Properties of Fulvic Acid Extracted from Excess Sludge and Its Inhibiting Effect on β-Hexosaminidase Release

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The physicochemical and biological properties of fulvic acid extracted and purified from excess sludge and solubilized excess sludge were studied. Solubilization was introduced to improve the recovery rate of fulvic acid from the sludge. The structural features of fulvic acid from excess sludge and solubilized excess sludge were characterized by using an elemental analysis, Fourier transform infrared spectroscopy and 1H-nuclear magnetic resonance spectroscopy, and were compared with fulvic acid extracted from peat which had an inhibitory effect on the type I allergy in our previous study. The results show that they had a higher aliphatic characteristic with lower oxygen group content than fulvic acid from peat, and that the aliphatic characteristic was further strengthened by the use of solubilization. The biological properties of fulvic acid from excess sludge and solubilized excess sludge showed an inhibitory effect on β-hexosaminidase release at the antigen-antibody binding stage and antigen-receptor binding stage by using rat basophilic leukemia cells.

Key words: fulvic acid; excess sludge; solubilization; antiallergic activity; antioxidative activity

Fulvic acid (FA) is in a class of compounds including humic substances together with hemic acid and humin, and is formed through the degradation of such organic substances as dead plants, microbes and animals by chemical and biological processes. FA is also found abundantly in peat, weathered coal and other humified substances. FA consists of a mixture of closely related complex aromatic polymers, and chemical and spectroscopic analysis have revealed the presence of aromatic rings, and phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl and alkoxyl groups. FA has various useful effects due to its functional groups. Studies on the physiological actions of fulvic acid exerted on the living body are gradually being carried out. For example, the antioxidative activity of FA extracted from peat has been reported. The possible application of coal-derived fulvic acid as an antimicrobial substance has been described, and the anti-inflammatory property of coal-derived fulvic acid has been also reported. Moreover, the Ministry of Health, Labour and Welfare of Japan designated fulvic acid as a food in 2004.

In our previous study reported in this journal, we have indicated that FA extracted from Canadian Sphagnum peat (CP-FA) had an inhibitory effect on chemical mediator release in rat basophilic leukemia (RBL-2H3) cells. We showed a method for inhibiting the onset of the type I allergy by performing a nonspecific hyporesensitization treatment with fulvic acid. We also provided a method for inhibiting the onset of the type I allergy by suppressing degranulation with fulvic acid, and issued an international patent on this study.

It has been described that humic substances were included in sewage sludge. Sewage sludge generally consists of primary sludge and excess sludge (ES). Primary sludge contains such inorganic materials as gravel, silt and clay, and contains a high level of heavy metals. Meanwhile, ES consists mainly of microorganisms which include a high proportion of organic matter and nutrients. It is thought that ES is more suitable than primary sludge as a resource of FA, so we extracted and purified FA from excess sludge.

Solubilization was introduced in the extraction process to improve the recovery rate of FA. It has been reported that from 11% to 66% of total organic carbon (TOC) was humic substances in groundwater, and it was found that FA constituted 7–72% of landfill leachate TOC. In our previous study, solubilized excess sludge (SS) contained soluble fractions having a high level of TOC. A high concentration of FA may be included in the TOC-rich fraction of SS. The structural features of FA extracted from ES (ES-FA) and SS (SS-FA) were analyzed by using an elemental analysis, fourier transform infrared (FT-IR) spectroscopy and 1H nuclear magnetic resonance (1H-NMR) spectroscopy. The in-

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Abbreviations: FA, fulvic acid; CP-FA, Canadian Sphagnum peat; RBL-2H3, rat basophilic leukemia; ES, excess sludge; TOC, total organic carbon; SS, solubilized excess sludge; ES-FA, FA extracted from ES; SS-FA, FA extracted from SS; FT-IR, fourier transform infrared; 1H-NMR, 1H nuclear magnetic resonance; TN, dissolved total nitrogen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC, inorganic carbon
hibitory effect of ES-FA and SS-FA on type I allergy by RBL-2H3 cells was also investigated as a case study for their utilization. To utilize ES as a new resource of FA, the physicochemical and biological properties of ES-FA and SS-FA were compared with those of CP-FA.

