez, CA, USA) against ultrapure water, and the dialysate was designated as the DAX-8-non-adsorbed fraction (>1 kDa). The fraction was concentrated to 10 mL using a rotary evaporator at 40 °C. The concentrated solution was divided into five 2-mL portions and freeze-dried. The constituents adsorbed onto the DAX-8 resin were eluted with 0.1 M NaOH and passed through a cation exchange resin (H^+ form) column. This was designated as the DAX-8-adsorbed fraction. The fraction was concentrated to 10 mL using a rotary evaporator at 40 °C, divided into five 2-mL portions, and freeze-dried. As a control treatment, 40 mg of the HA samples was dissolved in 40 mL of 0.1 M NaOH (without urea) and treated in the same manner, with the exception that the DAX-8-

Table 1 Elemental composition of the humic acid samples used

Humic acid	Ash[a] (g kg^{-1})	Elemental composition[b] (g kg^{-1})				
		C	H	N	S	O
Fujisaki	9.6	530	51.7	56.2	7.4	355
Dando	6.7	530	52.5	44.9	2.9	369
Takizawa	6.7	568	41.0	39.5	2.5	349

[a]On a moisture-free basis
[b]On a moisture and ash-free basis

non-adsorbed portion was used only for the analysis of organic carbon concentration without dialyzing and freeze-drying.

Determination of organic carbon

For the precipitated fraction, 1 mg of the freeze-dried sample was dissolved in 800 μL of 0.1 M NaOH and a part of the solution was mixed with four times the volume of 0.067 M potassium dihydrogen phosphate [15], vortexed, and then allowed to stand at room temperature overnight to remove inorganic carbon. The total organic carbon concentration of the mixture was determined using a total organic carbon analyzer (TOC-V_E; Shimadzu Co., Ltd., Kyoto, Japan). For the DAX-8-adsorbed and DAX-8-non-adsorbed fractions, a divided portion of the fractions was dissolved in 800 μL of 0.1 M NaOH and determined the concentration of total organic carbon in the same manner as described above.

Evaluation of humification degree

The absorbances of the precipitated and DAX-8-adsorbed fractions used for the analysis of total organic carbon content at 400 and 600 nm were determined using a V-630 spectrophotometer (JASCO Corp., Tokyo, Japan). The degree of humification of the precipitated and DAX-8-adsorbed fractions was evaluated based on the A_{600}/C and $\log(A_{400}/A_{600})$ values, where A_{400}, A_{600}, and C denoted the absorbance at 400 and 600 nm and the carbon concentration (mg mL^{-1}), respectively [15].

HPSEC

The molecular size distribution was estimated by HPSEC as described in our previous study [4]. For HPSEC, the freeze-dried samples were dissolved in 1 mL of 0.1 M NaOH and then neutralized by passing through a cation-exchange cartridge (H^+ form; Dionex OnGuard II H, Thermo Fisher Scientific K.K., Yokohama, Japan). The solution was adjusted to the same composition as the mobile phase and then filtered through a 0.45-μm membrane filter. A 100-μL portion of the sample solution was injected. The chromatograms were monitored by UV absorption using a photodiode array detector (MD-2018, JASCO Corp., Tokyo, Japan) and by fluorescence at excitation and emission wavelengths of 460 and 520 nm, respectively, using a fluorescence detector (FP-920, JASCO Corp., Tokyo, Japan). To estimate the molecular weight (MW), the column was calibrated using polyethylene glycols as the MW standards [7].

3-D EEM fluorescence spectroscopy

The freeze-dried sample was dissolved in 1 mL of 0.1 M NaOH and neutralized by passing through a cation-exchange cartridge (H^+ form; Dionex OnGuard II H, Thermo Fisher Scientific K.K., Yokohama, Japan). The solution was diluted with ultrapure water and added with 0.05 M phosphate buffer (pH 8.0) to a final concentration of 5 mg organic carbon L^{-1} in 0.01 M phosphate buffer. The 3-D EEM fluorescence spectra were recorded in a 1-cm quartz cell using a FP-8300 scanning spectrofluorometer (JASCO Corp., Tokyo, Japan) equipped with an automatic higher order diffraction cut filter. The spectra were recorded over the excitation and emission wavelength ranges of 200–550 nm and 250–600 nm, respectively, and then corrected for instrumental bias according to the manufacturer's method. Inner-filter effect was corrected using the following equation [16, 17]:

