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Molecular size fractionation of soil humic acids using preparative high performance size-exclusion chromatography

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ABSTRACT

High performance size-exclusion chromatography (HPSEC) is useful for the molecular size separation of soil humic acids (HAs), but there is no method available for various HAs with different chemical properties. In this paper the authors propose a new preparative HPSEC method for various soil HAs. Three soil HAs with different chemical properties were fractionated by a Shodex OHpak SB-2004 HQ column with 10 mM sodium phosphate buffer (pH 7.0)/acetonitrile (3:1, v/v) as an eluent. The HAs eluted within a reasonable column range time (12–25 min) without peak tailing. Preparative HPSEC chromatograms of these HAs indicated that non-size-exclusion effects were suppressed. The separated fractions were analyzed by HPSEC to determine their apparent molecular weights. These decreased sequentially from fraction 1 to fraction 10, suggesting that the HAs had been separated by their molecular size. The size-separated fractions of the soil HA were mixed to compare them with unfractionated HA. The analytical HPSEC chromatogram of the mixed HA was almost identical to that of the unfractionated HA. It appears that the HAs do not adsorb specifically to the column during preparative HPSEC. Our preparative HPSEC method allows for rapid and reproducible separation of various soil HAs by molecular size.

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1. Introduction

Fractionation techniques, such as size-exclusion chromatography (SEC), adsorption chromatography [1], and precipitation [2], have been used to reduce the heterogeneity of soil humic acids (HAs), because HA is a heterogeneous mixture of natural organic macromolecules. The SEC fractionates HAs based on differences in molecular size using various soft gels with different nominal fractionation ranges [3–6]. The SEC is useful for fractionation and characterization of HAs; however, the fractionation procedure is laborious and time-consuming.

The development of high performance size-exclusion chromatography (HPSEC) allowed for rapid and reproducible size fractionation of natural organic matter. In early research, Becher et al. [7], using HPSEC fractionation of chlorinated dissolved organic matter (DOM) in marsh water, observed that mutagenic activity was associated with the low molecular weight DOM fraction. Recently, Piccolo and coworkers developed a preparative HPSEC method for HAs isolated from different sources, and characterized the separated fractions of HAs using pyrolysis-gas-chromatography/mass spectrometry, ^{1}H and ^{13}C NMR spectroscopies, and bioactivity [8–10]. These studies demonstrated the usefulness of preparative HPSEC for HA characterization.

However, there are potential problems in the application of HPSEC for humic substances. It is well known that the HPSEC elution pattern of humic substances is affected by ionic interaction with and specific adsorption to the stationary phase of the HPSEC column [11,12]. The intensity of these interactions depends on the chemical properties of the humic substances, e.g. functional group composition and aromatic structure. Since humic substances isolated from different sources show different chemical properties [13,14], the source of humic substances significantly affects the HPSEC elution pattern and fractionation.

Preparative HPSEC methods for aquatic natural organic matters were developed and validated by measuring the molecular weight distribution of separated fractions [15,16]. Egeberg and Alberts [16] show that specific adsorptions of their aquatic sample to the stationary phase are minor problem, but some aromatic standard reagents strongly interact with the stationary phase. Therefore, it is assumed that the specific hydrophobic interaction would prevent the size-exclusion separation of humic substances that have high proportion of aromatic composition. It also suggests that the optimization of HPSEC conditions for various soil HAs appears to be

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more difficult than that of aquatic natural organic matter, because some soil HAs have a significantly higher proportion of aromatic composition [14,17].

Most of the recent studies on preparative HPSEC of soil HAs have been based on the method investigated by Conte and Piccolo [18], who used a silica-based gel filtration column with 0.05 M NaCl solution as an eluent. In their method, preparative HPSEC chromatograms of some HAs showed a peak at the exclusion limit and exhibited a peak tailing [9,19]. These peaks represent non-size-exclusion effects, which could be attributed to the wide diversity of the chemical properties of soil HA. Furthermore, their method requires a long chromatography time (2–3 h) and did not taken advantage of the speed of HPSEC.

We therefore consider that there is no validated HPSEC method available for the rapid fractionation of HAs isolated from various types of soil. In particular, Melanudand (Andosol) HA, which has a significantly high proportion of aromatic composition [14], shows peak tailing in a HPSEC chromatogram [20]. We previously investigated the effects of HPSEC conditions on the elution pattern of soil HAs and developed an analytical HPSEC method for soil HAs, including Melanudand soil HA [20]. In this study, the analytical method was scaled up for the preparative HPSEC of soil HA. We demonstrated the molecular size separation of Melanudand and Dystrochrept soil HAs, and validated the method by analysis of molecular weight distribution of size-separated fractions. The main purpose of this work was to demonstrate and propose a rapid preparative HPSEC method for size fractionation of various soil HAs.