Materials and Methods

Collection of sludge samples and solubilization conditions. ES was sampled from the return line of an aeration reactor at a municipal wastewater treatment plant in Japan. In this examination, the concentration of ES was 4200 mg/l, a total amount of 8 liters of ES being used and divided in 2 parts. One part was solubilized to verify the enhanced recovery efficiency by solubilization as a pretreatment for extraction, while the other part was just extracted. A beads-mill with a cylindrical container of the rotary drum type was selected as the solubilization method. ES and glass beads were put in the container as contact material, and ES was solubilized by the shearing force arising from collision and friction with the glass beads. The ES concentration was measured according to the standard method. TOC and dissolved total nitrogen (TN) were measured by a TOC-800 analyzer (YANACO) and TN-301P analyzer (YANACO) after passing through a filter paper with 1-μm pore size. The FA recovery rate was calculated by comparing dry weight of recovered FA and raw material.

FA extraction. The extraction and purification of FA from ES and SS were carried out according to the standard method of the International Humic Substances Society, with some modifications (Fig. 1). ES and SS were stirred for 4 h at pH 11 in 0.1 M NaOH. The residue (humin and other insoluble compounds) was separated from the supernatant by centrifugation (3000 rpm, 20 min). The supernatant was then acidified with 6 M HCl to pH 1.5, before the supernatant and precipitated fraction were separated by centrifugation. The supernatant was purified by column adsorption resin (XAD-7, Organo Co.), and the fractions retained by this resin were recovered with 0.1 M NaOH. The FA recovery rate was calculated by comparing dry weight of recovered FA and raw material.

Elemental analysis. The carbon (C), hydrogen (H), and nitrogen (N) contents of FA were analyzed by a 2400 CHN Elemental Analyzer (Perkin-Elmer). The sulfur (S) content was analyzed by the silver absorption method with an elemental analyzer (Mitamuraiken). The oxygen content was calculated by subtracting the C, H, N, and S contents from 100% and is expressed on a dry and ash-free (d.a.f.) basis.

FT-IR spectroscopy. Two milligram each of ES-FA and SS-FA were mixed with 100 mg of potassium bromide (KBr), and the mixture pressed into a disk. The pellets were then analyzed with an FT-IR-300 spectrum photometer from 400 to 4000 cm⁻¹ (Jasco).

¹H-NMR spectroscopy. ¹H-NMR spectra were measured and recorded in D₂O with a Bruker Avance 500 spectrometer. Chemical shift values (ppm) are reported in parts per million relative to the NMR solvents in D₂O (4.8 ppm).

Reagents. Eagle’s minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Japan). Fetal bovine serum was purchased from Hyclone Co. (Japan), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 2-morpholinoethanesulfonic acid (MES) were purchased from Dojindo (Japan). Dinitrophenylated bovine serum albumin (DNB-BSA) was obtained from Cosmo Biotechnology Co. (Japan), and anti-DNP-IgE, ketotifen and γ-glutamine were from Sigma (Japan). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries.

Cell and cell culture. RBL-2H3 cells were purchased from JCRB Bank, Japan. The cells were cultivated in MEM supplemented with 10% FBS and 2% of γ-glutamine at 37°C in a 5% CO₂ incubator.