$$\mathrm{Em_{real}} = \mathrm{Em_{obs}} \times 10^{b \times (Aex + Aem)}$$

where $\mathrm{Em_{obs}}$ was the observed fluorescence intensity, $\mathrm{Em_{real}}$ denoted the fluorescence in the absence of self-absorption, b was 0.5 cm, and the path length to the center of the cell for both excitation and emission, A_{ex} and A_{em}, denoted the absorbance at excitation and emission wavelengths, respectively. The Raman scatter effect was minimized by subtracting EEM spectrum of 0.01 M phosphate buffer (pH 8.0). The relative fluorescence intensity was expressed as the quinine sulfate unit (QSU) using the fluorescence intensity of a quinine sulfate solution (0.01 mg L^{-1} in 0.05 M H_2SO_4) at the excitation/emission wavelengths ($\lambda_{ex}/\lambda_{em}$) = 350/450 nm.

DRIFT spectroscopy

DRIFT spectra of the fractions were recorded using an FT/IR-4100 spectrometer (JASCO Corp., Tokyo, Japan) equipped with a DR-81 diffuse reflectance accessory (JASCO Corp., Tokyo, Japan). The freeze-dried samples were thoroughly mixed with 50–100 times the amount of potassium bromide (FT-IR grade; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in an agate mortar and pestle and placed in an aluminum sample cup. Spectra were collected from 4000 to 800 cm^{-1} and averaged over 100 scans and then transformed into Kubelka-Munk units [18]. The resolution was set at 4 cm^{-1}. To identify the principal bands that contribute to the more complex band resulting from overlapping features, Fourier self-deconvolution (FSD) was performed for the wavenumber region between 1800 and 800 cm^{-1}, using the JASCO FT-IR software provided with the spectrometer.

Results and discussion

Distribution of HA constituents among the fractions

The dissolution of HAs in 0.1 M NaOH alone and subsequent acidification resulted in the dissociation of acid-soluble constituents from the dark-colored precipitates (Fig. 1). The proportion of the organic carbon, recovered as precipitates, was 84 % for the Fujisaki HA sample, 84 % for the Dando HA sample, and 92 % for the Takizawa HA

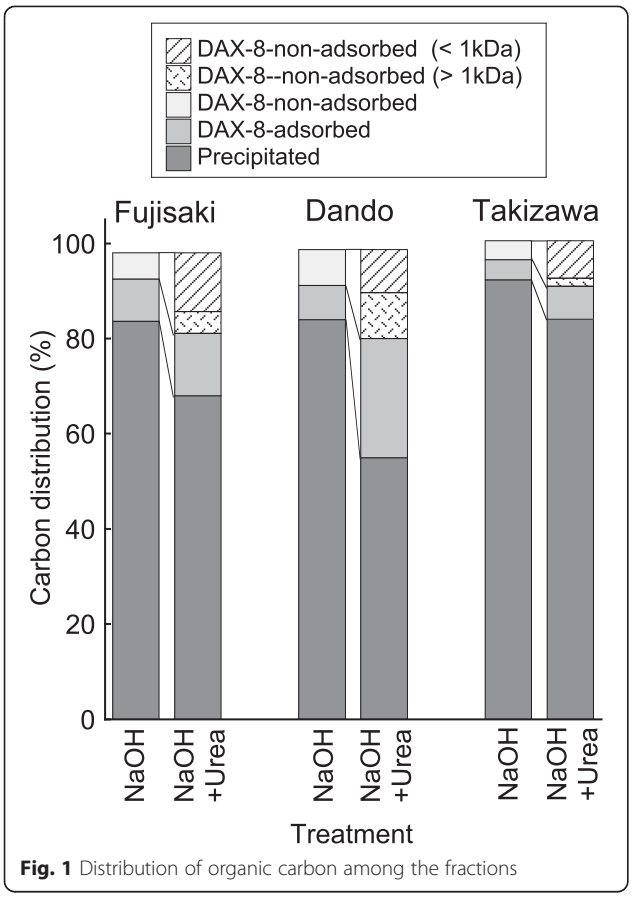

Fig. 1 Distribution of organic carbon among the fractions

sample. Among the acid-soluble constituents, the brown-colored constituents were recovered by adsorption onto the DAX-8 resin (DAX-8-adsorbed fraction). The organic carbon in the DAX-8-adsorbed fraction was approximately half of that in the acid-soluble constituents. Dissolution in alkaline concentrated urea enhanced the dissociation of acid-soluble constituents. The precipitated fraction accounted for 68 % of the total organic carbon in the Fujisaki HA sample, 54 % in the Dando HA sample, and 84 % in the Takizawa HA sample. The proportion of the brown-colored DAX-8-adsorbed fraction was significantly higher than when treated with 0.1 M NaOH. The DAX-8-non-adsorbed fraction was nearly colorless, and the proportion of the total organic carbon in this fraction was also increased by treatment with concentrated urea. However, a substantial part was lost during the dialysis (Fig. 1). The proportion of organic carbon in the unrecovered part was estimated to be 12, 9, and 8 % for the Fujisaki HA, Dando HA, and Takizawa HA samples, respectively.