2. Experimental

2.1. Humic acid (HA)

HAs were extracted from the A horizons of Hanaore (HO; Hyogo, Japan, Typic Dystrochrept, mixed forest), Sugadaira forest (SGM; Nagano, Japan, Typic Melanudand, broad-leaved forest), and Sugadaira grassland soils (SGG; Nagano, Japan, Typic Melanudand, grassland), using the International Humic Substance Society method with some modifications [21,22]. Liquid-state ^{13}C NMR characteristics of the HAs are listed in Table 1. The SGG HA shows a significantly higher proportion of aromatic carbon and lower proportions of alkyl carbon and *O*-alkyl carbon than the HO HA. The distribution of the carbon species of the SGM HA is intermediate between that of the HO and SGG HAs.

For the preparative fractionation, 800 mg of the HA was suspended in 40 mL milliQ water and 1 M NaOH solution was gradually added to dissolve the HA. The solution (pH 7.0–8.5) was shaken slowly under nitrogen overnight. Following adjustment of the solution volume to 45 mL with milliQ water, 15 mL of 40 mM sodium phosphate buffer (pH 7.0) and 20 mL of acetonitrile were added to the solution. Consequently, the HA concentration was 10 mg mL^{-1} and the composition of sample solution was the same as that of the HPSEC eluent. This solution was filtered through a 0.22 μm hydrophilic PTFE membrane filter (Omnipore, Millipore, Tokyo, Japan). The total injected mass of the HO, SGM, and SGG HAs were 3.2, 2.3, and 2.5 g, respectively.

2.2. Analytical and preparative high performance size-exclusion chromatography (HPSEC)

We previously developed an analytical HPSEC method for various soil HAs using a Waters 600E system controller, 717 plus autosampler, and 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA) [20]. In short, the method is as follows: column, Shodex OHpak SB-805 HQ column (Showa Denko, Tokyo, Japan; 0.8 mm I.D. × 300 mm; 0–4000 kDa for pullulan) with a Shodex OHpak SB-G guard column; column temperature, 40 °C; eluent, 10 mM sodium phosphate buffer (pH 7.0) + 25% acetonitrile (v/v); standard, sodium polystyrene sulfonate (PSSNa); flow rate, 0.8 mL min^{-1}; injection volume, 30 μL; detection, 260 nm. Blue Dextran (2000 kDa) and acetone were used for the determination of the void volume (V_0) and total permeation volume ($V_0 + V_i$), respectively.

The preparative HPSEC was carried using the same instruments, settings, and eluent as described above, but a Shodex OHpak SB-2004 HQ column (Showa Denko, Tokyo, Japan; 20 mm I.D. × 300 mm; 0–1000 kDa for pullulan) preceded by a Shodex OHpak SB-LG guard column (8.0 mm I.D. × 50 mm) was used at a flow rate of 3.0 mL min^{-1} and a detection wavelength of 650 nm. Although detection wavelength affects the shape of the HPSEC chromatogram of humic substances [23], 650 nm was used as a detection wavelength to estimate molecular size distribution. Two milliliters of HA solution was injected into the preparative column and fractionated into 10 fractions using a fraction collector (SF-2120, Advantec, Tokyo, Japan).

An exclusion peak of the HO HA was fractionated as fraction 1, and the eluate of the main broad peak of the HO HA was fractionated equally into nine fractions based on peak area (650 nm). Since the absorbance at 650 nm of the chromatogram peak of the SGM and SGG HAs exceeded the absorbance detector limit, the collection periods for the size fractions were calculated from a refractive index chromatogram using a Waters 2410 refractive index detector at a flow rate of 1.5 mL min^{-1} (this was the maximum tolerated flow rate of the detector). The eluates of SGM and SGG HAs were fractionated according to retention times that equally divided the peak into ten areas based on the peak area (refractive index), except for an exclusion peak of the SGM HA due to the low yield.

2.3. Isolation and HPSEC analysis of separated fractions of HA

The collected fractions were evaporated to remove acetonitrile. For the estimation of the molecular weight distributions, a portion of the solution was diluted 50 times with HPSEC eluent and analyzed by HPSEC. The whole HAs were also analyzed as described previously [20]. The molecular weight at peak maximum (M_p), weight-(M_w) and number-averaged molecular weights (M_n) were calculated by Waters Millennium 32 Chromatography Manager version 3.06 software.

The residual evaporated solution was acidified to pH 1.0 with 6 M HCl and centrifuged. Precipitated HA was dialyzed in deionized water (Spectra/Por CE membrane, molecular weight cut-off = 500 Da, Spectrum, Houston, TX, USA) and then freeze-dried.

2.4. HPSEC analysis of mixed solution of the separated HO HA fractions

To validate the effects of the fractionation procedure on the molecular distribution of the HA, fractions 2–10 of HO HA were combined and the analytical HPSEC chromatogram of the mixed sample was compared with that of the whole HO HA. The freeze-dried HO HA fraction (2.5 mg) was suspended in milliQ water (2.5 mL) and dissolved with 0.1 M NaOH. After gentle shaking overnight, the solution was made up to 25 mL (0.1 mg mL^{-1}) with 10 mM sodium phosphate buffer (pH 7.0). Absorbance of the solution at 600 nm was determined using a spectrophotometer (V-530, Jasco, Tokyo, Japan), and then the solutions of fractions 2–10 were mixed so that the contribution of each fraction to the absorbance would be equal in the mixed solution. This mixed solution was diluted to 50 times with the HPSEC eluent and analyzed by HPSEC.

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