MTT assay. The MTT assay is a sensitive and quantitative colorimetric method that is used to determine the cytotoxicity of potential medicinal agents and other toxic materials. RBL-2H3 cells were seeded on to 96-well plates (Falcon Co.) at 5.0 × 10⁴ cells/well in 100 μl of a medium. The cells were incubated (37°C, 5% CO₂) overnight to allow them to attach to the wells. After incubation, the cultured cells were washed with PBS, and 100 μl of ES-FA or SS-FA dissolved in the medium was added to obtain final concentrations of 0.1, 1, 10 and 100 μg/ml. The cells were incubated for 24 h, and then 10 μl of 5 mg/ml of the MTT solution was added to each well. The cells were incubated for another 24 h to allow MTT to be metabolized. After that, 100 μl of 10% sodium dodecyl sulfate (SDS) was added, this being followed by another 24 h of incubation to completely dissolve the formazan produced by the cells. The absorbance of the converted dye was measured at a wavelength of 570 nm using a PowerScan HT multidetection micro-plate reader (Dainippon Pharmaceutical Co.). Blanks were prepared at the same time to correct for the absorbance caused by sample color and by the inherent ability of a sample to reduce MTT in the absence of the cells.

The optical density of the formazan produced by the untreated control cells is considered as representing 100% viability.

β-Hexosaminidase inhibition assay at the different stages. To determine the effect of ES-FA and SS-FA on the antigen-antibody binding stage, a β-hexosaminidase inhibition assay with RBL-2H3 cells was performed according to the method described by Kawasaki et al. The assay at the antigen-antibody binding stage used RBL-2H3 cells that were seeded on to a 96-well plate at 5.0 × 10⁴ cells/well in 100 μl of the medium. The cells were incubated and sensitized for 24 h at 37°C and 5% CO₂ with 0.3 μg/ml of anti-DNP-IgE. The cells were then washed twice with PBS to eliminate free IgE. After incubating the cells at 37°C for 10 min in 60 μl/well of a release mixture (116.9 mm NaCl, 5.4 mm KCl, 0.8 mm MgSO₄·7H₂O, 5.6 mm glucose, 25 mm HEPES, 2.0 mm CaCl₂, and 1 mg/ml BSA at pH 7.7) containing 5 μl/well of ES-FA or SS-FA (0.001, 0.01, 0.1, 1 or 10 μg/ml), the cells were exposed to 5 μl of serum well of 4 μg/ml of DNP-BSA in PBS, before being incubated at 37°C for 1 h. As a positive control, 3 μl of ketotifen was used. After the plates had been put on ice for 10 min to terminate the reaction, 20 μl of the supernatant was transferred to another plate; 80 μl of a substrate solution (5 μM 4-nitrophenyl N-acetyl-β-D-glucosami- nide in a 50 mM C₆H₄O₂ buffer at pH 4.5) was then added to the supernatant with subsequent incubation at 37°C for 30 min. After that, 100 μl/well of a stop buffer (0.1 M NaHCO₃/Na₂CO₃ at pH 10) was added, and the absorbance at 405 nm was obtained by the multi-
Assay for DPPH scavenging activities. This assay was conducted to determine the antioxidative activity of ES-FA and SS-FA. The DPPH scavenging activity was examined according to the method described by Oki et al.\textsuperscript{17} To each well of a 96-well plate, a solution of 400 μM DPPH (50 μl) was added to a mixture of 200 μM MES buffer (50 μl), 20% ethanol (50 μl) and 80% ethanol (50 μl) containing a varying amount of the test compound (10, 20, 30, 40 or 50 mM) at room temperature for 20 min. Blanks were prepared at the same time to correct for the absorbance caused by the sample color. After that, the absorbance of the mixture was measured at 520 nm by a Power Scan HT microplate reader. The percentage of the scavenging activity toward DPPH radicals by the test sample was calculated using the following equation:

\[
\text{DPPH radical scavenging activity (\%)} = \left( 1 - \frac{A_{520} (\text{test sample})}{A_{520} (\text{blank})} \right) \times 100
\]

Statistical analysis. Student’s t-test was used to determine the statistical significance between treatment groups in each experiment. Differences were considered statistically significant at a value of \( p < 0.05 \).