Our previous study has demonstrated that acid-soluble constituents were separated from HAs during preparative PAGE in the presence of concentrated urea [7]. This study further revealed that the dissociation of acid-soluble constituents from HAs occurred when the HAs were dissolved in alkaline concentrated urea solution, followed by the acidification of the solution. Moreover, it was confirmed that the brown-colored acid-soluble constituents, as well as the nearly colorless acid-soluble constituents, were dissociated from HAs by concentrated urea treatment. Concentrated urea is considered to disrupt the hydrogen-bonding and hydrophobic interactions [1, 12, 19]. Therefore, the dissociation of HA constituents observed in this study can be attributed to the disruption of the hydrogen-bonding and hydrophobic interactions triggered by concentrated urea.

Humification degree of the fractions

In order to evaluate the humification degree of the precipitated and DAX-8-adsorbed fractions, A_{600}/C and $\log(A_{400}/A_{600})$ values were used (Fig. 2). An increase in the A_{600}/C value and a decrease in the $\log(A_{400}/A_{600})$ value were known to indicate an increase in the degree of humification of HA [15]. The degree of humification used here is synonymous with the degree of darkening [15, 20]. Kumada [20] classified HAs into four types according to their optical properties: type A HAs are the most humified, type B HAs are the intermediates between type A and type Rp, type Rp HAs are the least humified, and type P HAs are moderately humified, as indicated in Fig. 2.

The DAX-8-adsorbed fraction exhibited a lower A_{600}/C value and a higher $\log(A_{400}/A_{600})$ value compared to the corresponding whole HA sample irrespective of treatment with concentrated urea. The reverse was true for the precipitated fraction. These results indicated that the acid-soluble constituents of HA were characterized by a low degree of humification and that their dissociation from HA resulted in an increase in the humification degree of precipitated constituents. These findings were in agreement with our previous study, where preparative PAGE of HAs was carried out in the presence of concentrated urea [7]. The degree of humification of the precipitated fraction was significantly higher when treated with alkaline concentrated urea. This was attributed to the higher dissociation of acid-soluble constituents in the presence of concentrated urea.

Molecular size distribution of the fractions

The molecular size distributions were estimated using HPSEC with UV detection at 280 nm (Fig. 3). The molecular size distributions of the whole samples of Fujisaki and Dando HAs were similar but differed largely from that of Takizawa HA. An intense peak was observed at the void volume (V_0), and a broad peak was eluted in the MW region of 2–20 kDa for the Fujisaki and Dando HA whole samples. In contrast, for the Takizawa HA, a broad peak was eluted at a MW of 2 kDa. Thus, the

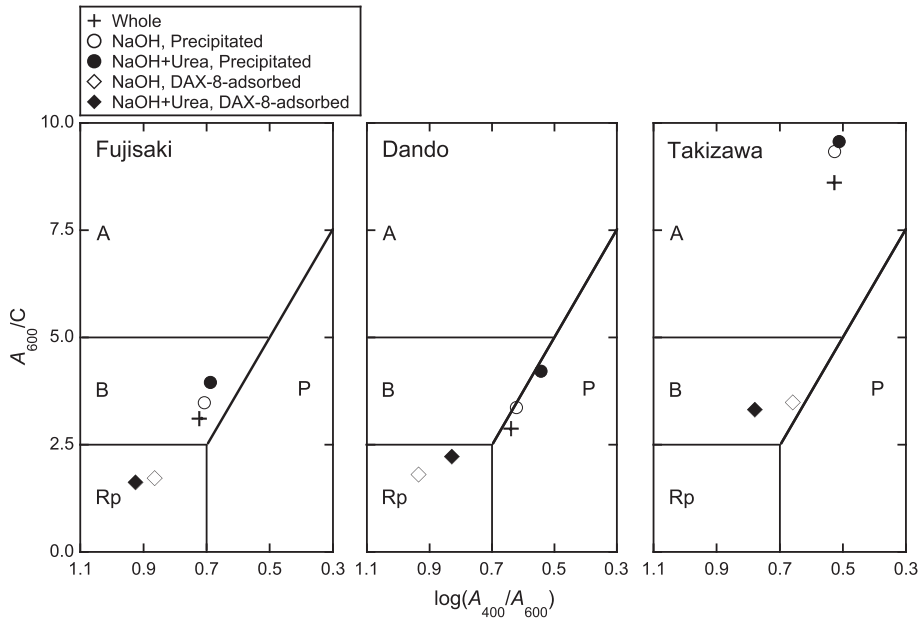

Fig. 2 $\log(A_{400}/A_{600})$ versus A_{600}/C diagram of precipitated and DAX-8-adsorbed fractions. The symbols (*A*, *B*, *P*, and *Rp*) in the figure indicate the types of HAs in Kumada's classification system [20]. The degree of humification is higher in the order *A* > *B* or *P* > *Rp*

Takizawa HA consisted mainly of relatively small molecular size constituents compared to the Fujisaki and Dando HAs.

For control treatment, the molecular size distribution of the precipitated fraction was similar to that of the corresponding whole HA, while the peak of DAX-8-adsorbed fraction was observed at a MW of 2 kDa irrespective of the HAs used. The molecular size of the DAX-8-adsorbed fraction was similar to those of fulvic acids (FAs) [21]. Higher molecular size distributions were obtained from the precipitated fractions treated with alkaline urea compared to those having received the treatment with 0.1 NaOH alone. This was attributed

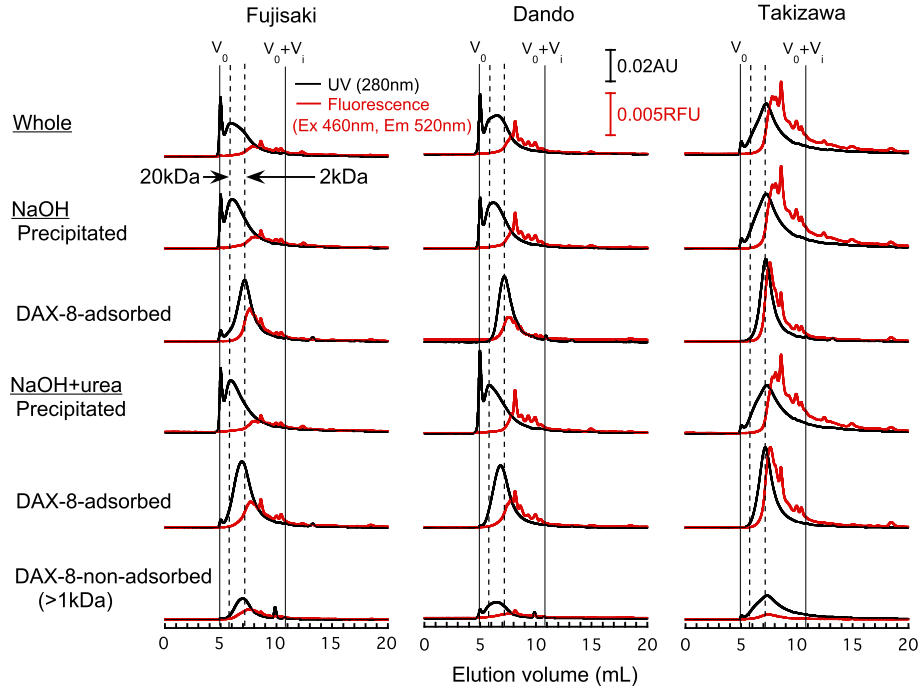

Fig. 3 Size exclusion chromatograms (UV detection at 280 nm; fluorescence detection at excitation 460 nm and emission 520 nm) of whole humic acid samples and their fractions normalized to the concentration of 100 mg carbon L^{-1}. V_0 void volume, $V_0 + V_i$ total effective column volume

to the higher dissociation of DAX-8-adsorbed and DAX-8-non-adsorbed fractions when treated with concentrated urea. The molecular size of the DAX-8-adsorbed fraction was similar to (in the case of Takizawa HA) or larger than (Fujisaki and Dando HAs) those observed for the control treatment. The DAX-8-non-adsorbed fraction (>1 kDa) showed a similar molecular size to that observed for the DAX-8-adsorbed fraction; however, the intensity of the peak was observed to be relatively lower. The molecular sizes of the acid-soluble constituents dissociated by concentrated urea treatment were similar to those dissociated by preparative PAGE in the presence of concentrated urea [7].

When detected by fluorescence at $\lambda_{ex}/\lambda_{em}$ = 460/520 nm, the peaks for whole HAs were eluted over a wide range of elution volumes (Fig. 3), as observed in previous studies [21, 22]. However, the intensities of the fluorescence-detected peaks varied with the HA samples (lowest in the Fujisaki HA and highest in the Takizawa HA).