Results and Discussion

Properties of the sample sludge and FA recovery rate

Chemical processing such as ozone oxidation and alkali treatment, and physical processing such as supercritical water oxidation and ultrasonic waves have been developed as solubilization techniques.\textsuperscript{18,19} We chose the beads-mill from these which is one of the physical processing methods for pretreating before alkali extraction. We considered that alkali extraction would be enhanced, because the contact area with the alkaline solution will be greater by the pulverization of microorganisms with the beads-mill. This technique has a stable solubilization effect because of the mechanical treatment, and its operation and maintenance are easy. Moreover, possibility of heat degradation and heat denaturation of FA is less than by heat treatment such as supercritical water oxidation. The properties of ES and SS are shown in Table 1. The sludge concentration decreased from 4200 mg/l to 3108 mg/l after solubilization. The TOC concentration of ES was 5.2 mg/l, but of SS was 547.5 mg/l. While the TN concentration of ES was 8.8 mg/l, of SS it was 144.5 mg/l. These figures indicate that the sludge floc had been disintegrated, and that various cytoplasmic materials had been released into the liquid as a result of cell wall disintegration. Expression of these acids might indicate that microorganisms had been degraded into low-molecular-weight substances. The FA recovery rates of ES and SS were 0.8% and 3.5%, respectively. The FA recovery efficiency was enhanced by increasing the soluble fraction with solubilization. In our previous study, the FA recovery rate of CP was 4.8%, and the recovery rate of SS-FA approached that of CP-FA by solubilization. The exploitation of wetlands, which is a source of supply of peat, has recently been severely restricted for the ecological preservation of wetlands by the Ramsar Convention.\textsuperscript{20} From the viewpoint of conservation of nature, the utilization of excess sludge as a resource of humic substances could be a valid method.

Elemental analysis

Table 2 shows the elemental analysis and atomic ratio of ES-FA and SS-FA. The data for CP-FA are shown for comparison. ES-FA and SS-FA had higher H and N contents, and lower O contents than CP-FA. It is suggested that the higher H content of ES-FA and SS-FA was due to their greater aliphatic characteristic from the FT-IR results, and their higher N content related low humification degree of ES-FA and SS-FA. Sposito et al. have reported that FA extracted from a sewage sludge-soil mixture contained S from 4.5% to 11.6%.\textsuperscript{21} However, the S amount in our FA was very low. This result may indicate one of the factors to show the safety of our FA, because there have been concern that an excessive S component indicates cell cytotoxicity. In a comparison between ES-FA and SS-FA, SS-FA had less C than ES-FA. One of the reasons for the lower C could be carbon mineralization, because the inorganic carbon (IC) of ES was 13.6 mg/l, and of SS was 38.0 mg/l (Table 1).

To obtain more detailed information on FA, the atomic ratios of H/C and O/C were calculated from the elemental analysis results. It has been reported that the H/C ratio decreases with increasing degradation of aliphatic content, and that the H/C ratio is negatively correlated with the degree of humification. Humification indicates the generation and increase of the dark colored organic material of humic substances, and is an important indicator of the degree of aromatic condensation.\textsuperscript{22,23} Those reports indicate that ES-FA and SS-FA have a low degree of humification and contain lower ratio of aromatic carbon than CP-FA. The O/C ratio is representative of the degree of O-alkyl and carboxylic

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recovery rate (%)</th>
<th>Elemental composition (% d.a.f.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td>ES-FA</td>
<td>0.8</td>
<td>44.8</td>
</tr>
<tr>
<td>SS-FA</td>
<td>3.5</td>
<td>37.7</td>
</tr>
<tr>
<td>CP-FA</td>
<td>4.8</td>
<td>47.8</td>
</tr>
</tbody>
</table>

Each value of represents the mean of two measurements.

Table 1. Main Properties of ES and SS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sludge concentration (mg/l)</th>
<th>TOC (mg/l)</th>
<th>IC (mg/l)</th>
<th>TN (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES</td>
<td>4200</td>
<td>5.2</td>
<td>13.6</td>
<td>8.8</td>
</tr>
<tr>
<td>SS</td>
<td>3108</td>
<td>547.5</td>
<td>38.0</td>
<td>144.5</td>
</tr>
</tbody>
</table>
acid composition.\textsuperscript{24,25} Compared to that of CP-FA and ES-FA, the high O/C ratio of SS-FA suggests that it contained a high proportion of $O$-alkyl and carboxylic acid functional groups.