Fluorescence-detected peaks were observed for all the fractions (Fig. 3), with the peak intensity being relatively low in the DAX-8-non-adsorbed fraction. This indicated that the fluorescent substances were mainly partitioned into both the precipitated and DAX-8-adsorbed fractions. The fluorescence-detected peaks of the DAX-8-adsorbed and DAX-8-non-adsorbed fractions were eluted in advance of that of the precipitated fraction. Therefore, the elution profile of the precipitated fraction lacked the largest molecular size components, compared to the corresponding whole HA. This indicates that the molecular sizes of the fluorescent substances in the DAX-8-adsorbed and DAX-8-non-adsorbed fractions were higher than those in the precipitated fractions.

Fluorescent properties of the fractions

3-D EEM fluorescence spectroscopy was utilized to investigate the fluorescent properties of whole HAs and their fractions. The 3-D EEM contour plots are shown in Fig. 4, and the fluorescence maxima and their relative intensities have been summarized in Table 2.

The fluorescence peaks at $\lambda_{ex}/\lambda_{em}$ = 265–275/505–540 nm (H1) and 430–460/510–540 nm (H4) were observed for all the whole HA samples, with the relative fluorescence intensities varying with each HA. In addition, the fluorescence peak was observed at $\lambda_{ex}/\lambda_{em}$ = 360–365/505 nm (H3) for the Fujisaki and Dando HA samples and at $\lambda_{ex}/\lambda_{em}$ = 310/510 nm (H2) for the Dando HA sample. The relative fluorescence intensities (QSU) were significantly high in the Takizawa HA compared to the other HAs. This is in agreement with the results of HPSEC with fluorescence detection. Our previous studies [4, 7, 21–23] showed that the fluorescent substances were considerably more in Andosol HAs than in HAs prepared from the other types of soils.

For the control treatment, the fluorescence maxima and relative intensities of the precipitated fractions were nearly identical to those observed for the whole HA samples. In contrast, the positions of fluorescence peaks of the DAX-8-adsorbed fraction (F1–3) did not coincide with those of the precipitated fraction and the whole HA, with the excitation and emission wavelengths being shorter for the former compared to the latter. The positions of the fluorescence maxima resembled those of FAs [24]. However, the fluorescence maxima and relative fluorescence intensities of the DAX-8-adsorbed fraction were similar between the different HA samples.

For the treatment with concentrated urea, the fluorescence maxima and relative intensities of the precipitated fraction were observed to be identical to those of the whole HA and the precipitated fraction of the control treatment. The fluorescence maxima and relative fluorescence intensities of the DAX-8-adsorbed fraction were similar to those observed in the DAX-8-adsorbed fraction from the control treatment. However, a fluorescence peak at $\lambda_{ex}/\lambda_{em}$ = 275/315 nm (P) was observed for the DAX-8-adsorbed fraction of the Dando HA sample, which was attributed to the fluorescence of protein-like substances [25, 26].

The position of the major fluorescence maximum of DAX-8-non-adsorbed fraction varied for each HA sample: it was observed at $\lambda_{ex}/\lambda_{em}$ = 270/465 nm (F2) for the Fujisaki HA sample, at 215/430 nm (F1) for the Dando HA sample, and at 225/440 nm (F1) for the Takizawa HA sample. All spectra were associated with a secondary maximum (P) at $\lambda_{ex}/\lambda_{em}$ = 280/330 nm (Fujisaki HA) or 270/330 nm (Dando and Takizawa HAs). These secondary maxima were attributed to the fluorescence of protein-like substances [25, 26].

Richard et al. [12] fractionated a soil HA sample by ultrafiltration, in the presence of 7 M urea. They reported that the fraction with molecular size 0.5–1 kDa exhibited an emission maxima at a shorter wavelength compared to the fractions with molecular size >1 kDa. This indicated that the relatively smaller HA constituents with an emission maximum at a shorter wavelength were dissociated in the presence of concentrated urea, an observation that was confirmed by the present results.

Infrared spectroscopy of the fractions

Figure 5 shows the DRIFT spectra of the whole HAs and their fractions. The spectrum of the precipitated fraction was similar to that of the corresponding whole HA, irrespective of treatment with concentrated urea. In contrast, the spectra of the DAX-8-adsorbed and DAX-8-non-adsorbed fractions were different from their whole samples and precipitated fractions. Absorption peaks at 2850 and 2920 cm^{-1}, assigned to the symmetric and asymmetric methylene stretching bands in aliphatic chains

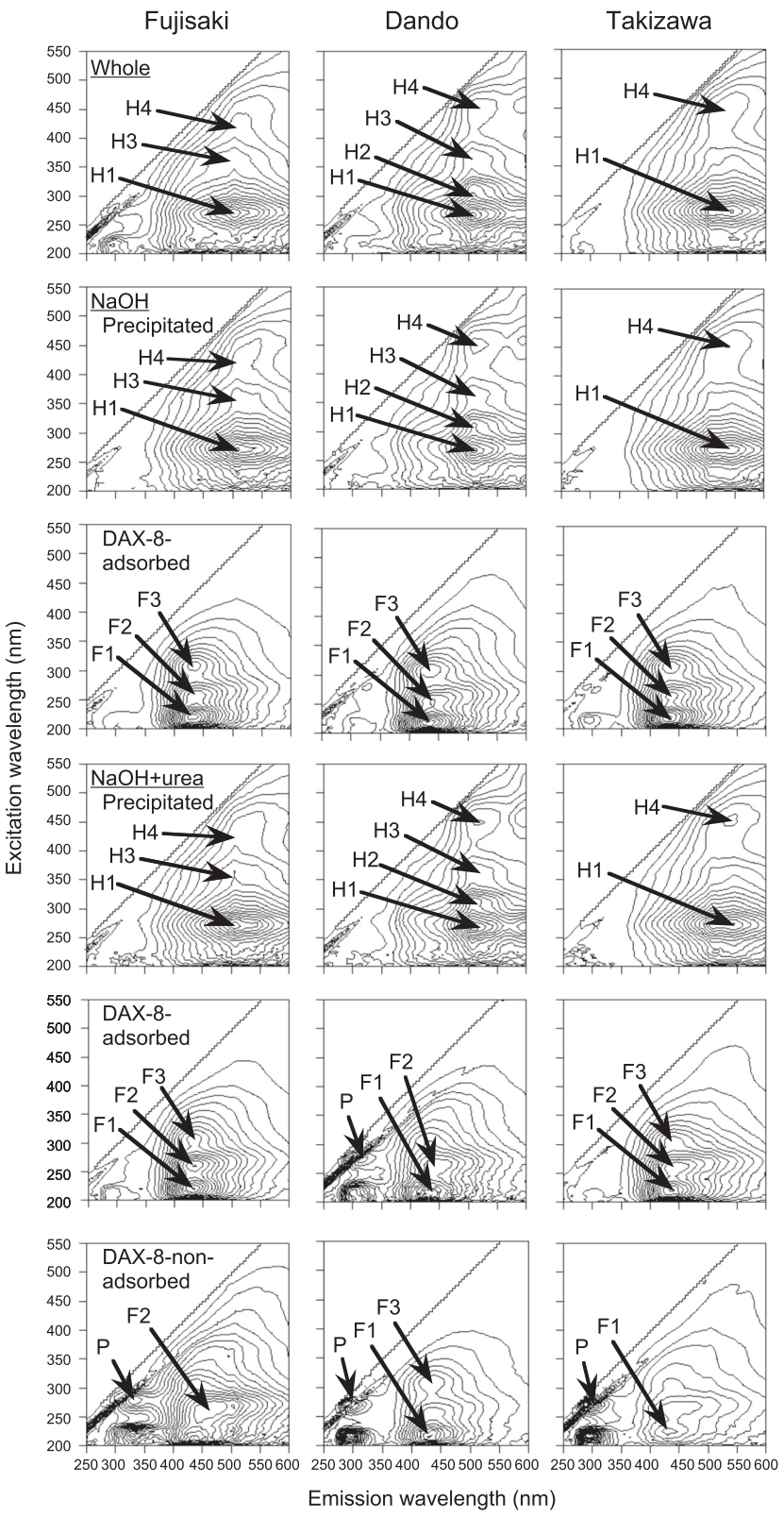

Fig. 4 Three-dimensional excitation-emission matrix (3-D EEM) fluorescence spectra of whole humic acid samples and their fractions. Emission intensities were normalized on the maximum. *Arrows* indicate the positions of fluorescence peaks. *H1–4* humic acid-specific fluorescence peaks, *F1–3* fulvic acid-like fluorescence peaks [24], *P* protein-like fluorescence peaks [25, 26]