\textit{FT-IR spectroscopy}

The infrared spectra of ES-FA and SS-FA are shown in Fig. 2 with the infrared spectrum of CP-FA being shown for comparison. These infrared spectra were identified from data in the study by Stevenson and Goh.\textsuperscript{26} The infrared spectra of ES-FA and SS-FA had strong absorbance at 3400, 2920, 1720, 1510, and 1400–1200 cm$^{-1}$. The wide absorption band at 3400–3200 cm$^{-1}$ is attributed to an intermolecular OH stretch, the band at 2850–2920 cm$^{-1}$ being due to aliphatic C–H stretching vibration, indicating the presence of methyl and methylene groups. The shoulder at 1720 cm$^{-1}$ attributed to the C=O stretching vibration of COOH groups was found in each wave of ES-FA and SS-FA. The band near to 1620 cm$^{-1}$ is attributed to structural vibration of aromatic C=C, H-bonded C=O of quinones, or H-bonded and conjugated ketones. Disappearance of the absorption near 1540 cm$^{-1}$ is attributed to amide groups in the peptide linkages of protein. In the case of FA from ES and SS, this absorption might have been caused by microorganism cell protein.

The results show that ES-FA and SS-FA had a comparatively similar structure to that of CP-FA, although we compared our FA spectra with some spectra of FA extracted from natural sources.\textsuperscript{27} We found that the component ratio of functional groups indicated difference, the absorption at 2920 cm$^{-1}$ being relatively stronger in ES-FA and SS-FA. Compared to CP-FA, ES-FA and SS-FA revealed their greater aliphatic characteristic due to the different intensities of oxidation and humification processes undergone by the materials. The 1620 cm$^{-1}$ band of ES-FA and SS-FA was considerably weaker than that of CP-FA. The absorption of the 1620 cm$^{-1}$ band is one of the indicators for estimating the degree of humification. These results show that ES-FA and SS-FA had less polycondensation than CP-FA. CP-FA is a humic substance that has been broken down into smaller, more fulvic subunits by bacterial enzymes, and decarboxy and oxidation reactions with time. On the other hand, these results might naturally follow because ES-FA and SS-FA had a short-term humification process in the activated sludge reactor. The results of FT-IR are consistent with the results obtained from the elemental analysis.

\textit{1H-NMR spectroscopy}

The $^1$H-NMR spectra of ES-FA and SS-FA are shown in Fig. 3. $^1$H-NMR was used to elucidate the structure of their molecules. The peak characterization of the ES-FA and SS-FA spectra was similar. They consisted of peaks in the region of 7.0–8.1 ppm (aromatic H), strong peaks of 3.3–4.6 ppm (carbohydrate H), and peaks in region of 0.8–3.3 ppm (methylene and aromatic CH$_2$, CH$_3$). For a more effective comparison, the proton chemical shift was quantitatively classified into four categories for protons in different chemical environments,\textsuperscript{28,29} the results being presented in Table 3. The resonance of SS-FA showed a significantly higher percentage (19.48%) of protons on the terminal CH$_3$, CH$_2$ and CH of methylene chains (region I) than that of ES-FA (2.98%), and the content of the proton ratio of CH$_3$, CH$_2$ and CH protons to aromatic or carboxyl groups (region II) of SS-FA were also higher (20.48%) than that of ES-FA (13.48%). On the other hand, the percentage of protons on the $\alpha$-carbon to oxygen function or carbohydrates (region III) in ES-FA accounted for 33.28%, as compared to 20.88% in SS-FA. The percentage of
aromatic protons (region IV) of ES-FA was also high (49.90%), compared to that of SS-FA (39.17%). This molecule of SS-FA contained a larger numbers of protons of aliphatic components (region I and region II) than that of ES-FA. This result shows that the aliphatic character of ES was further strengthened by the use of solubilization, this being consistent with the results obtained from the elemental analysis and FT-IR.