Table 2 Positions and relative intensities of the fluorescence peaks

Fraction	Peak[a]	Fujisaki		Dando		Takizawa	
		$\lambda_{ex}/\lambda_{em}^{b}$	QSU	$\lambda_{ex}/\lambda_{em}^{b}$	QSU	$\lambda_{ex}/\lambda_{em}^{b}$	QSU
Whole	H1	270/505	65	265/505	54	275/540	134
	H2	–	–	310/510	36	–	–
	H3	365/505	27	360/505	23	–	–
	H4	430/510	18	460/515	16	450/540	48
NaOH							
Precipitated	H1	270/505	54	270/510	56	275/545	180
	H2	–	–	310/510	38	–	–
	H3	365/505	22	365/505	23	–	–
	H4	430/505	16	450/510	18	450/535	57
DAX-8-adsorbed	F1	220/430	107	220/430	100	220/430	104
	F2	255/435	77	255/440	71	255/435	80
	F3	310/435	57	310/435	55	310/435	59
NaOH + urea							
Precipitated	H1	270/505	48	270/510	69	275/545	181
	H2	–	–	310/510	48	–	–
	H3	360/505	21	365/505	27	–	–
	H4	430/505	16	450/510	21	450/540	59
DAX-8-adsorbed	F1	220/435	105	210/435	96	220/435	154
	F2	260/435	89	260/440	72	260/440	127
	F3	305/435	58	–	–	305/440	81
	P	–	–	275/315	32	–	–
DAX-8-non-adsorbed (>1 kDa)	F1	–	–	215/430	30	225/440	29
	F2	270/465	31	–	–	–	–
	F3	–	–	310/435	15	–	–
	P	280/330	14	270/310	12	270/310	26

QSU quinine sulfate unit
[a]Indicated in Fig. 4
[b]Excitation/emission wavelengths (nm)

[27], respectively, were observed in most of the spectra. The peaks were intense in the spectra obtained for the Fujisaki and Dando HAs, especially in the precipitated and DAX-8-non-adsorbed fractions. Absorption bands in the wavenumber region between 800 and 1800 cm^{-1} were observed to be overlapping. Therefore, the FSD was applied in order to enhance the resolution of the absorption bands (Fig. 6).

For the whole HAs, the peak observed at 1720 cm^{-1}, assigned to the C=O stretching of carboxyl group, was more intense in the Takizawa HA. On the other hand, the peaks at around 1670 and 1540 cm^{-1}, attributed to the amide I and amide II bands of proteinous moieties [28], and at 1510 cm^{-1} (vibrations of aromatic moieties in lignin) [29–31], 1410 cm^{-1} (the symmetric carboxylate stretching) [28], and 1080 and 1030 cm^{-1}, attributed to C–H stretching of polysaccharide moieties [28], were observed to be more intense in the Fujisaki and Dando HA samples.

The control and concentrated urea-treated precipitated fractions exhibited similar spectra to those displayed by the corresponding whole HA. In contrast, the control-treated DAX-8-adsorbed fraction showed a similar spectrum, irrespective of the type of HA. The spectrum was characterized by a more intense peak representing the carboxyl group at 1720 cm^{-1} and a less intense peak at 1600 cm^{-1}, attributed to the C=C stretching in aromatic rings [28] compared to the whole HAs. These spectral characteristics were similar to that observed for FA [27, 32]. The DAX-8-adsorbed fraction from the concentrated urea treatment displayed spectra with more intense peaks at 1670 and 1540 cm^{-1} due to the amide I and amide II bands, compared to those shown by the DAX-8-adsorbed fractions from the control treatment. The spectra of DAX-8-non-adsorbed fraction were characterized by prominent amide peaks at 1670 and 1540 cm^{-1} and by intense polysaccharide peaks at 1080 and 1030 cm^{-1}. The presence of proteinous

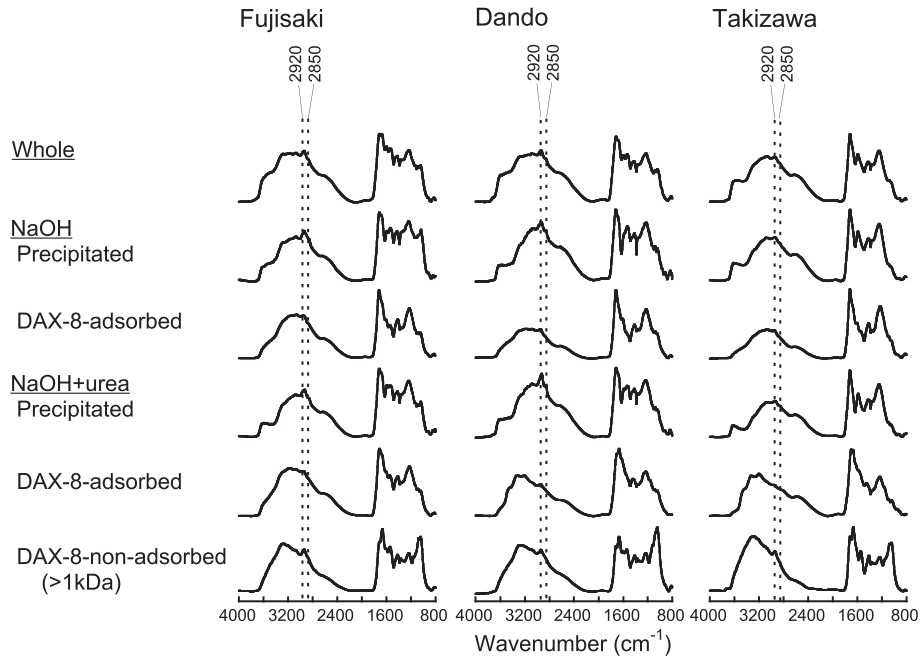

Fig. 5 Diffuse reflectance infrared Fourier transform (DRIFT) spectra of whole HA samples and their fractions

moieties in the acid-soluble constituents is in agreement with the results of 3-D EEM fluorescence spectroscopy.

Conclusions

The acid-soluble constituents of soil HAs obtained by treatment with concentrated urea were characterized by lower degrees of humification and smaller molecular sizes and displayed FA-like fluorescence. These features indicate that the acid-soluble constituents of soil HAs dissociated by concentrated urea treatment expressed similar properties to FAs. This was supported by the results of infrared spectroscopy. Infrared and fluorescence spectroscopies

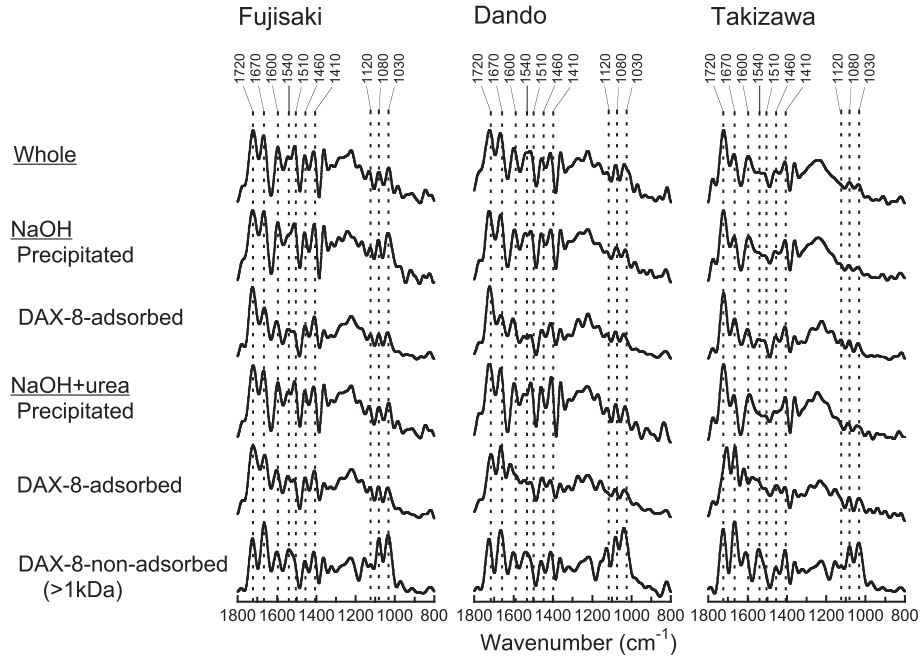

Fig. 6 Fourier self-deconvolution (FSD) spectra of whole humic acid samples and their fractions

revealed that the acid-soluble constituents contained proteinous moieties and the DAX-8-non-adsorbed fraction was rich in polysaccharide moieties. In contrast, the humification degree of the precipitated fraction increased due to the separation of acid-soluble constituents. The precipitated fraction still contained smaller molecular size fluorescent substances. The present findings suggest that soil HAs are composed of dark-colored acid-insoluble constituents, FA-like acid-soluble constituents, protein-like constituents, and polysaccharides bound by weak linkages.

Abbreviations

3-D EEM: three-dimensional excitation-emission matrix; DRIFT: diffuse reflectance infrared Fourier transform; FA: fulvic acid; FSD: Fourier self-deconvolution; HA: humic acid; HPSEC: high-performance size exclusion chromatography; MW: molecular weight; PAGE: polyacrylamide gel electrophoresis.

Competing interests

The author declares that he has no competing interests.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research (No. 26450072) from the Japan Society for the Promotion of Science (JSPS).

Received: 28 November 2014 Accepted: 10 June 2015
Published online: 30 June 2015

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