Cytotoxic effect of ES-FA and SS-FA on RBL-2H3 cells

The MTT assay results for the effect of ES-FA and SS-FA on RBL-2H3 cells are shown in Fig. 4. RBL-2H3 cells were treated for 24 h at final concentrations of SS-FA and ES-FA of 0.1, 1.0, 10.0 and 100 mg/ml. Neither ES-FA nor SS-FA exhibited cytotoxicity at any concentration.

Effect of ES-FA and SS-FA on β-hexosaminidase release at the antigen-antibody binding stage

Type I allergy occurs due to environmental substances known as allergens such as food, dust, medicine, cosmetics and pollen. This class of antigen induces the production of antigen-specific IgE antibodies that bind to FcεRI receptors on mast cells or basophiles. The early phase reaction in type I allergy occurs within minutes, and then mediators such as histamine and β-hexosaminidase are released from the cells. These mediators induce vasodilatation, mucous secretion, and bronchoconstriction. RBL-2H3 cells are considered as a good tool for studying the effect of unknown compounds on the β-hexosaminidase release activity, because the cells display characteristics of mucosal-type mast cells and express several hundred thousand IgE receptors on the membrane surface. We assayed the inhibitory effect on β-hexosaminidase release of ES-FA and SS-FA by using the RBL-2H3 cells.

The inhibitory effect on β-hexosaminidase release of ES-FA and SS-FA on RBL-2H3 cells is shown in Fig. 5. The β-hexosaminidase release from IgE-sensitized RBL-2H3 cells was induced by DNP-BSA as a stimulatory antigen at the antigen-antibody binding stage (control). ES-FA and SS-FA at concentrations of 0.001–10 μg/ml were used; both ES-FA and SS-FA had an inhibitory effect on β-hexosaminidase release, the highest inhibition rate of ES-FA being 20.3% at 1 mg/ml compared to the control, and of SS-FA being 27.7% at 0.1 μg/ml. The inhibitory effect of SS-FA was thus more than ES-FA. Even though ES-FA and SS-FA didn’t cause cytotoxicity at the tested concentrations used during the MTT assay, the β-hexosaminidase inhibitory effect of ES-FA and SS-FA was not dose dependent, because the macromolecular conformation of FA varies according to such as factors as the concentration, composition and pH of the solution. These observations suggest that several stimulation mechanisms were simultaneously and intracellularly involved in response to ES-FA and SS-FA.

### Table 3. Hydrogen Distribution Calculated from ¹H-NMR Data for ES-FA and SS-FA

<table>
<thead>
<tr>
<th>Chemical shift region δ (ppm)</th>
<th>Assignment</th>
<th>Relative contribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. (0.4–1.7)</td>
<td>Terminal CH₃, CH₂ and CH of methylene chains, etc.</td>
<td>2.98</td>
</tr>
<tr>
<td>II. (1.7–3.3)</td>
<td>CH₃, CH₂ and CH proton α to aromatic or carboxyl groups, etc.</td>
<td>13.84</td>
</tr>
<tr>
<td>III. (3.3–4.6)</td>
<td>Protons on carbon α to oxygen, carbohydrate, etc.</td>
<td>33.28</td>
</tr>
<tr>
<td>IV. (6.5–8.1)</td>
<td>Aromatic protons (including quinone, phenol, etc.)</td>
<td>49.90</td>
</tr>
</tbody>
</table>

Fig. 4. Cytotoxic Effect of ES-FA and SS-FA on RBL-2H3 Cells by an MTT Assay.

The percentage cell viability was calculated relative to the untreated control. The cells (5.0 x 10⁴ cells/well in 100μl) were incubated with ES-FA and SS-FA at 37°C for 24 h in 5% CO₂.

Fig. 5. Inhibiting Effect of ES-FA and SS-FA on the β-Hexosaminidase Release from RBL-2H3 Cells at the Antigen-Antibody Binding Stage.

The cells (5.0 x 10⁴ cells/well in 100μl) were preincubated with ES-FA and SS-FA at 37°C for 10 min prior to their incubation with DNP-BSA. Results represent one trial (n = 4–6). Four additional trials showed similar results. *Significantly different from the control (DNP-BSA; p < 0.05, Student’s t-test).
Inhibiting Effect of ES-FA and SS-FA on the β-Hexosaminidase Release from RBL-2H3 Cells at the Antibody-Receptor Binding Stage.

The cells (5.0 × 10^4 cells/well in 100 μl) were preincubated with ES-FA and SS-FA at 37°C for 1 h prior to their IgE sensitization, and then preincubated with ES-FA and SS-FA at 37°C for 24 h prior to their DNP-BSA stimulation. Results represent one trial (n = 4–6). Three additional trials showed similar results. *Significantly different from the control (DNP-BSA; p < 0.05, Student’s t-test).

Effect of ES-FA and SS-FA on β-hexosaminidase release at the antibody-receptor binding stage

The β-hexosaminidase release from IgE-sensitized RBL-2H3 cells was induced by DNP-BSA as a stimulatory antigen at the antibody-receptor binding stage. The inhibitory effects on β-hexosaminidase release of ES-FA and SS-FA at final concentrations of 0.001–10 μg/ml on RBL-2H3 cells is shown in Fig. 6. The highest inhibition rate of ES-FA was 21.5% at 0.1 μg/ml, and for SS-FA it was 18.7% at 0.01 μg/ml. The β-hexosaminidase inhibitory effect of SS-FA was thus more than SS-FA.

In our previous study, we investigated the inhibitory effect of various types of Tunisian olive oil on the chemical mediator release and cytokine production by basophilic cells. It was found that hydroxytyrosol-rich olive oil had a high inhibitory effect at the antibody-receptor binding stage. It is known that hydroxytyrosol has radical scavenging activity. Suzuki et al. have also reported that the radical scavenging activity is one of the factors involved in antiallergic activity. We then confirmed the antioxidation activity of ES-FA, SS-FA, and CP-FA by an assay of the scavenging activity toward DPPH radicals. As shown in Fig. 7, the DPPH radicals were dose dependently scavenged by CP-FA, ES-FA, and SS-FA. These results show that the scavenging activity follows the order (from strongest to weakest) of CP-FA > ES-FA > SS-FA. One reason for this might be that the quinone group and ketone group content of CP-FA was highest in the three types of FA by FT-IR, and that ES-FA had more phenol groups than SS-FA by the 1H-NMR results. It is well known that quinones, ketones and polyphenols play an important role in antioxidation activity. These results for the DPPH radicals are consistent with the results obtained from 1H-NMR and FT-IR.

In conclusion, FA extracted from ES and SS had higher aliphatic characteristics and a lower oxygen group content than that of CP-FA. Solubilization increased the recovery rate, and induced a difference in the chemical structures of ES-FA and SS-FA. The molecule of SS-FA contained larger aliphatic components (including methylene) than ES-FA, and ES-FA had more quinone and phenol groups than SS-FA. Moreover, ES-FA and SS-FA had an inhibitory effect on β-hexosaminidase release by IgE-sensitized, antigen-stimulated RBL-2H3 cells. The results of our studies suggest excess sludge as a new resource for FA production. For example, excess sludge-derived FA can be widely applied to quasi-medical drugs for the purpose of preventing and/or inhibiting the onset of the type I allergy in the form of a dermatological agent, poultice, etc.; and as a cosmetic having the function of preventing and/or inhibiting the onset of the type I allergy in the form of bath salts and skin care cosmetics (e.g., body wash, cream, and shampoo). The detailed mechanism behind the antiallergic effect of excess sludge-derived fulvic and a safety review are subjects of a future study. However, we hope that recycling activity will grow in the future to minimize raw material consumption and the disposal of waste into the environment.

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